ADVACES IN CLINICAL CHEMISTRY

Volume 4

Harry Sobotka &
C. P. Stewart

ADVANCES IN CLINICAL CHEMISTRY

VOLUME 4

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Advances in CLINICAL CHEMISTRY

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FOREWORD TO THE SERIES

A historian of science in years to come may well be astonished at the explosive burst of scientific activity round about the middle of the twentieth century of our era. He will be puzzled by the interrelationship between the growth of population and the rise of the standard of living; he will be interested in the increased percentage of scientists among the population, their greater specialization and the resulting fragmentation of science; he will analyze the economic and the psychological motivation of scientists; he will compare the progress of knowledge with the broadness of the current of scientific publication.

Living as we do in the midst of these events, we are hardly aware of their relatively rapid rate. What we notice is a doubling of the scientific output every ten years, regardless of contemporary political events. It is this climate which has engendered the appearance of series of reviews in dozens of disciplines. It may be with yearning or with a feeling of superiority, that we look back at such annual compendia as "Maly's Jahresberichte der Thierchemie" of one hundred years ago, which encompassed the annual progress in the zoological half of biochemistry within 300 to 400 pages.

Nowadays, that number of pages would not suffice to record the complete annual increment of knowledge in a single specialized division of the subject such as Clinical Chemistry. Media already existing furnish a comprehensive list of publications and an encyclopedic summarization of their contents; the present series of "Advances in Clinical Chemistry"—like other "Advances" series—attempts something different. Its aim is to provide a readable account of selected important developments, of their roots in the allied fundamental disciplines, and of their impact upon the progress of medical science. The articles will be written by experts who are actually working in the field which they describe; they will be objectively critical discussions and not mere annotated bibliographies; and the presentation of the subjects will be unbiased as the utterances of scientists are expected to be—*sine ira et studio.*

The bibliography appended to each chapter will not only serve to document the author's statements, it will lead the reader to those original publications in which techniques are described in full detail or in which viewpoints and opinions are expressed at greater length than is possible in the text.

The selection of the subjects in the present and in future volumes will include discussion of methods and of their rationale, critical and com-

parative evaluation of techniques, automation in Clinical Chemistry, and microanalytical procedures; the contents will comprise those borderline subjects, such as blood coagulation or complement chemistry, which are becoming more chemical with increasing knowledge of the underlying reactions; in some instances the discussion of a subject will center around a metabolic mechanism or even around a disease entity.

While recognizing that the elaboration and testing of methods is of the greatest importance in a subject, part of whose function is to provide reliable, accurate diagnostic and prognostic procedures, the new series will take cognizance of the fact that Clinical Chemistry plays an essential part in the progress of medical science in general by assisting in elucidating the fundamental biochemical abnormalities which underlie disease. The Editors hope that this program will stimulate the thinking of Clinical Chemists and of workers in related fields.

HARRY SOBOTKA
C. P. STEWART

PREFACE TO VOLUME 4

Volume 4 of this series covers again aspects of Clinical Chemistry ranging from discussions of analytical methods to reviews on the biochemistry of disease, centering around the physiology and pathology, for instance, of a hormone or of a vitamin, and including the pertinent chemical procedures. Recent developments in immunoelectrophoresis, microliter analysis, and flame photometry are treated with a view to their concrete applications.

We wish to express our gratitude again to the authors and to the publisher, and we thank our colleagues for suggestions and criticisms, past and future.

November, 1961

HARRY SOBOTKA
C. P. STEWART

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FLAME PHOTOMETRY

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1. Introduction

It is the object of this review to provide a critical account of modern flame photometry in the light of the basic principles of the method. No attempt has been made to provide a catalog of the innumerable articles appearing on the subject, but it is hoped that this chapter will help clinical chemists to decide whether flame photometry is applicable to a particular problem and perhaps to suggest ways of overcoming difficulties with the method.

1.1. Historical; Background

Herschel (H1) studied the emission of salts introduced into a flame; he recorded [quoted by Mavrodineanu (M2)] that:

"Salts of soda give a copious and purely homogeneous yellow.

Salts of potash give a beautiful pale violet.

Salts of lime give a brick red, in whose spectrum a yellow and bright green line are seen.

Salts of strontia give a magnificent crimson. If analyzed by the prism two definite yellows are seen, one of which verges strongly to orange.

Salts of magnesia give no colour.

Salts of lithia give a red (on the authority of Dr. Turner's experiment with the blow-pipe).

Salts of baryta give a fine pale apple-green. This contrast between the flames of baryta and strontia is extremely remarkable.

Salts of copper give a superb green, or blue green.

Salts of iron (protoxide) gave white, where the sulphate was used . . . .

The colors thus communicated by the different bases to flames afford in many cases a ready and neat way of detecting extremely minute quantities of them; . . ."

Talbot (T1) had previously studied the flame spectra of lithium and strontium, but the full potentialities of Herschel's observations for qualitative analysis were first realized in the work of Kirchhoff and Bunsen (K1). These authors discovered cesium in 1860 and rubidium in the following year by observation of their flame spectra.

Janssen (J1) suggested that spectral analysis, until then used only for qualitative observations, was suitable also for quantitative work. He felt that such a development would be particularly advantageous in the case of elements like sodium which were difficult to determine by classic procedures. His suggestions bore fruit 3 years later when Champion *et al.* (C1) constructed an instrument for the determination of sodium in plant ash. A solution of plant ash was introduced into the flame by means of a platinum wire and the emission intensity measured by comparing it by means of a visual photometric attachment with light from a reference constant-intensity sodium flame. This "spectronatromètre" was the first flame photometer; and when one considers that it was capable of an accuracy of between 2 and 5 %, it is interesting that it was not for more than 70 years that the method was applied to clinical problems.

However, the principles of flame photometry were not fully developed until the brilliant work of Lundegårdh (L1, L2). This worker devised for the first time a satisfactory method of introducing solutions into the

flame at a constant rate. This was by means of a concentric atomizer which dispersed the solution into droplets which were then led through a spray chamber to remove the larger particles before passing into the flame. Instead of the Bunsen flame used by the earlier workers, Lundegårdh substituted an air-acetylene flame and photographed the emission on a photographic plate after it had been dispersed by means of a spectograph. He also developed procedures in which the intensity of an emission line was recorded directly by a galvanometer connected to the amplified output from a photocell which had been placed so as to receive an appropriate portion of the flame spectrum dispersed by a prism. Lundegårdh was able to measure almost half of the elements in the periodic table, and his work is the foundation of all modern work on flame photometry. It is only in the last few years that substantial advances have been made beyond the techniques employed by Lundegårdh.

Schuhknecht (S1) produced a greatly simplified instrument by substituting a filter for the monochromator used by Lundegårdh, and this type of apparatus is probably the most widely used today. But for the outbreak of war in 1939 this simple method would certainly have been rapidly adopted by clinical chemists, but it was not until the work of Barnes and associates (B1) and Domingo and Klyne (D2) after earlier work by Ells and Marshall (E2), Ells (E1), Griggs *et al.* (G2), and Cholak and Hubbard (C2) that the method became widely adopted.

In modern laboratories sodium and potassium are almost exclusively determined by flame photometry and it seems likely that the same will shortly become true of calcium and magnesium and possibly of iron, copper, chromium, manganese, cobalt, lead, and zinc.

2. The Flame Photometer

The flame photometer consists essentially of an atomizer, a burner, some means of isolating the desired part of the spectrum, a photosensitive detector, sometimes an amplifier and, finally, a method of presenting the desired emission, whether by galvanometer, null meter, or chart recorder.

2.1. The Atomizer

Two main types are in common use: (*a*) an atomizer which produces an aerosol which passes through a spray chamber before reaching the flame (Fig. 1); (*b*) an atomizer which sprays directly into the flame. This type is sometimes an integral part of the burner, as with the Beckman or Zeiss atomizer-burner (Fig. 2). Atomizers of type (*a*) are usually

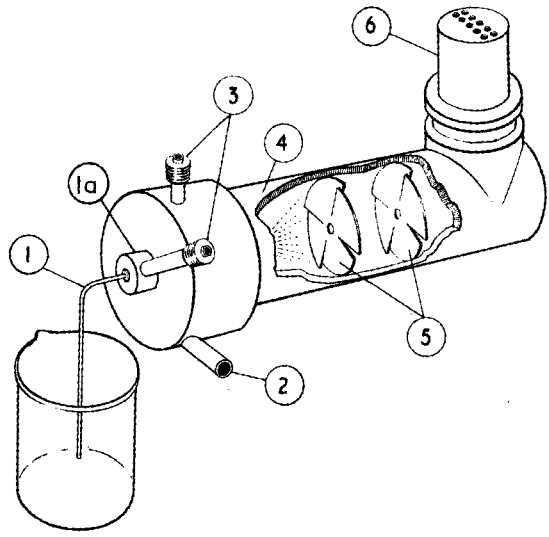

FIG. 1. An atomizer of the aerosol-producing type; aerosol passes through a spray chamber before reaching the flame. (1) Inlet capillary; (1a) metal atomizer; (2) drainpipe; (3) fuel and air inlets; (4) spray chamber; (5) baffles; (6) burner. (By permission of Evans Electroselenium Ltd., Halstead, Essex, England.)

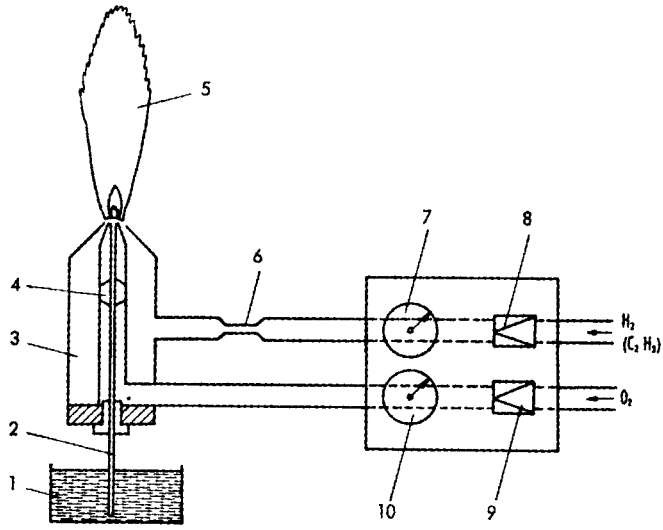

FIG. 2. Schematic diagram of burner of Zeiss flame spectrophotometer. (1) Sample; (2) cannula; (3) fuel gas inlet pipe; (4) guide piece for cannula; (5) flame; (6) throttling device for regulation of pressure indicator; (7) pressure gage; (8 and 9) pressure regulators; (10) pressure gage. (By permission Carl Zeiss, Germany, through Degenhardt & Co. Ltd., London, W.1.)

concentric, as shown in Fig. 1, but are sometimes constructed of two capillaries at right angles (Fig. 3). The spray chamber-atomizer is generally employed with simpler instruments and cooler flames, while the integral atomizer-burner is usually used when hotter flames such as oxyacetylene are burned.

In the author's opinion the integral type is much superior for most purposes. Unlike the spray chamber atomizer, the integral type reaches equilibrium almost instantly when solutions are sprayed and, if correctly designed, is extremely robust in use. A further important distinction is

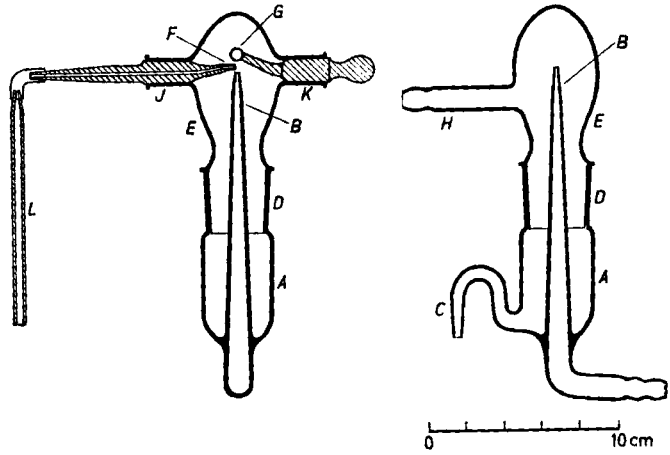

FIG. 3. Atomizer constructed from two capillaries at right angles. Atomizer (two vertical cross sections at right angles): (*A*) lower part carrying air inlet (*B*) and drain for waste (*C*). (*A*) is joined by the ground glass joint (*D*) to the upper part (*E*), which carries the solution inlet (*F*), the baffle plate (*G*), and the outlet to the burner (*H*). (*F*) and (*G*) are mounted on ground glass joints (*J*, *K*). (*F*) is joined by a piece of narrow rubber tubing to the vertical tube (*L*) which dips into the solution to be analyzed. (By permission of Domingo and Klyne (D2).)

that organic solvents can be used only in the integral atomizer without difficulty and without producing an unstable flame. On the other hand, flames cooler than air-hydrogen are not usable with type (*b*), while the spray chamber-atomizer can be used with any flame.

2.2. THE BURNER

As will have been gathered from the preceding description, two main types of burners are employed. The Meker type burner is most often used for cooler flames. In this type the flame gases are mixed inside the burner tube and are prevented from striking back by a grid at the mouth of the tube. Different grids are employed for different gas mixtures, but

the orifices in the grid for the hotter flames are generally inconveniently small so that frequent cleaning may be necessary; the integral atomizer-burner has already been mentioned.

2.3. The Optical System

Flame photometers can be divided into two groups on the basis of their optical systems. In group 1, the required part of the spectrum is selected by means of absorption or interference filters. These instruments are suitable only for the determination of sodium and potassium in biological fluids, whatever the claims of manufacturers. In group 2, emissions are isolated by means of a prism or diffraction-grating monochromator. When used with a higher temperature flame, such as oxy-acetylene, instruments of this type are capable of determining sodium, potassium, calcium, and magnesium in all biological fluids and tissues; they are also capable of determining many other elements of biological importance, such as iron, manganese, and cobalt, after a suitable preliminary extraction into organic solvents.

2.4. Photosensitive Detectors

Three types are used. (*1*) *Barrier-layer cells.* These are satisfactory only for simple filter instruments. (*2*) *Vacuum phototubes.* These tubes require an external power supply, unlike barrier-layer cells, and their output is usually amplified before measurement. (*3*) *Photomultiplier tubes* are easily the most satisfactory detectors for use in flame photometry. The photocurrent is amplified inside the tube in such a way that much lower light levels can be detected and measured accurately than is possible with vacuum phototubes with amplifiers. A stable source of high voltage up to perhaps 2000 volts is required to operate the photomultiplier tubes, but these tubes are almost universally used in high-performance instruments and are essential if the advantages of using narrow band width are to be obtained.

2.5. Measurement of Emission

The emission of the selected element may be presented in several ways. Easily the most satisfactory is a direct-reading galvanometer. While most simple instruments use this method, some of the more expensive instruments use a nullpoint method. In the author's opinion this latter method of measuring emissions is less suitable for the best flame photometric work.

For more complete and advanced studies, recording devices which

record the emission while the wavelength is continuously varied give much more information than is easily obtained by other means.

2.6 Internal Standard Instruments

Internal Standard Instruments. In some instruments a double-beam principle is employed. An internal standard element, such as lithium, is added to a constant concentration to all unknown and standard solutions. Dual optical paths are employed and the internal standard emission, after

Fig. 4. A flame photometer with double monochromator. (By permission of Carl Zeiss, Germany, through Degenhardt, London, W.1.)

isolation by a suitable optical system, is focused on a photocell. The current produced from this cell is opposed to the current from a separate photocell which responds to the isolated emission from the element being measured. The opposing currents are balanced by means of an accurate potentiometer, the potentiometer readings to produce balance when known solutions are sprayed is noted, and the unknown concentrations are deduced. This method is effective in eliminating or minimizing spray interference (see below) but is incompletely effective in the other types of interference met with in biological samples. Unfortunately, it is just these types of interference which occur most often and which present

the greatest problems. In the author's opinion the extra cost entailed in the provision of internal standard operation would be better expended by providing improved optical resolution and more sensitive photocells.

2.7 Commercial Flame Photometers

There are many excellent commercial instruments on the market, and it should be fairly simple to select a suitable model from the considerations mentioned in the preceding section.

When the requirement is for many routine analyses of sodium and potassium, a simple filter flame photometer burning a low temperature flame should be purchased. Many such models are on the market. On the other hand, if analysis for calcium and magnesium in biological fluids is also required, then only a fairly complex instrument with monochromator, photomultiplier, and high-temperature flame is satisfactory (Fig. 4). Compromise instruments between these two extremes lose the simplicity of the first type without gaining the versatility of the second.

3. Fundamental Principles

3.1. The Flame

A thorough study of flames is described by Gaydon and Wolfhard (G1); for practical analytical purposes their most important characteristic is their temperature. This is because the proportion of atoms which are excited in the flame depends critically upon the flame temperature as well as on the characteristics of the element. Table 1 lists the temperatures of flames which have been used in flame photometry. When an integral atomizer-burner is used, aspiration of an aqueous solution results in some lowering of flame temperature, perhaps by as much as

TABLE 1
Flame Temperatures

Fuel-oxidant mixture	Temperature (°C)
Coal gas-air	1840
Propane-air	1925
Butane-air	1930
Acetylene-air	2050
Hydrogen-air	2115
Hydrogen-oxygen	2690
Acetylene-oxygen	3110
Hydrogen-perchloryl fluoride	3300
Hydrogen-fluorine	4000
Cyanogen-oxygen	4850

200° in the case of an acetylene-oxygen flame. In cooler flames or with the spray chamber-atomizer, cooling is probably much less. One of the cooler flames is optimum for the determination of sodium and potassium; where calcium and magnesium or other biological elements are also to be determined then acetylene-oxygen is the flame of choice.

3.2. Emission Spectra

When salt in solution is sprayed into a flame a whole complex series of reactions rapidly ensues: the water envelope of the droplet is evaporated, and the resulting solid salt particle is heated and then vaporized into gaseous salt molecules. These molecules are then dissociated by the heat of the flame into free atoms; some of these atoms recombine reversibly with other components of the flame, such as OH, and still others lose an electron and become ionized. In this mixed flame population of molecules, atoms and ions, each may absorb energy from the flame and pass to a higher or excited state. This absorbed energy may later be released as light when it forms the characteristic emission spectrum of the element. When an atom absorbs energy from the flame and becomes excited, one of its outer valence electrons is displaced further from the nucleus to a position of higher energy. According to modern theory, energy can only be absorbed by the atom in discrete amounts, so that only certain definite higher energy states can occur. The atom cannot exist in a state of energy intermediate between the ground state and the next higher energy level. The difference between these two energy levels in each atom is usually expressed in electron volts (an electron volt $= 1.6 \times 10^{-12}$ erg) and is defined as the excitation potential. This absorbed energy may be emitted as light and from the relation

$$E_f - E_0 = h\nu$$

where E_f = energy of higher level; E_0 = energy of lower level; h = Planck's constant, and ν = frequency of radiation, it follows that each transition is associated with the emission of light of a definite wavelength. It is also apparent from this equation that higher energy transitions will result in emissions at the blue and ultraviolet end of the spectrum, while lower energy transitions will occur at the longer wavelengths. Molecules and ions may also absorb energy from the flame, and in the case of ions energy transitions will, like atomic transitions, result in emission concentrated over a very narrow band width. In the case of molecules, however, the changes in electronic energy of the constituent atoms are usually accompanied by changes in the energy of vibration of the constituent atoms or of the rotation of the whole molecule. These

energy changes in vibration-rotation may also result in emission of light, and the sum of these emissions is a broad band extending through a considerable spectral range.

There exists an upper limit of energy for each atom, and when this is reached the atom becomes ionized; the energy required for ionization is known as the ionization potential. With the cooler flames only a very small proportion of atoms in the flame is ionized, but in the hotter flames this is not true, and in some cases ions may constitute the greater proportion of the element in the flame.

Whether one considers atoms or ions, the proportion of atoms which is in the excited state is extremely small. In the emission of a spectral line due to the transition from an excited state j of excitation energy E_j to a ground state of energy $E_0 = 0$, if P_j and P_0 are the statistical weights for the excited and ground states, respectively, the number of atoms in the excited state N_j is related to the number of atoms in the ground state N_0 by the relation

$$N_j = N_0 P_j/P_0 \exp(-E_j/kT)$$

(W1). Table 2 is taken from Walsh's paper and shows the small proportion of atoms in the excited state for various elements in transitions to the ground state (resonance lines). This table shows the dependence of the number of atoms in the excited state, and therefore of the emission and sensitivity of the method, on the flame temperature.

3.3. Absorption Spectra

Walsh (W1) pointed out that if one could measure some function of the unexcited atoms in the flame, rather than of the much smaller proportion of excited atoms as in emission flame spectrophotometry, one should have a potentially sensitive method. He showed that this was possible if light of the appropriate wavelength were directed through a flame and onto a photocell, after passing through a monochromator. Atoms in the ground state in the flame absorb light of the same wavelength as is emitted in transitions *to* the ground state, and the proportion of light absorbed will bear a relationship to the atoms in the ground state and therefore, except where ionization is appreciable, to the total number of atoms in the flame and hence to the total concentration of the element in solution. Light of the appropriate wavelength is obtained from a hollow-cathode tube, and the absorption of this wavelength is measured using an apparatus of the type shown in Fig. 5. Any light emitted at this same wavelength by the excited atoms in the flame can be eliminated by modulating the monochromatic light source and arranging that only

TABLE 2

VALUES OF N_j/N_o FOR VARIOUS RESONANCE LINES[a]

Resonance line	P_j/P_o	N_j/N_o			
		T = 2000°K	T = 3000°K	T = 4000°K	T = 5000°K
Ca 8521 Å	2	4.44×10^{-4}	7.24×10^{-3}	2.98×10^{-2}	6.82×10^{-2}
Na 5890 Å	2	9.86×10^{-6}	5.88×10^{-4}	4.44×10^{-3}	1.51×10^{-2}
Ca 4227 Å	3	1.21×10^{-7}	3.69×10^{-5}	6.03×10^{-4}	3.33×10^{-3}
Zn 2139 Å	3	7.29×10^{-15}	5.58×10^{-10}	1.48×10^{-7}	4.32×10^{-6}

[a] By permission from Walsh (W1).

light modulated at this frequency will be detected by the recorder. The absorption recorded is much less dependent on temperature than is emission because the Doppler broadening which decreases peak absorption increases only as $T^{1/2}$

$$[\text{cf. } N_j/N_0 = P_j/P_0 \exp(-E_j/kT)].$$

This is a most elegant and attractive method, which is likely to be used increasingly in flame photometric analysis. As will be discussed later, interference effects are not inherently absent with this method; anionic interference is equally or more troublesome with this method than with emission flame work, while cationic interference is less prominent, not because of the inherent nature of the method, but because of the lower flame temperatures usually used with this method. Its attraction is that it has some claims to be an absolute method in certain cir-

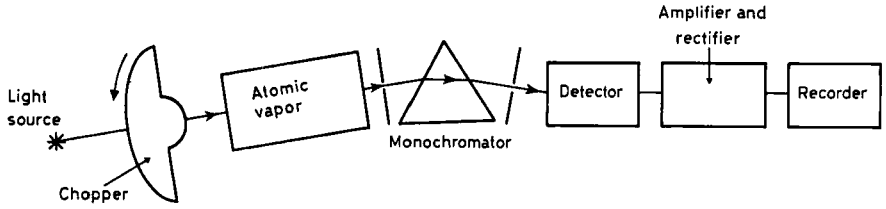

FIG. 5. Apparatus for measuring atomic absorption. (By permission of Russell *et al.* (R1, p. 318).)

cumstances, but its great practical advantage is in its ability to measure elements whose excitation potential is too high (i.e., 5.5 ev or above) for effective measurement in emission work even using an oxygen-acetylene flame with an organic solvent. Thus elements such as zinc and cadmium, with resonance lines in the extreme ultraviolet (and therefore involving high-energy transitions) have the same order of sensitivity as sodium with this method, although the excitation potential of the sodium line at 589 is much lower (2.1 ev). This is in marked contrast with emission spectra, where zinc is almost impossible to measure.

However, the method is so far in a relatively undeveloped stage, and in the author's opinion the best instrument for exploitation of this principle has still to be designed. Further research into the use of this method with hotter flames seems desirable; most applications so far described have depended on Meker-type burners with a spray chamber atomizer with its attendant disadvantages. The method should be equally applicable to the integral atomizer-burner, and it seems possible that a monochromator instrument of this type using either absorption or

emission, whenever appropriate, would allow the use of these two principles in a complementary manner.

3.4. Organic Solvents

Organic solvents give a greatly increased sensitivity in emission work and are most successfully used with an integral type of atomizer. The organic solvents exert their enhancing effect either in aqueous-organic solvent mixtures or still more when the organic solvent is used in 100 % concentration. While several factors are of importance, the main reason for this increased sensitivity is the increased flame temperature possible with organic solvents, so that temperatures 200–300° higher than is possible with aqueous solvents can be attained. The exponential dependence of sensitivity in emission work on flame temperature makes this increased flame temperature very significant. This subject has been most fully studied by Dean (D1). Table 3 records the enhancement possible when organic solvents are used in place of aqueous solutions. By dissolving a suitable complexing agent in an organic solvent, for example, 4-methyl-2-pentanone, many elements can be extracted directly into the organic solvent, which is then directly sprayed into the flame. This procedure is

TABLE 3
Emission Intensity of Certain Elements in Water and Organic Solvents[a]

Element	Wavelength (mμ)	Excitation potential (ev)	Sensitivity (μg/ml)/(% T)		Enhancement
			Water	Organic	
Al	396.2	3.14	67.0	0.5[b]	134-fold
Cr	425.4	2.91	5.0	0.1[b]	50
Cu	324.7	3.81	0.6	0.06[c]	10
Fe	372.0	3.3	2.5	0.125[b]	20
LaO	560	2.21	0.5	0.05[b]	10
Pb	405.8	4.37	14.0	1.3[d]	11
Li	323.3	3.83	46.0	2.08[b]	22
	460.3	4.52	12.5	0.7[b]	18
	610.4	3.87	4.4	0.6[b]	8
	670.8	1.85	0.067	0.007[b]	9
Mg	285.2	4.34	1.0	0.1[b]	10
Mn	403.4	3.08	0.20	0.018[b]	11
Na	330.2	3.75	12.5	0.9[b]	14
	818	3.61	1500	125[b]	12

[a] By permission from Dean (D1, p. 63).
[b] 4-Methyl-2-pentanone.
[c] Chloroform.
[d] Gasoline.

an extremely powerful method which will certainly be more widely used in the future; determinations of very small quantities can be made by this means of iron, chromium, aluminium, lanthanum, magnesium, copper, yttrium, nickel, manganese, and others. This type of procedure is the method of choice at the moment for trace analysis because the sensitivities obtained are rarely exceeded by the absorption method; the latter is somewhat less convenient in use where many elements are to be determined. This situation will probably change when new instruments are developed which allow the use of either absorption or emission and either aqueous or organic solvent, as seems appropriate.

3.5. Interference

An interferent is a substance or factor which distorts the relationship in pure solution and low concentration between salt concentration and emission. Interferents can be classified as follows.

3.5.1. *Spectral Interference*

(*a*) *Background interference.* All metals in large amounts give rise to a continuous spectrum. Thus, sodium radiates from 360 to 602 mμ, potassium from 340 to 570 mμ, and lithium from 320 to 460 mμ (D1). This type of interference can be minimized by using more selective filters or narrower monochromator band widths. In extreme cases, chemical separation may be required.

(*b*) *Overlapping or adjacent emissions.* When the emissions of elements present in solution are closer together than can be separated by the optical system used, again the only effective remedy is to use more selective filters, or narrower band widths. Thus, the manganese emission at 403 mμ is difficult to separate from the potassium emission at 404 mμ, while the magnesium emission at 285.2 mμ cannot readily be separated from the sodium emission at 285.3 mμ, save with very high resolutions. In some cases the emissions actually overlap, so that complete separation is impossible whatever the optical means employed. Thus, calcium, which has band systems of CaOH in the green and red portions of the spectrum, will give rise to severe interference with sodium when calcium is present in excess because the CaOH molecules also emit at the sodium wavelength. In such cases chemical separation is the only solution, although interference can sometimes be minimized by choosing flame conditions least suitable to excitation of the interferent. For example, in a cool flame calcium in excess will interfere less with sodium than in a hotter flame. It is sometimes also possible to minimize interference of this nature without chemical separation by

adding substances which will depress the emission of interferent. In the example discussed here, calcium interference with sodium can be minimized by adding excess of phosphate or aluminium, with which calcium forms difficultly vaporizable compounds.

3.5.2. *Cationic Interference*

The ionization of an element in the flame can be considered as a dissociation process:

$$A = A^+ + e^-$$

where A is an atom in the flame, A^+ its positive ion and e^- a free electron. This reaction has an equilibrium constant K:

$$K = \frac{[A+]\,[e-]}{[A]}$$

Ionization in various flames for alkali and alkaline earth metals is shown in Table 4 (a and b). As the dissociation of an atom into ion and electron has a dissociation constant, it follows that increase in the flame content of electrons will depress the ionization. This is accomplished when another element, also capable of ionization in a flame, is present in solution. This depression of ionization will lead to an increase in the number of atoms in the ground state, with a consequent increase in the number of excited atoms. The resultant increase in emission intensity of the element being measured is referred to as cationic interference or enhancement. Since this phenomenon depends on ionization, it will not occur in cooler flames or with elements of high ionization potential. Thus, sodium and potassium which have fairly low ionization potentials may be determined together without cationic interference in the coal gas-air flame, although in the air-acetylene flame the presence of sodium will enhance potassium emission and vice versa (Figs. 6 and 7). This type of interference is quite common and is to be distinguished from spectral interference. The effects can be readily minimized by adding excess of interferent, so that ionization is fully depressed and independent of any further variation in interferent concentration; or when convenient, the interference can be entirely removed by a suitable selection of flame temperature. Thus, sodium and potassium cause mutual cationic interference in air-acetylene but not in coal gas-air flames, while sodium markedly enhances calcium emission in an oxygen-acetylene flame but to a small or negligible extent in an air-acetylene or oxygen-hydrogen flame. This type of interference has been claimed to be absent from atomic absorption spectrophotometry, but this is rather a reflection of the fact that the absorption method has been used with elements with higher ionization potentials in relatively cool flames rather than to an

inherent characteristic of the method. One can predict that if the absorption method is used in a flame such as oxygen-hydrogen with either rubidium or potassium, then enhancement effects should readily be observed. A further method of overcoming this type of interference is by adding an excess of an easily ionizable element such as cesium; this is effective whether the interfering element is cesium or some other element of low ionization potential.

TABLE 4a

PER CENT IONIZATION OF ALKALI AND ALKALINE EARTH METALS IN FLAMES[a]

Element	Ionization potential (ev)	Air-propane	Hydrogen-oxygen	Acetylene-oxygen
Lithium	5.37	< 0.01	0.9	16.1
Sodium	5.12	0.3	5.0	26.4
Potassium	4.32	2.5	31.9	82.1
Rubidium	4.16	13.5	44.4	89.6
Cesium	3.87	28.3	69.6	96.4
Calcium	6.11	< 0.01	1.0	7.3
Strontium	5.69	< 0.1	2.7	17.2
Barium	5.21	1.0	8.6	42.8

[a] By permission from Dean (D1, p. 42).

From W. H. Foster, Jr., Ph.D. Thesis, Massachusetts Institute of Technology, May, 1959.

3.5.3. *Anionic Interference*

This is the most persistent and difficult type of interference met with in flame photometry. It is due to the formation of difficultly vaporizable compounds of certain metals with certain anions. Thus, calcium emission is depressed by the presence of phosphate, sulfate, and dichromate. Aluminate anions, silicate, iron, and beryllium also cause depression of calcium emission. The mechanism with all these substances is the formation of a compound of the type Ca-I-O of high melting point and low volatility (where I is interferent). Unlike cationic interference, which is most marked in the hotter flames, this type of interference is most troublesome in cooler flames. It is equally troublesome in emission or absorption work. This type of interference can be overcome either by adding excess of the interfering ion, such as phosphate (M1), or by adding a substance which complexes with the metal being determined and prevents the formation of the refractory compounds. Ethylenediamine tetraacetate has been successfully used in this way by Willis (W2). In biological materials this type of interference is unimportant with sodium and potassium, very marked with calcium, and slight or

TABLE 4b

FRACTION OF METALS IONIZED[a]

Metal	Ionization potential	Pressure[b] (atm)	Temperature 1500°K	2000°K	2500°K	3000°K	3500°K
Potassium	4.32	10^{-2}	2.7×10^{-6}	2.5×10^{-4}	4.7×10^{-3}	0.028	0.11
		10^{-4}	2.7×10^{-5}	2.5×10^{-3}	0.047	0.27	0.75
		10^{-6}	2.7×10^{-4}	0.025	0.42	0.94	0.99
Sodium	5.12	10^{-2}	1.2×10^{-7}	2.7×10^{-5}	7×10^{-4}	6.2×10^{-3}	0.031
		10^{-4}	1.2×10^{-6}	2.7×10^{-4}	7×10^{-3}	0.062	0.30
		10^{-6}	1.2×10^{-5}	2.7×10^{-3}	0.07	0.53	0.95
Calcium	6.11	10^{-4}	5.5×10^{-8}	3.0×10^{-5}	1.4×10^{-3}	0.019	0.12
		10^{-6}	5.5×10^{-7}	3.0×10^{-4}	0.014	0.19	0.77

[a] By permission of Gaydon and Wolfhard (G1, p. 283).

[b] This is the flame metal content expressed as a partial pressure.

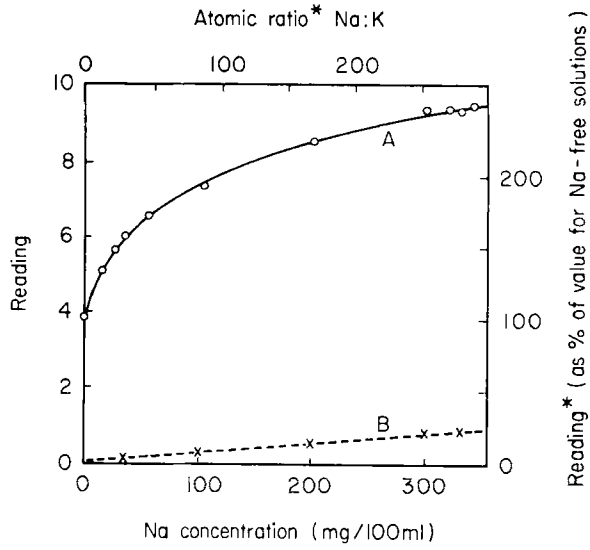

FIG. 6. Flame photometer readings for potassium; effect of sodium. Curve *A*, solutions containing 2 mg K/100 ml (as KCl) + Na (as NaCl), as shown by abscissas; Curve *B*, solutions containing no K, but Na, as shown by abscissas. Asterisks denote ordinate and abscissa for curve *A* only. (By permission of Domingo and Klyne (D2).)

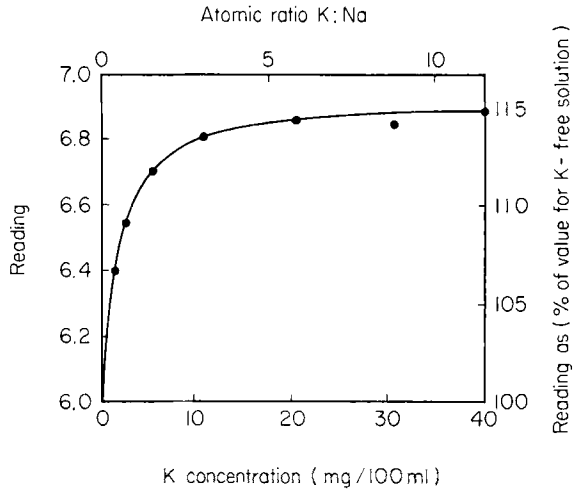

FIG. 7. Flame photometer readings for sodium; effect of added potassium. In all solutions 2 mg Na/100 ml (as NaCl); K (as KCl) added as shown by abscissas. (By permission of Domingo and Klyne (D2).)

absent with magnesium. Phosphate does not depress magnesium emission provided a hot flame such as oxygen-acetylene is used (A1). It should be noted that it is often impossible to select a flame which will minimize anion interference without incurring the risk of cationic enhancement.

3.5.4. *Spray Interference*

If there is a difference in physical properties between the test solution and the standards it is likely that erroneous results will be obtained. This is quite well recognized and can be readily overcome. Together with variation in flame characteristics between test and standard reading, this is the only type of interference in which a lithium internal standard is really effective.

3.5.5. *Self-absorption*

This refers to the failure of emission to increase in proportion to solution concentration above a certain limiting concentration. It is due to the absorption by atoms in the ground state in the cooler outer part of the flame of light emitted by excited atoms in the central hotter part of the flame. This effect is generally seen only with transitions from the ground state, and higher level transitions ending in a state above the ground state do not suffer from this type of interference. This is because in the outer part of the flame only atoms in the ground state are present and higher energy states do not occur.

This type of interference is not troublesome with sodium, potassium, or calcium but does occur with magnesium in the oxygen-acetylene flame at concentrations above 0.1 meq/liter. Error is avoided by preparing standards sufficiently close together to eliminate any error by interpolating readings, by working at concentrations at which self-absorption does not occur, or by using emissions involving higher energy level transitions.

4. Practical Applications

The determination of sodium and potassium in biological fluids and tissues is so widely practiced that no detailed discussion is necessary. A simple filter instrument with absorption or interference filters is perfectly adequate for most purposes. However, the determination of calcium and magnesium in biological fluids and tissues has proved more difficult and merits further discussion.

4.1. Calcium

Calcium has a higher excitation potential than sodium and potassium (Table 5), and consequently a higher flame temperature is necessary for its determination. In addition, anionic interference is particularly trouble-

TABLE 5

DETAILED FLAME SPECTRA[a]

Element[b]	Wavelength[c] (mμ)	Emitter[d]	Excitation potential (ev)
Na	285.3*r*	I	4.06
(5.14;33.3)	330.2*r*	I	3.75
	330.3*r*	I	3.75
	568.3	I	4.03
	568.8	I	4.03
	589.0*r*, *x*	I	2.10
	589.5*r*, *x*	I	2.10
	818.3	I	3.61
	819.5	I	3.61
K	344.6*r*	I	3.59
(4.34;20.6)	344.7*r*	I	3.59
	404.4*r*	I	3.06
	404.7*r*	I	3.06
	693.9	I	3.40
	696.4	I	3.40
	766.5*r*,*x*	I	1.61
	769.9*r*, *x*	I	1.61
Ca	422.7*r*, *x*	I	2.93
(6.11;3.12)	393.4*r*	II	3.15
	396.8*r*	II	3.12
	622	CaOH	1.97
	554	CaOH	
Mg	277.7	I	7.17
(7.64;4.42)	277.8	I	7.17
	278.0	I	7.17
	278.1	I	7.17
	278.3	I	7.17
	285.2*r*, *x*	I	4.34
	333.0	I	6.43
	333.2	I	6.43
	333.7	I	6.43
	382.9	I	5.85
	383.2	I	5.85
	383.8	I	5.85
	279.6*r*	II	4.43
	280.3*r*	II	4.42
	371	MgO	3.49
	383	MgO	3.38

[a] Reprinted from "Biochemists Handbook," 1961, edited by C. Long; by permission of the publishers, E. N. Spon & Co. Ltd., London.

[b] The ionization potential of the neutral atom followed by the excitation potential of the singly ionized atom (expressed in ev) are given in parentheses.

[c] *r* represents a transition to ground state. The most suitable wavelengths for analysis are given as *x*.

[d] I represents the neutral atom, II the singly ionized atom.

some in biological fluids from the presence of phosphate. The preponderance of sodium with its much greater emission intensity makes it quite unsound to use the CaOH band emissions in the green or red portion of the spectrum if one wishes the method to be applicable to urine as well as to plasma. The constant amount of sodium in plasma makes it possible to use for plasma analysis the band emissions of calcium at the red end of the spectrum with a constant correction for the spectral interference from sodium. This interference cannot be overcome even with the use of a monochromator. However, it is much more satisfactory to measure the calcium emission in the blue at 422.7 mμ, using a monochromator instrument with photomultiplier and a flame not cooler than air-acetylene. At this wavelength sodium spectral interference is eliminated with a suitable band width (0.3 mμ or less, using an oxygen-acetylene flame and Zeiss integral atomizer-burner). Phosphate interference may be overcome either by addition of excess phosphate (M1) or by complexing with ethylenediamine tetraacetate (W2). Either the absorption (W2) or emission methods may be used; the emission method is perhaps more advantageous, as hotter flames with their lesser susceptibility to anionic interference are generally used with the latter method. With suitable precautions, calcium may be determined in all biological fluids and tissues with the same ease and accuracy as sodium and potassium.

4.2. Magnesium

The determination of magnesium has presented more difficulty than calcium and this for several reasons: the excitation potential of the magnesium line 285.2 mμ is 4.3 ev compared with values of 2.1, 1.6, and 2.9 for the emissions of sodium at 589 mμ, potassium at 766.5 mμ, and calcium at 422.7 mμ, respectively. For emission work it is therefore necessary to use a hot flame such as oxygen-acetylene to attain sufficient sensitivity. Further, even the emission obtained with an oxygen-acetylene flame is small and requires high sensitivity for its detection and measurement. In addition, a monochromator of very high resolution is necessary if even partial separation of the emission of magnesium at 285.2 mμ is to be achieved from the adjacent sodium emission at 285.3 mμ (Table 6). This severe spectral interference is commonly neglected, but it can be overcome by the use of sufficiently narrow band width (approximately 0.04 mμ using the Zeiss burner and oxygen-acetylene flame with double monochromator MM 12). Anionic interference is not troublesome with magnesium using high-temperature flames, and Alcock *et al.* (A1) described methods suitable for the determination of magnesium in all biological fluids and tissues.

TABLE 6

FLAME LINES AND BANDS OF ANALYTICAL IMPORTANCE[a,b]

Wavelength (mμ)	Element	Wavelength (mμ)	Element	Wavelength (mμ)	Element
213.9	Zn	374.8	Fe	484*b*	Al
228.8	Cd	374.9	Fe	493.4	Ba
253.7	Hg	375.8	Fe	495*b*	B
285.2	Mg	377.6	Tl	497*b*	Ti
285.3	Na	378.6	Ru	500*b*	Zn
303.4	Sn	383*b*	Mg	510*b*	Be
307.6	Zn	385.6	Fe	518*b*	Ti
317.5	Sn	386.0	Fe	520.5	Cr
324.8	Cu	387.3	Co	520.6	Cr
326.1	Cd	387.4	Co	520.8	Cr
327.4	Cu	396.2	Al	521*b*	B
328.1	Ag	403.3	Ga	535.0	Tl
330.2	Na	403.5	Mn	540*b*	Mo
330.3	Na	404.4	K	548*b*	B
338.3	Ag	404.7	K	550*b*	U
340.5	Pd	405.8	Pb	552*b*	Dy
341.2	Co	407.8	Sr	553.6	Ba
341.5	Ni	410.2	In	554*b*	Ca
343.5	Rh	417.2	Ga	560*b*	La
344.6	K	420.2	Rb	562*b*	Pr
344.7	K	421.6	Sr	565*b*	Tb
349*b*	Sn	422.7	Ca	570*b*	Gd
350.2	Co	425.4	Cr	570*b*	Dy
350.3	Rh	427.5	Cr	571*b*	Pr
351.5	Ni	429.0	Cr	576*b*	V
352.4	Ni	430.4	Nd	589.0	Na
352.5	Ni	438*b*	La	589.5	Na
353.0	Co	442*b*	La	600*b*	Mo
360.5	Cr	444*b*	Y	600*b*	Tb
361.0	Pd	450*b*	Nb	622*b*	Ca
363.5	Pd	450*b*	Gd	653*b*	Sm
364*b*	Te	451.1	In	660*b*	Nd
368.4	Pb	455.4	Ba	670.8	Li
369.2	Rh	455.5	Cs	681*b*	Sr
371*b*	Mg	460.7	Sr	715*b*	Ti
372*b*	Te	460.9	Sc	766.5	K
372.0	Fe	462*b*	Gd	769.9	K
372.3	Fe	462*b*	Nb	780.0	Rb
372.8	Ru	466.2	Eu	794.8	Rb
373.3	Fe	467*b*	Al	818.3	Na
373.5	Fe	471*b*	Be	819.5	Na
373.7	Fe	472.3	Bi	852.1	Cs
374.3	Fe	481*b*	Ce	873*b*	Ba
374.6	Fe	483*b*	Y	894.3	Cs

[a] The emissions are arranged in order of wavelength. Inclusion does not necessarily mean that the emission is suitable for quantitative measurement of the element concerned. Band emissions are given at the most sensitive wavelength and are marked *b*.

[b] Reprinted from "Biochemists Handbook," 1961, edited by C. Long; by permission of the publishers, E. N. Spon & Co. Ltd., London.

4.3. Determination of Magnesium and Calcium in Biological Fluids and Tissues

The methods in use in the author's laboratory for determination of calcium and magnesium in plasma and urine and in fecal and tissue ash are described below in detail. Willis (W2) has shown that absorption methods are also suitable. The methods described here have proved reliable in practice over several years of intensive use.

A Zeiss spectrophotometer PMQ II with flame attachment is used with double quartz monochromator MM 12 replacing the single monochromator normally used with the instrument. The burner is modified slightly by enlarging the fuel inlet to 1.3 mm. An oxygen-acetylene flame is used for both magnesium and calcium. The wavelength settings are 285.2 and 422.7, while the acetylene pressures are approximately 200 mm and 150 mm of water for magnesium and calcium, respectively. Slit widths of 0.02 mm are used in each case, and the instrument is operated at full sensitivity.

The point of peak emission is accurately located by rotating the wavelength selector while spraying a suitable standard solution before analysis for each element. The instrument is set to zero with the flame off but with full sensitivity turned on. The flame background reading is recorded after turning on the flame and while deionized water is sprayed; the deflection of a 14 m*M* NaCl solution is next recorded followed by readings for one of the working standard solutions (0.20 meq/liter for Mg and 0.50 meq/liter for Ca). Conditions are satisfactory when the ratio of emission due to working standard to emission due to flame background is 1.0 or more for Mg and 5.0 or more for Ca. The deflection due to 14 m*M* NaCl should not exceed 2% of the 0.2 meq/liter standard for Mg nor 1% of the 0.5 meq/liter standard for Ca.

Stock Solutions

All solutions are made with deionized distilled water and are prepared from AnalaR chemicals (British Drug Houses, Poole, England) with the exception of the magnesium and calcium stock solutions. The calcium solutions are made from "Specpure" calcium carbonate (Johnson, Matthey & Co. Ltd., London) dissolved in the minimum volume of concentrated HCl. The magnesium stock solution is prepared from "Specpure" magnesium sulfate. Polythene containers are used to store all solutions.

The following solutions are used:

Magnesium Stock Solution I. A solution of 25 m*M* $MgSO_4 \cdot 7H_2O$, the concentration of which is checked by titration with disodium ethylene-

diamine tetraacetate which has been standardized with calcium stock solution and with standard zinc acetate solution.

Magnesium Solution II, 8 m*M* $MgCl_2$.

Mixed Salt Solution A, 30 m*M* KCl, 5 m*M* K_2SO_4, 1.4 *M* NaCl, 50 m*M* KH_2PO_4.

Mixed Salt Solution B, 50 m*M* KCl, 5m*M* K_2SO_4, 50 m*M* NaCl, 150 m*M* KH_2PO_4.

Calcium Stock Solution, 25 m*M* $CaCl_2$. This is prepared from "Specpure" calcium carbonate in appropriate amount by dissolution in HCl.

Perchloric Acid, $HClO_4$, 60% (w/w).

Hydrochloric Acid, HCl (sp. gr. 1.18).

Phosphoric Acid, H_3PO_4 (sp. gr. 1.75).

Phosphate Solution, 44.4 m*M* KH_2PO_4.

Sodium Solutions, 200 m*M* NaCl; 14 m*M* NaCl.

Magnesium Standards for Plasma, Urine, and Feces. Solutions are prepared by adding 10 ml mixed salt solution A, 10 ml calcium solution, and an appropriate volume of magnesium solution I to 700 ml water in a liter flask. Perchloric acid (50 ml) is now added, and the volume is made up to 1 liter. A range of standards at intervals of 0.05 m*M* is prepared from 0.05 to 0.40 m*M* with respect to magnesium concentration.

Calcium Standards for Plasma, Urine, and Feces. Ten milliliters of mixed salt solution A, 10 ml magnesium solution II, and an appropriate volume of calcium solution are added to 700 ml water in a liter flask. Perchloric acid (50 ml) is now added, and the volume is made to 1 liter. A range of standards at intervals of 0.05 m*M* is prepared from 0.05 to 0.5 m*M* with respect to calcium concentration.

Magnesium Standards for Soft Tissues. Standard solutions are prepared to cover the range 0.125–0.50 m*M* with respect to magnesium concentration at intervals of 0.125 m*M*. Ten milliliters of salt solution B, 2 ml calcium solution, and appropriate volume of magnesium solution I are added to approximately 700 ml water in a liter flask; HCl (100 ml) is added and the volume is made up to 1 liter.

Calcium Standards for Soft Tissues. Standard solutions are prepared to cover the range up to 0.10 m*M* with respect to calcium at intervals of 0.01 m*M*. Ten milliliters of salt solution B, 10 ml magnesium solution I, and an appropriate volume of calcium solution are added to approximately 700 ml water in a liter flask; HCl (100 ml) is added and the volume is made up to 1 liter.

Combined Diluting and Deproteinizing Fluids. (*1*) Phosphate solution (10 ml) is diluted to approximately 700 ml in a volumetric flask, mixed with 55.5 ml $HClO_4$, and made up to 1 liter. This solution is used for

plasma or serum. (*2*) Phosphate solution (10 ml) and sodium solution (25 ml 200 m*M* NaCl) are diluted to approximately 700 ml in a volumetric flask. Perchloric acid (55.5 ml) is added and the volume is made up to 1 liter. This solution is used for analysis of urine and fecal ash. (*3*) H_3PO_4 (1 ml) is added to HCl (100 ml) and the volume is made up to 1 liter. This solution is used for soft tissue analysis.

Procedure

The same dilution is used for analysis of both calcium and magnesium in plasma or serum, urine, tissue ash, and, where possible, in fecal ash. Occasionally a further dilution of the fecal ash solution may be appropriate where one element is in unusual concentration.

Readings are obtained for those standards just above and just below each unknown sample reading, and the results are calculated by interpolation.

The normal (95%) ranges for plasma or serum obtained with minimal stasis from fasting subjects is 1.5–1.8 meq/liter for magnesium and 4.9–5.5 meq/liter for calcium. If heparin is used for an anticoagulant, it should be purified by ion exchange resins, as samples of heparin are often contaminated with calcium.

All glassware should be specially acid-washed and finally rinsed in distilled water. All contact with filter paper or rubber stoppers should be avoided.

The precision of a single routine estimation of calcium or magnesium by this procedure is between 1 and 2%.

Plasma or Serum. One volume of plasma or serum is added drop by drop to 9 volumes of combined diluting and deproteinizing fluid (*1*). The tube is capped with "parafilm" (A. Gallenkamp & Co. Ltd., London, E. C. 2), and the contents are mixed by gently inverting the tube several times. It is then centrifuged, and the clear supernatant is used.

Urine. Urine is diluted 10- or 20-fold with diluting fluid (*2*). Twenty-four-hour urine specimens should be collected in a Winchester bottle containing 10 ml concentrated HCl in order to prevent precipitation of magnesium and calcium.

Feces. Specimens are homogenized, and a sample is dried at 105° for 16 hours. A known weight (approximately 1.0 g) is ashed in a platinum crucible at 400°. The ash is dissolved in *N*HCl (200 ml HCl/g dry feces). The solution is thoroughly shaken to ensure complete dissolution. The solution is further diluted as required with diluting fluid (*2*).

Soft Tissues. Soft tissues are dried to constant weight at 105° and then ground in an agate mortar with several changes of a mixture of equal

volumes of ethyl ether and light petroleum (b.p. 40–60°). Obvious tendon is removed in the case of muscle. The powder is placed in the oven at 105° for 12 hours. A known weight (approximately 20 mg) of the dry fat-free tissue powder is weighed into a platinum crucible and ashed at 400°. The ash is dissolved in diluting fluid (3) (4 ml/20 mg tissue powder). The ash solution is suitable for the determination of sodium and potassium by flame photometry as well as for calcium and magnesium.

Microanalysis. The methods described are directly applicable even when only a small volume of plasma or urine is available. Magnesium or calcium can easily be determined on 25 microliters of plasma with the same precision as in the macro-procedure. This small volume contains approximately 2.5 μg of calcium and 0.5 μg of magnesium.

Twenty-five microliters of plasma are pipetted into a microcentrifuge tube of approximately 0.4 ml capacity (Beckman Instruments, Inc., Spinco Division, Palo Alto, California). Then 250 μl of diluting fluid (1) are added and the tube centrifuged in a microcentrifuge (Beckman Instruments, Inc.). The supernatant fluid is analyzed by aspirating directly from the micro-tube above the packed protein precipitate. It is convenient to make an adaptor which will hold the small tube in the sample turntable (Fig. 4) in the correct position for spraying.

The procedure may be still further scaled down in the case of calcium. One or two microliters of plasma (0.1 or 0.2 μg of calcium) are pipetted into the micro-tube. Then 250 μl of diluting fluid (2) are added and after centrifugation the supernatant fluid is analyzed as described above.

5. Working Conditions

There are several essentials for the satisfactory operation of a flame photometer when many analyses are to be undertaken.

1. A period of at least several weeks must be allowed between the receipt of a flame photometer from the manufacturers and the undertaking of clinical analysis. This is an absolute essential if the analyst is to become thoroughly familiar with the working of his instrument.
2. The flame photometer must be carefully maintained. It is the author's experience that an integral burner atomizer should be thoroughly and carefully cleaned every day. In addition, one or two spare burners should be available. These should be ordered when the flame photometer is being purchased.
3. All reagents should be stored in polythene vessels.
4. An ample supply of deionized distilled water should be available.
5. Control solutions of composition unknown to the analyst should be

analyzed with every batch. Where the control solution gives a result outside of the chosen confidence limits, the analysis should be repeated and if the error persists the test solutions should not be reported and the analyses should be discarded until the cause of the error has been definitely proven.

6. For satisfactory flame analysis it is essential that the analyst or his supervisor should be conversant with the theoretical basis of the method. Rule-of-thumb working will sooner or later result in serious errors being made.

6. Conclusion

Relatively few reliable texts are available on the subject of flame photometry, but recently a comprehensive textbook (D1) has appeared. A full bibliography is given in this book and the whole subject is thoroughly covered. The reviewer has drawn heavily on this work in preparing this review. For more detailed theoretical considerations the reader should consult Mavrodineanu and Boiteux (M3).

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THE NONGLUCOSE MELLITURIAS

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1. Introduction

1.1. Scope and General Comments

This discussion of the more unusual types of melliturias will deal with the nonglucose melliturias exclusive of galactosemia. Because studies relating to the latter have become quite extensive and the interrelationships of galactose with other metabolic pathways are becoming more clearly delineated, separate treatment is required.

Knowledge of the existence of melliturias resulting from sugars other than glucose dates back many years, but only in recent years have reliable techniques become available for the detection and identification of simple sugars in biological material. This was especially true when

two or more sugars were present in the urine in varying amounts. With the application of paper chromatography to the separation of sugars, this technique was soon applied to urine, and has developed into the most useful method available (P1).

Several factors have impeded the progress of detection and study of patients with the rarer forms of mellituria besides the notorious difficulty and unreliability of the methods applicable to the identification of the sugars themselves. Nonglucose melliturias are relatively infrequent; many of the recognized melliturias are harmless and require no special management, while those with significant clinical symptoms have been so poorly understood in the past that rational therapy was not possible; finally, our knowledge of the normal metabolism of the various sugars is so new, that until recent years a study of the unusual melliturias could accomplish little more than classification; hence, the problem was not as intellectually challenging as it is today.

1.2. Sugars Which Have Been Identified in Urine

Several sugars are excreted by normal individuals in very low concentrations. In 18 normal adults, Tower *et al.* (T4) found glucose, xylose, arabinose, and ribose in all; lactose and galactose were found in some individuals, and the amounts were variable; glucuronic acid and deoxyribose could be identified occasionally; fructose could be identified only after the urine was concentrated. Futterman and Roe (F5) found xylulose and ribulose in human and rat urine which was 300 times concentrated. Bickel (B4), on the other hand, found much higher concentrations of sugars in the urine of infants, both premature and normal. The sugar concentration decreased to the adult level after about the tenth day of life. In this neonatal period lactose, pentose, glucose, and galactose are found in order of decreasing concentration.

Other sugars must be included when considering mellituria from all causes: maltose and sucrose. The structural formulas of these sugars are shown in Fig. 1.

1.3. Abnormal Melliturias

The term "abnormal mellituria" may connote either an unusually large quantity of a sugar normally present or the presence of one not ordinarily detectable in urine.

The melliturias are commonly subdivided into those resulting from ingestion of the particular sugar, described as "alimentary," and those which are "metabolic," of endogenous origin.

We shall group mellituria into four categories:

1. Urinary sugars found in normal individuals.
2. Mellituria secondary to hepatic or renal damage.
3. Mellituria resulting from abnormalities in the gastrointestinal tract.

In these, urinary sugar is usually found when the individual is on a

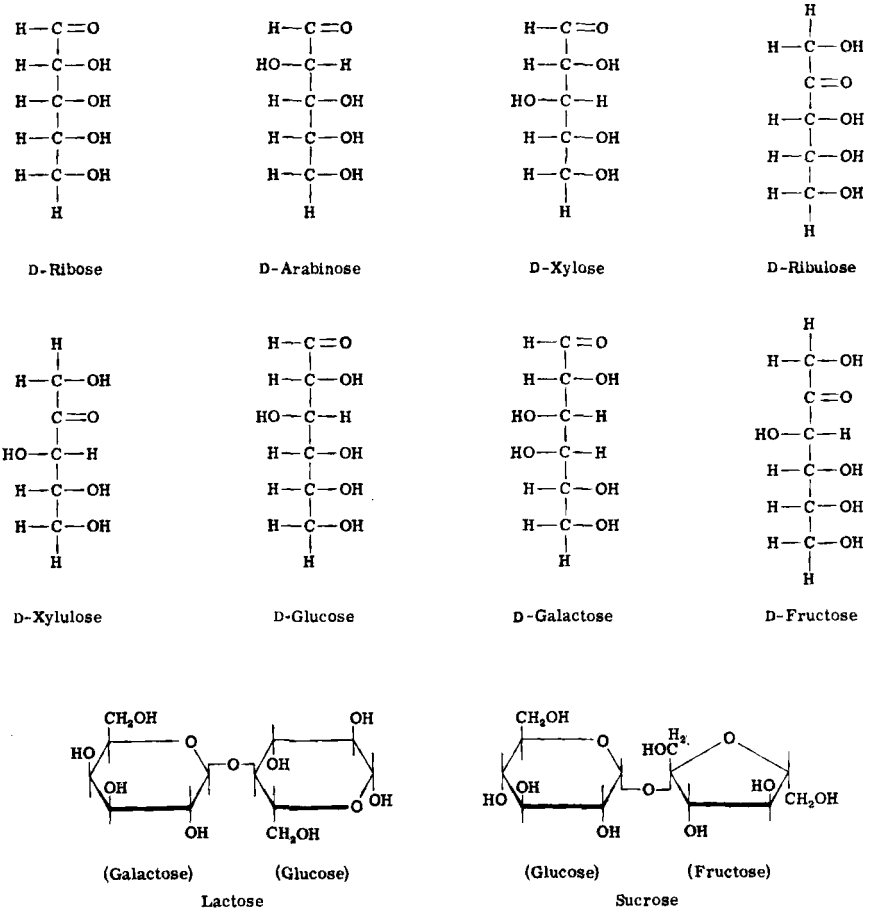

FIG. 1. Formulas of sugars which have been identified in the urine. The monosaccharides are represented in the straight chain form. The disaccharides are illustrated in the ring form. All sugars in solution are chiefly in the ring form.

normal diet or following a sugar-loading test. The mechanisms involved may reflect extremes of normal variation, deficiency of intestinal enzymes for hydrolyzing the sugar (if it is not a monosaccharide), or possibly abnormalities of the transport mechanism.

4. Heritable abnormalities resulting in a deficiency of an enzyme,

primarily in the liver, which is necessary for the normal metabolism of the sugar.

The melliturias of groups one and two are secondary, incidental, and asymptomatic per se. Some of the melliturias in the last two groups are benign, but others are serious, even fatal, if not correctly diagnosed and properly treated.

1.4. Metabolism of Sugars

The simple sugars glucose, galactose, fructose, and pentoses are not acted on by the digestive enzymes. Only glucose and galactose are absorbed from the intestine largely by active transport systems (C3). Fructose is partially converted to glucose in the process of being absorbed; approximately 1/6 is converted to glucose in man (M1). The disaccharides lactose, maltose, and sucrose are normally hydrolyzed in the intestine to the constituent monosaccharides, which are then absorbed. It is apparent that a small amount of the ingested disaccharide may be absorbed by normal individuals (B4, T4).

The first step in the metabolism of glucose is the phosphorylation in the 6 position by ATP, catalyzed by hexokinase. Hexokinase is capable of catalyzing the phosphorylation of galactose and fructose, but the affinity of glucose for the enzyme is so much greater that this reaction is probably not significant when glucose is present. There are specific kinases which catalyze phosphorylation of galactose and of fructose on the carbon-one position. Each phosphorylated sugar then enters the Embden-Meyerhof metabolic pathway at a different stage. It becomes apparent, then, that a deficiency of a single enzyme, properly located, could give rise to an inability to metabolize one monosaccharide, but leave the metabolism of the other two undisturbed. Hers and Kusacka (H3) demonstrated the specific fructokinase and showed that an aldolase split fructose-1-phosphate to dihydroxyacetone phosphate and glyceraldehyde. Glyceraldehyde is then phosphorylated by ATP in the presence of triose kinase to glyceraldehyde-3-phosphate. Both moieties then enter the Embden-Meyerhof pathway at the triose level (Fig. 2).

The metabolism of pentoses has been studied in detail only recently, chiefly of ribose and to a lesser extent deoxyribose. Horecker and his coworkers (H7) have considered the steps in the hexose monophosphate shunt, and the pentose oxidative or Warburg-Dickens-Lipmann pathway. It appears that this pathway is the source of the pentose moiety of nucleotides, ribonucleic acid, and deoxyribonucleic acid. It is also undoubtedly the source of the small amounts of ribulose in normal urine.

There is no clear evidence on the reactions involved in the utilization

of ingested pentoses. Xylose is not utilized, which is the basis for its use as a test substance for malabsorption (B3). It is clear that a diminished fruit intake will significantly reduce the amount of urinary arabinose and xylose but have little effect on ribose.

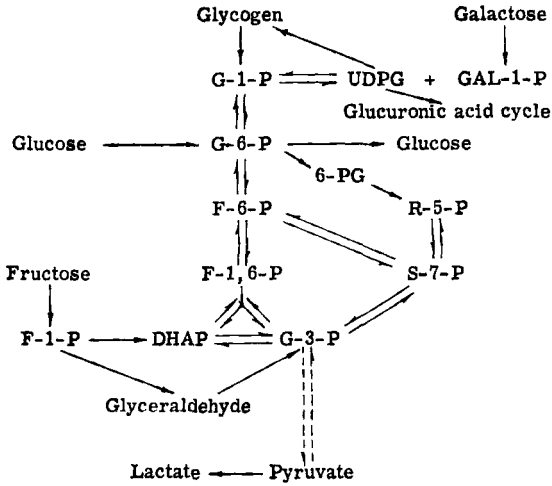

FIG. 2. A schematic representation of carbohydrate metabolism. The figure indicates the relationship of the point of entry of glucose, galactose, and fructose, showing that separate enzyme systems are involved. The pentose oxidative cycle is abbreviated by the symbols:

$$\text{6-PG} \longrightarrow \text{R-5-P} \longrightarrow \text{S-7-P}$$

Many reactions involved in this pathway are omitted. The point of origin of the glucuronic acid cycle from UDPG is indicated (see Fig. 3). The metabolism of pyruvate may proceed in any of several different directions, viz., amino acids, fatty acids, sterols, steroids, and the tricarboxylic acid cycle. Specific enzymes catalyze each conversion indicated by arrows. Broken arrows indicate that several steps are involved.

Abbreviations used: Gal-1-P, galactose-1-phosphate; UDPG, uridine diphosphate glucose; G-1-P, glucose-1-phosphate; G-6-P, glucose-6-phosphate; F-6-P, fructose-6-phosphate; F-1,6-P, fructose-1,6-diphosphate; G-3-P, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; F-1-P, fructose-1-phosphate; 6-PG, 6-phosphogluconic acid; R-5-P, ribulose-5-phosphate; S-7-P, sedoheptulose-7-phosphate.

The glucuronic acid oxidation pathway is the most recently studied metabolic route involving pentose. It is this pathway, illustrated in Fig. 3, which is most pertinent to the understanding of the defect in patients with familial pentosuria (T3, T4).

Glucose-1-phosphate and uridine triphosphate in the presence of UDPG pyrophosphorylase form uridine diphosphate glucose (UDPG) and pyrophosphate (see Fig. 2). Glucuronic acid is derived from UDPG

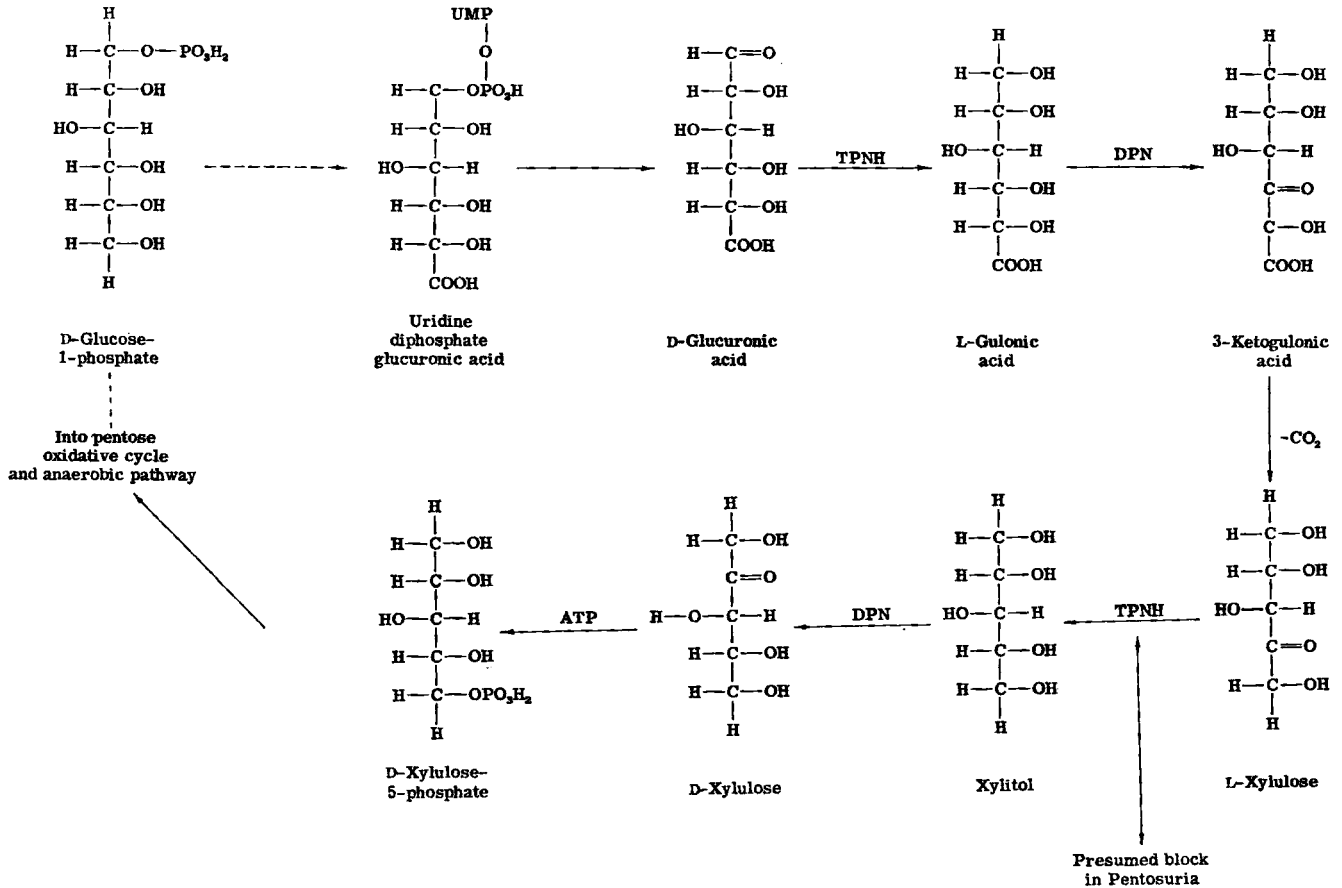

FIG. 3. Steps in the glucuronic acid oxidative pathway. The site of the apparent enzymatic deficiency in individuals with familial pentosuria is indicated.

through several steps, and its metabolic fate is indicated in Fig. 3. Xylulose-5-phosphate, the terminal substance in this specific series of reactions, may undergo further oxidation or may return to the Embden-Meyerhof pathway to re-form glucose-1-phosphate.

The disaccharides are not directly metabolized to a significant extent by the tissues of the body. Their utilization requires that they be split in the intestine to the constituent monosaccharides in order to be utilized.

2. Methods

2.1. Screening Methods

The first step in the study of mellituria of any type is the detection of the presence of an increased amount of sugar in the urine. Though it is obvious, one cannot emphasize too strongly or too frequently the necessity of using a nonspecific reagent for the screening procedure in the routine testing, i.e., the Benedict test or its equivalent (H1) should be used and not the glucose oxidase reaction. Use of the latter on a routine basis has been the cause of confusion and missed diagnoses in patients with nonglucose melliturias. This can have serious consequences in patients with galactosemia and some of the types of melliturias described below.

The finding of a urine which has a positive Benedict reaction, but a negative glucose oxidase reaction, is the simplest screening method for the detection of nonglucose melliturias.

2.2. Preservation

Urine specimens which are collected for identification and quantitation of the sugar(s) present can be adequately preserved by adding toluene to the container (4–5 ml for a 24-hour collection) and keeping the urine cool until processed. It is important that the studies be started as soon as the collection is completed because urine becomes alkaline on standing and epimerization occurs (P3). Immediate freezing of the specimen following collection is satisfactory if the identification procedures cannot be done immediately.

2.3. Conventional Techniques

2.3.1. *Qualitative Benedict Reaction*

This is a nonspecific reaction for reducing substances. If the test is carefully performed under controlled conditions, it can detect as little as 30 mg/100 ml of glucose. Fructose and ketopentoses will effect reduction when the reaction mixture is heated to 55°C for 10 minutes, whereas glucose, galactose, and lactose will not (L3).

2.3.2. *Determination of Optical Rotation with the Polarimeter*

Fructose is levorotatory, whereas the other sugars usually encountered are dextrorotatory or have no significant rotation (H1).

2.3.3. *Osazone Preparation*

The appearance of the crystals and the melting point of the osazones are used to identify the sugar (H1). Phenylhydrazine gives the same osazone with fructose and glucose. The methylphenylfructosazone crystals, which form after methylphenylhydrazine and fructose are acidified with acetic acid and heated in a boiling water bath for 5–10 minutes, appear after 15 minutes, and crystallization is complete within 2 hours. The glucose gives the same osazone, but crystallization occurs after 5 hours standing (H1). Interfering substances occur in urine which often make preparation of the osazone extremely difficult. Moreover, the technique requires a relatively high concentration of the sugar and is particularly unsatisfactory when several sugars are present.

2.3.4. *Isolation as the Benzimidazole Derivative*

This method involves the oxidation of aldoses to aldonic acids by potassium hypoiodite in methanol and the condensation of the isolated potassium salt of the aldonic acid in hot acid with *o*-phenylenediamine (M3). The specific aldobenzimidazole can be identified by the solubility in water, optical rotation in acid, and melting point. This method is not as simple as chromatography. It requires dehydration of the sample and a relatively high concentration of the sugars to be identified.

2.3.5. *Fermentation of the Sugar by Yeast*

This technique was extensively used in the past, especially to distinguish glucose from galactose. Fales (F1) has utilized this technique in conjunction with paper chromatography, thus segregating glucose, mannose, and fructose as the fermentable sugars and lactose, galactose, and arabinose as the nonfermentable sugars. The specimens are chromatographed before and after fermentation by yeast. The fermentation method appears simple, but actually inaccurate results may be obtained if conditions are not maintained constant.

2.4. Quantitative Colorimetric Methods

2.4.1. *Pentoses*

The cystine-H_2SO_4 reaction is a very good colorimetric method for determining pentoses quantitatively (D3). Roe's (R5) method is probably the best method for quantitative determination of aldopentoses. This method uses 2% *p*-bromoaniline in acetic acid saturated with

thiourea. The reagent is added to the sample to be analyzed, and the mixture is heated in a 70°C water bath for 10 minutes and then cooled. The tubes are allowed to stand in the dark for 70 minutes before they are read in a spectrophotometer at a wavelength of 520 mμ. The *p*-bromoaniline reagent is strongly corrosive and appropriate care must be taken. Glucose and galactose interfere but not significantly at concentrations of less than 20 mg/100 ml.

The orcinol reaction (A1) is simple, and further evidence of identification can be gained by determining the absorption spectrum of the color obtained by the reaction. The reaction is not specific for pentoses. Bial's test (H1) for pentoses in the urine represents the qualitative version of this reaction.

2.4.2. *Glucose*

The glucose oxidase reaction, first described by Keilin and Hartree (K1), is specific for glucose, and several laboratories have developed modifications which couple the reaction to a chromogenic hydrogen acceptor which makes the reaction a simple, useful technique which can be measured with a spectrophotometer. High concentrations of uric acid, ascorbic acid, and bilirubin give "false positive" reactions.

2.4.3. *Galactose*

Galactose is usually estimated quantitatively by the difference in the glucose oxidase determination and the total reducing substance as determined either by the copper reduction method (N1) or the anthrone reaction (A1).

2.4.4. *Fructose*

The most commonly used method for the quantitative determination of fructose is that of Roe *et al.* (R6). This consists of the addition of 1 ml 0.1% resorcinol in glacial acetic acid containing 0.25% thiourea and 7 ml 30% HCl to a 2-ml sample to be tested and heating the solution for 10 minutes in an 80°C water bath to develop the color, which is read at a wavelength of 520 mμ. The resorcinol is stable for at least a month. Because of its flexibility and the fact that the color can be developed in a 37°C water bath, we prefer the method of Pogell. This method has been modified to yield a stable colored product (D2). The test tube with a 1-ml sample is placed in an ice bath, and 1 ml cold 0.04% skatole in 95% ethanol and then 8 ml cold 30% HCl are added. The contents are mixed and incubated at 38°C. The reaction is stopped by removing the tubes to an ice bath and immediately adding 10 ml chloroform to extract the color. The time of incubation can be varied to accommodate the con-

centration of fructose in the sample. The color increases with the time of incubation.

The qualitative Seliwanoff test for fructose in the urine utilizes the resorcinol reaction (H1).

2.4.5. *Lactose*

Lactose can be quantitated by determining the increase in glucose following acid hydrolysis at 100°C for 20 minutes, using the glucose oxidase method (S1a). The method of Timmell *et al.* (T1), which employs 0.4% *o*-aminodiphenyl in glacial acetic acid, can be used to measure the lactose concentration in a solution. The amount of glucose present in the sample can be determined by the glucose oxidase method and its contribution to the final color developed deducted. The reaction mixture must be heated to 100°C for 90 minutes for complete color development with lactose. Fructose does not react with this reagent but aldoses do.

2.4.6. *Sucrose*

Sucrose is a nonreducing sugar. It can be estimated by acid hydrolysis followed by determination of glucose and/or fructose. It may also be determined in a like manner and with greater specificity following the action of the enzyme saccharase. The methods employed for fructose can be used for sucrose. Raybin's reagent is specific for sucrose, but it is not very sensitive (R2).

2.5. Paper Chromatography

2.5.1. *Summary of Principles*

A description of the principles of paper chromatography is not within the scope of this summary. Suffice it to say that the compounds to be separated have varying degrees of affinity for the cellulose fibers of the paper and differential solubilities in the solvent system used; hence, different compounds will travel at different rates with relation to the rate of travel of the solvent. The ratio between the rates of travel of the solvent and the particular compound in question is constant under standard conditions and is designated the R_f of that compound:

$$R_f = \frac{\text{distance traveled by compound}}{\text{distance traveled by solvent}}$$

It is, however, constant only under specified standard conditions, and in practice comparison with authentic standard substances is necessary.

2.5.2. *Paper Chromatography of Sugars*

Paper chromatography was first applied to the separation of sugars by Partridge (P1). Isherwood and Jermyn (I2) have studied the relation-

ship between the structure of sugars and their R_f values, which varies with different solvents, depending on the water content of the solvent except when, as in the case of phenol, the solvent forms complexes with the sugars. It was shown that the relative R_f values of different sugars in nonphenolic solvent systems depend only on the configuration of the OH groups of the ring.

Separation and identification can usually be improved by running the paper with a particular solvent system, drying the paper, and then running the chromatogram in another solvent system perpendicular to the first (two-dimensional chromatography). This is obviously more time consuming than running a one-dimensional chromatogram. The difficulty in the chromatography of urinary sugars lies in the fact that glucose and galactose are not satisfactorily separable. We have found ethyl acetate:pyridine:water (4:10:3) very satisfactory for the detection and identification of the sugars usually found in urine, and glucose and galactose are separated. This system has the added advantage that it is not very sensitive to salts, whereas the systems described by Partridge (P1) required that the urine be deionized before application on paper. Hiatt (H4) has found conversion of xylulose to xylose when the former was passed through ionexchange resins. For more extensive treatment of the principles and application of chromatography to the identification of sugars, useful books are available (B5, L6).

The following system has been used in this laboratory and found satisfactory. One-tenth volume of 0.1 *N* oxalic acid is added to an aliquot of fresh urine to remove bivalent anions, and the mixture is allowed to stand 15 minutes. Any precipitate is removed by centrifugation. The amount of urine used for paper chromatography is determined by the reduction of Benedict's solution. With normal urine 150 μl must be streaked at the origin; 25 μl suffices if there is a "one-plus reaction" to Benedict's solution, and correspondingly smaller amounts if there is a high concentration of reducing material. Whatman No. 1 paper strips, 1 inch wide, cut to a satisfactory length for the tank to be used, are satisfactory. Standards are run separately from the urine but under the same conditions; and once the sugar has been tentatively identified, the urine is run again adding a known standard to the area of application of the urine which corresponds to the sugar suspected; if the standard and the "unknown" sugar are in fact identical, only one spot is produced on the chromatogram. Some investigators prefer a wider sheet of filter paper which permits several samples and standards to be run, side by side, on the same paper.

In order to determine the R_f of a sugar, the chromatogram must be removed from the tank before the solvent has reached the end of the

paper. Better separation can be achieved, however, if the solvent is allowed to run off the end of the paper, and the distance traveled by the standards is used for reference. It is convenient to let the chromatograms run overnight and remove and dry them under the hood in the morning. After drying, the chromatogram is sprayed with one of several mixtures which will react with the sugars and develop a color. Aniline hydrogen phthalate, now commercially avaliable in a spray bottle, and aniline oxalate (prepared by adding 0.9 ml aniline to 100 ml 0.1 *N* oxalic acid) are similar in the color produced with different sugars and in stability of the reagent. The chromatogram is sprayed and then heated at 105°C for 10 minutes in an oven. Fructose and the disaccharides give a yellow color, glucose and galactose a brown color, and pentoses a pink color. Fructose and sucrose do not react as readily with aniline hydrogen phthalate as with aniline oxalate. Both of these reagents are more reactive if the solvent system in which the chromatogram is run contains pyridine. An orcinol spray consisting of 2% orcinol in 2 *N* HCl, followed by heating to 105°C for 3 minutes, can be used for ketoheptoses. A naphthoresorcinol reagent, prepared by mixing equal volumes of 0.2% naphthoresorcinol in ethanol with 2% trichloroacetic acid in water, is useful for identifying ketoses. Several other solvent systems and spray reagents have been described (B5, K2, L6).

2.6. Ionophoresis of Sugar

Ionophoresis has been employed for the separation of sugars and has the advantage of speed; the separation requires only 2 hours (C2).

2.7. Criteria for Identification of Sugars

When positive identification of a sugar is required, several criteria of identification must be applied. Chromatography employing two different solvent systems and the use of different spray reagents, which permit differentiation based on differences in color developed with particular sugars, can give important confirmatory evidence in identification. Finally, a specific colorimetric reaction or the isolation and characterization of the osazone or benzimidazole derivative should be used to substantiate the identification.

3. Clinical Conditions Associated with Mellituria

3.1. Pentosuria

Tower *et al.* (T4) have reported a very thorough study of pentose excretion in normal individuals and in patients with neuromuscular disorders. Small quantities of pentose—a mixture of arabinose, xylose, and

ribose—were excreted in the urine of normal individuals, the concentration being about 6 mg/100 ml of urine. Total pentose excretion was found to be relatively constant for a given individual, but ingestion of a large quantity of fruit significantly elevated the concentration of xylose and arabinose (but not ribose) in the urine. Tower was unable to demonstrate xylulose or ribulose in normal urine even when concentrated to ¼ its original volume, but Futterman and Roe (F5) found both in urine concentrated 300 times.

Increased pentose excretion has been shown to follow experimental trauma, after craniofacial injuries in man, in response to cold exposure, and following the administration of thyroid hormone or cortisone. Drug-induced pentosuria has been known for a long time, but it has not been well studied. Morphine is the best-known inducer of pentosuria; antipyretics have a similar effect. Urinary pentose increases in response to fever and during allergic responses (T4).

Orr and Minot (O1) reported increased urinary excretion, by patients with muscular dystrophy, of a compound which they believed to be ribose-5-phosphate. Others have not been able to confirm this observation; however, Tower (T4) found that ribose excretion in patients with muscular dystrophy tended to be somewhat higher than that in normal individuals. Increased excretion of ribose has also been reported in the urine of patients with neoplasia (L7).

Essential pentosuria was first described in 1892 (S2), and it was one of the "inborn errors of metabolism" discussed by Garrod (G1). It is distinguished from the other pentosurias by the nature of the pentose excreted (L-xylulose), the much larger amount of pentose excreted (1.1—3.7 g/24 hours) (L4), the constancy of the excretion irrespective of diet, and its occurrence primarily in individuals of Eastern European Jewish origin. Touster's (T2, T3) and Hiatt's (H4) studies of a patient with essential pentosuria indicate that there is a deficiency (or absence) in these individuals of the enzyme necessary for the conversion of L-xylulose to xylitol (Fig. 3). Direct demonstration of the enzyme deficiency has not been accomplished.

Clinically, essential pentosuria is a benign disease and affected individuals have a normal life expectancy (L1). The only associated affliction is that of being mistakenly diagnosed and even treated for diabetes mellitus.

The best study of the genetics of the condition is that of Lasker *et al.* (L5), who concluded that the condition is inherited and transmitted as an autosomal recessive characteristic. The condition occurs almost exclusively in Jews, but one exception has been reported (B2). The inci-

dence among Central and Eastern European Jews is estimated at 1:1500, giving a frequency of 5% carriers in this group (R4). Roberts (R4) reports the condition in a mother and her two daughters and cites reports by Schultz and Macumber, each finding the condition in two generations with one parent affected. Hiatt (H4) has been successful in distinguishing heterozygous from normal individuals by determining the increase in xylulose excretion in the urine following a loading dose of glucuronolactone (L4, T3).

3.2. Fructosuria

Fructose is found in many plants and is an important portion of dietary carbohydrate. Most commonly it is ingested as free fructose or sucrose. Fructose is not actively transported by the intestinal mucosa, and variable proportions are converted to glucose in the process of absorption; in man, about one-sixth is converted (M1). Most of the metabolism of fructose occurs in the liver. If a renal threshold for fructose exists, it is very low.

Bickel (B4) found increased excretion of fructose in premature and newborn infants, up to 70 mg/100 ml. After the first 10 days of life, the amount of fructose was similar to that found in adults. Of 1000 pediatric patients whose urine was examined, he found 12 with elevated fructose excretion. The diagnoses included hepatitis and cirrhosis, Wilson's disease, mercury poisoning, severe general infection, and fructose intolerance. Tower *et al.* (T4) did not find fructose in the untreated urines of 18 normal adults, but was able to demonstrate it after the urines had been concentrated.

Benign or essential fructosuria is a harmless and a symptomatic familial condition which is important clinically primarily as a source of improper diagnosis of diabetes mellitus. The correct diagnosis is reached by identifying the sugar in the urine, demonstrating a normal glucose curve, an abnormal fructose tolerance test (peak over 20 mg/100 ml), and the disappearance of the fructose in the urine when fructose is removed from the diet. Insulin has no effect on the mellituria. Studies conducted with these patients demonstrated no rise in blood lactate or pyruvate, no drop in serum phosphorus, nor any elevation of RQ when fructose was administered to these individuals (H2, L8, S1). The fructosuric individual excretes about 20% of a fructose load in the urine. The remaining 80% is unaccounted for. The condition is quite rare; the estimated incidence is 1:130,000 in the general population (L2).

The enzyme presumed to be deficient in benign fructosuria is the fructokinase. The reasoning is based on the fact that there is no demonstrable utilization, no drop in serum phosphorus when fructose is administered,

nor associated symptoms; hence, it is unlikely that metabolic products accumulate within the cells. Lasker has presented evidence that benign fructosuria is an autosomal recessive condition (L2).

Froesch *et al.* (F3, F4) and Wolf *et al.* (W3) described a condition in which the affected individual is exquisitely sensitive to fructose. Administration of the conventional loading dose of fructose in one patient resulted in a drop of the blood glucose from 65 mg/100 ml to 8 mg at 90 minutes, while the fructose level in the blood had risen to 138 mg %. The serum inorganic phosphate dropped sharply, and symptoms of hypoglycemia were marked. Fructose was excreted in the urine in large amounts, and there was a concomitant amino aciduria, proteinuria, and rise of bilirubin in the serum. A first affected sibling, whose condition was not discerned until 8 months of age by the mother, is mentally retarded; whereas the abnormality was appreciated immediately in the next affected sibling and, the offending sugars being withheld, this second child remained mentally normal. The authors postulate a deficiency in these patients of either fructose-1-phosphate aldolase or glyceraldehyde kinase (see Fig. 2), with an intracellular accumulation of fructose-1-phosphate. The symptoms and the accumulated product are reminiscent of galactosemia (S3). The incidence of the disease in the families studied suggests that like benign fructosuria (L2) it is an autosomal recessive condition (F4).

Chambers and Pratt (C1) reported the case of a 24-year-old female who had vomiting and dyspepsia following fructose or cane sugar ingestion. Her fructose tolerance curve was not abnormal, and insufficient studies were done to permit diagnosis.

Increased fructose concentrations are normally found in the premature and full-term infant during the first 10 days of life. Thereafter, increased fructose excretion may follow a large intake of fructose or fructose-containing saccharides or in association with certain conditions affecting either the liver or kidneys. The amount of fructose excreted in these instances is small relative to excretion found in the fructosurias resulting from the enzymatic defects. Benign fructosuria requires diagnosis only to prevent the mistaken diagnosis of diabetes. Hereditary fructose intolerance requires prompt recognition in order to prevent hypoglycemia and perhaps mental retardation.

3.3. Lactosuria

Lactose is a reducing disaccharide, glucose-β-galactoside, found principally in milk. Normally, there is no significant absorption of lactose from the intestinal tract, and before utilization the disaccharide must be

hydrolyzed to its constituent monosaccharides, a process catalyzed by the enzyme lactase (a β-galactosidase) found in the *succus entericus.* Lactosuria may result from alimentary absorption of the intact disaccharide or arise endogenously, for lactose is produced by the mammary glands during pregnancy and lactation. Lactosuria in the lactating goat has been eliminated by amputation of the udder. It has been previously noted that lactose is one of the sugars which may occur in the urine of normal individuals in small amounts (B4, T4).

Lactosuria in pregnant and lactating women was demonstrated before the end of the nineteenth century. Watkins (W1) made a study of lactose in the urine of pregnant women and concluded that there was no renal threshold for galactose or lactose. Flynn *et al.* (F2) studied the urine of a large number of nonpregnant women and of women at different stages of pregnancy and during the *post-partum* period. They found that 79% of pregnant women had a positive Benedict reaction in the urine by the fortieth week of pregnancy and 97.6% had a reducing substance in their urine by the fourth day after delivery. Antenatally, twice as many women had lactose as glucose in the urine at the level tested. Postnatally, the overwhelming majority had only lactose.

Bickel (B4) had demonstrated that normal newborn and premature infants on milk formulas have a significant lactosuria lasting about 2 weeks. It is assumed that increased permeability and inadequate lactase production might explain these findings.

Bickel found increased lactose in 13 out of 1000 urines tested by the paper chromatographic technique. These patients had severe diarrhea, steatorrhea, neonatal sepsis, or hiatus hernia. It is assumed that increased permeability is responsible. Loss of lactase as a result of increased intestinal motility and shortened emptying time of the intestine should be considered in the diarrheas. Suppressed synthesis of lactase is a possible mechanism in diarrhea, steatorrhea, and sepsis. Moncrief and Wilkinson (M2) also found lactosuria in their patients with sucrosuria, mental retardation, and hiatus hernia. In some instances the lactosuria was as impressive as the sucrosuria. The mechanism for the mellituria associated with hiatus hernia is not apparent. More recently, Inall (I1) reported an infant with chronic diarrhea and vomiting and failure to gain weight. The urine was found to contain 1.2 g lactose/24 hours, 300 mg sucrose, and no amino acids. The infant recovered completely when placed on a strict lactose-free regimen for 5 weeks. He was then able to take a normal diet without ill effect, and no lactose or sucrose could be demonstrated in the urine. One is tempted to speculate that the hydrolytic enzymes merely appeared in the intestine at a later age than is usual. It

is noteworthy that in contrast to the cases discussed below, there was no amino aciduria nor was there a familial pattern.

In 1958, Durand (D4) reported studies of lactosuria in a 13-month-old girl, the daughter of consanguineous parents, who was weak, poorly nourished, failed to thrive, had chronic diarrhea resistent to therapy, renal acidosis, and intermittent proteinuria; post-mortem examination at 15 months of age revealed atrophic enteritis, hepatic and adrenal atrophy, as well as degeneration of the convoluted tubules. Holzel *et al.* (H6) described siblings who failed to thrive and had chronic diarrhea when on a diet containing lactose; withdrawal of lactose resulted in resumption of normal growth. More recently, Darling *et al.* (D1) described three siblings and a cousin who manifested vomiting, failure to thrive, diarrhea, proteinuria, and amino aciduria; lactosuria was demonstrated in two of these four patients, all of whom died. The three groups of investigators postulated absence of lactase in the intestinal tract. The importance of a proper diagnosis is indicated by the fact that 6 out of 8 of the reported cases have died.

Very small amounts of lactose are found in the urine of normal individuals when lactose is included in their diet, and under this dietetic condition increased amounts are excreted by the premature and full-term infant for the first 10 days of life. Lactose, probably of endogenous origin, is commonly in amounts sufficient to be detected by the usual reduction methods in the urine of pregnant and lactating women. Indentification of lactose in the urine of women may be important to prevent a mistaken diagnosis of diabetes or, conversely, to ensure a proper search in "glycosuria." Severe illness, such as diarrhea and sepsis, often results in increased lactose excretion. Evidence is accumulating to indicate that lactose excretion is increased in a relatively high percentage of patients with upper gastrointestinal anomalies.

One patient is reported with apparent lactose intolerance who recovered from this intolerance after a brief respite from lactose ingestion. This suggests that the appearance of lactose in the *succus entericus* may be delayed and symptomatic.

3.4. Sucrosuria

Sucrose is a nonreducing disaccharide derived from glucose and fructose. Sucrose is a plant sugar and is not metabolized by the cells of the animal body, so that if sucrose as such gains access to the blood, it is excreted quantitatively in the urine. It is normally hydrolyzed in the intestinal tract, and the constituent monosaccharides are absorbed. It is

reported that small doses of hydrazine phosphate cause an alimentary sucrosuria (R1) such as occurs in normal individuals following a load of sucrose (E1), and there appears to be no familial pattern in the response to a loading dose of sucrose (P2). Von Reuss (V1) reported sucrosuria in infants with gastroenteritis. In 1954, Moncrieff and Wilkinson (M2) reported young children who had, in common, hiatus hernia, mental retardation, and sucrosuria. Their studies revealed other sugars also to be elevated in the urine when present in the diet. Lactose excretion was of the same order of magnitude as the sucrose. Woodruff (W4) reported 12 patients with mental retardation, hiatus hernia, and sucrosuria. The sucrosuria may have had a relation to the gastrointestinal anomaly, but the mental retardation in the 12 patients was due to varied and unrelated causes. Woodruff noted that many patients with hiatus hernia who were tested did not show sucrosuria after a loading dose of sucrose. Perry (P2) tested a number of mentally retarded patients, family members in some instances, as well as normal controls; there was no difference in frequency of sucrosuria in the control and in the mentally retarded group, and no apparent familial correlation of response was apparent. Durand (D4) was able to demonstrate sucrosuria in 3 of 6 children with anatomical abnormalities of the stomach, but none was mentally retarded. At present, insufficient data exist to permit a final verdict on the acceptability of the triad of mental retardation, hiatus hernia, and sucrosuria as a syndrome.

Elmer *et al.* (E1) present one patient with exogenous sucrosuria who is of particular interest. This 25-year-old female was well until the gradual onset of generalized weakness, thirst, loss of weight, and persistent skin infections. She was investigated for presumed diabetes, but she was found to have no reducing substance in her urine and a normal glucose tolerance curve. Sucrosuria was suspected because of the failure to find reducing substance in the urine and a urinary specific gravity of 1.070, in the absence of protein. An inordinately high specific gravity which is not immediately explicable is frequently the leading clue to the diagnoses of sucrosuria. It is apparent in this case that the condition was not entirely benign and required dietary regulation to relieve the symptoms and restore health.

Recently, Weijers *et al.* (W2) have reported three patients with a clinical constellation resembling the type of patient with lactosuria first described by Durand (D4). The patients had diarrhea when sucrose was added to the diet. The stools contained a very high concentration of lactic acid and increased concentrations of butyric acid, propionic acid, and formic acid while the patients were on the diet with sucrose. These

abnormalities and the symptoms abated when saccharase was also given with the food. This is the most concrete evidence to date that these syndromes are in fact due to a deficiency of the suspected enzyme in the intestine. This group, however, did not report having tested the urine for sucrose.

There are several reports of patients with sucrosuria not related to dietary intake (B1, E1, H5, R1, R3). This is quite surprising, for sucrose is considered to be produced only by plants; no one has yet demonstrated sucrose synthesis by mammalian tissue. All of the reported patients with endogenous sucrosuria have shown this abnormality following pancreatic disease. Subacute pancreatitis, produced by instillation of turpentine into the pancreatic duct of animals, was followed by the appearance of readily detectable amounts of nonreducing, hydrolyzable carbohydrate in the urine within 24 hours; whereas a similar injection into the common duct had no such effect. Chronic pancreatitis produced by passing a silk thread into the duct of Wirsung caused an increase in sucrose in the urine which disappeared if the pancreas was subsequently excised. Partial pancreatectomy is said to result in sucrosuria (R1).

Elmer *et al.* (E1) report two patients with endogenous sucrosuria, using methods of identification which were above reproach. They observed specific gravities of the urine from 1.056 to 1.145. The sucrose was identified in the blood and urine by acid hydrolysis and determination of the products and by measuring the effect of incubation with saccharase. Both patients suffered initially from pancreatitis. They also had a moderate hyperglycemia when the sucrose was elevated. The maximal values of blood sucrose were 35 and 60 mg %, respectively, and urine sucrose was about 65 g/24 hours and 150 g/24 hours, respectively. The second patient had symptoms suggesting diabetes, to wit, thirst, loss of weight, and generalized weakness. The symptoms were accentuated during bouts of pancreatic colic. Neither patient had an increased renal excretion between attacks of pancreatic colic.

Sucrosuria may be asymptomatic and be demonstrated only after a loading dose of sucrose, a phenomenon found in many normal individuals. It may be more frequent when gastric anomalies are present. Alimentary sucrosuria can apparently be associated with two distinct symptom complexes: (1) that resembling diabetes and (2) that associated with diarrhea. The latter appears to be due to saccharase deficiency. Endogenous sucrosuria has been reported only in association with pancreatic disease and may symptomatically mimic diabetes.

3.5. Maltosuria

But a single case of maltosuria is reported in the literature (G2). The identification of maltose is based on the osazone, but the study is inadequate. The preliminary studies of Weijers *et al.* (W2) would suggest that maltosuria, secondary to maltase deficiency, exists and will be demonstrated conclusively in the near future. These patients can be expected to suffer from chronic diarrhea or vomiting or both.

4. Summary

A brief summary of the nonglucose melliturias, exclusive of galactosuria, is presented. The metabolism of the different sugars is summarized, and methods for identification are enumerated. Mellituria is categorized into the following groups:

1. Urinary sugars found in normal individuals. All of the sugars discussed may be found in the urine of normal individuals under particular circumstances, but the quantity is usually quite small.

2. Mellituria secondary to hepatic, renal, or gastrointestinal damage. This is considered to be a secondary phenomenon and noncontributory to the symptoms of the patient. Several different mechanisms are considered possible, either singly or in combination. These include alteration of the transport mechanisms, diminished capacity of the organs, primarily of the liver, to metabolize the sugar, and suppression of synthesis or activation of hydrolytic enzymes in the intestine.

3. Increased urinary excretion of sugars resulting from enzymatic abnormalities in the gastrointestinal tract. This type of mellituria is demonstrable following ingestion of the particular sugar, either as a food constituent or as a loading dose. The mechanisms involved reflect the extreme of normal secretion of the appropriate hydrolytic enzyme in the intestine, a genetically determined deficiency of hydrolytic enzymes, or abnormality of transport mechanisms.

4. Heritable abnormalities resulting from a deficiency, primarily in the liver, of an enzyme which is required for the normal catabolism of the sugar. This results in accumulation of the sugar in the blood and excretion into the urine.

The syndromes resulting from, or associated with, different types of mellituria are just beginning to be appreciated. It is difficult at this time to put some of the reported results in the proper perspective. New and improved techniques in many fields of investigation, however, will now permit us to approach the mechanisms involved more rationally. We can expect these conditions to provide interesting information and provocative study in the future.

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ORGANIC ACIDS IN BLOOD AND URINE

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1. Introduction

1.1. Limitation of the Subject and Terminology

When considering organic acids, one must first give a definition of these substances.

According to the chemical definition, the group should include all acids containing carbon atoms in their molecules (except carbonic acid), but it is customary in biochemistry to exclude amino acids and fatty acids.

We shall adopt this convention and therefore exclude the last-mentioned groups of acids from this review and shall furthermore exclude other nitrogen-containing acids, such as pyrrolidone-carboxylic acid, indoles, and other metabolites of tryptophan (e.g., anthranilic, hydroxyanthranilic, kynurenic, xanthurenic, and nicotinic acids), imidazolic metabolites or histidine (e.g., β-imidazolylpyruvic and urocanic acids), acidic guanidine derivatives, and carbamylated acids, as well as acidic com-

pounds with a pyrimidine, purine, or pterine nucleus. Nitrogen-containing conjugated aromatic acids will, nevertheless, be considered, since they are closely related to the corresponding free aromatic acids. Finally, phosphorus-containing acids (mainly phosphorylated acidic intermediates of glycolysis) will also be excluded.

This review will therefore deal with organic acids present in blood and urine that contain only carbon, hydrogen, and oxygen atoms, with the exception of fatty acids, but including conjugated aromatic acids, even though they contain other atoms besides carbon, hydrogen, and oxygen.

Some of the results concerning organic acids in blood deal with whole blood, others with plasma or serum. Since large differences exist between the concentration of some acids in red cells and plasma, it would seem better to study the concentration of the acids in plasma and cells separately rather than in whole blood. Some acids are, however, so unstable that the separation of the plasma from the cells might cause losses of these acids; in these particular cases, it is advisable to study whole blood rather than plasma.

As in the field of amino aciduria, the data concerning organoaciduria[1] are best expressed by the quantity (mg) eliminated in 24 hours.

During chromatographic studies of urinary organic acids of adult subjects, the volume of urine to be subjected to chromatography is often determined through its creatinine content. In such cases, it is necessary to use a technique for creatinine determination with sufficient specificity, some acids interfering with the determination of creatinine by the standard picric acid method (M13). In any case, while the determination of the creatinine content permits the determination of the volume of urine to use in chromatographic techniques, the same objections as those indicated by Bigwood *et al.* (B17) in considering amino aciduria prevent the use of creatinine as a general reference base in expressing data on organic acids in urine.

1.2. Historical Development and Scope

Although limited as defined above, the field of organic acids is very wide, since it includes aliphatic and aromatic acids of considerable biochemical importance. The study of these acids in biological fluids is essential for the knowledge of many metabolic sequences which have been established only *in vitro* or which are still uncertain. It provides, furthermore, an aid to the detection and comprehension of many path-

[1] The term "organoaciduria" will be used here to indicate the total elimination of organic acids, whether normal or pathological, and not, as some authors do (L17), to indicate a pathological increase of this elimination.

ological conditions, as will be shown below. Data concerned with organic acids of normal and pathological blood and urine are, however, far less numerous than those concerned with amino acids.

The study of amino acids is indeed facilitated by different factors: the number of naturally occurring amino acids is relatively limited; techniques of a fairly good selectivity are available for the characterization of amino acids; finally, the concentration of amino acids in blood and urine is not notably influenced by the diet, within the limits of normal variations in protein intake (B17). Such factors do not exist in the case of organic acids: a very large number of such acids must be considered as potential constituents of blood and urine; no selective method of characterization is available; finally, their concentration depends largely, for many, upon diet. Despite these differences, a parallelism can be shown between the historical development of our knowledge in the two fields.

The aim of the first studies of organic acids in blood and urine was to determine the amount of these acids; titrimetric methods, whose specificity is doubtful, were generally used in such cases. At the same time, special techniques (most of them colorimetric) were described for a few particular organic acids. During the last 10 years, our knowledge of organic acids has been considerably increased, mainly through the development of chromatographic techniques applied to the determination of these substances in blood and urine. These techniques have allowed the detection of a great number of such acids which were not previously known to be present in these biological fluids.

Paper chromatography, which is not unduly time consuming and which is easy to use for serial determinations, has been used by most workers in the field and has given very important qualitative or semiquantitative information. Other workers, however, have used column chromatography on silical gel or ion exchange resins, mainly for the isolation of the tricarboxylic acid cycle acids. Such techniques give more accurate quantitative results than those using paper chromatography, but they are difficult to use as routine methods, as the great number of organic acids present in biological fluids makes their separation quite difficult; the peaks isolated during such column chromatography generally contain more than a single acid and must therefore be fractionated again by a new chromatography under different experimental conditions.

Fractionation of organic acids by electrophoresis has also been studied by some workers and has been found very useful for separating some special acids.

During the same period, new techniques for the quantitative determination of some of the organic acids have been described. In addition to

colorimetric methods, fluorimetric and enzymatic methods of a very high specificity allow the determination of some important acidic metabolites. The combined use of paper chromatography and these techniques for the detection of the organic acids present in blood or urine permits one to obtain evidence of identity and also reliable quantitative data about some of the organic acids.

2. Titrimetric Methods for the Determination of Organic Acids as a Whole

As the titrimetric methods for the determination of organic acids as a whole lack specificity, the results obtained by such techniques are of limited interest; we shall therefore survey only some of the main advances made with their aid.

Van Slyke and Palmer (V2) described in 1920 a titrimetric method for the determination of organic acids in urine. They precipitated as calcium salts the carbonic, phosphoric, and oxalic acids and then determined the quantity of hydrochloric acid necessary to bring the pH of the filtrate from 8 to 2.7. Some later authors improved that technique (G14, W14), and others (D16, W13) have extracted the urinary organic acids with an organic solvent before titrating them. Such techniques have shown that there is an increase in the urinary organic acids after fasting as well as in hepatic failure, diabetes mellitus, some infectious diseases, and some particular congenital tubular renal insufficiencies (D6, L17).

Total organic acids have also been determined in blood serum by techniques adapted from that described by Van Slyke and Palmer for urine, the titration being done mostly with the aid of a pH meter rather than with pH indicators (C17, D4, G2, M11, N8, P3). Just as in the case of urine, other authors extract the organic acids into an organic solvent before titrating them (B7).

An increase in blood serum organic acids has been found especially during uremic acidosis (B7), diabetes mellitus with acidotic coma (R1), and toxicosis in infants (K23).

3. Methods Involving Fractionation of the Organic Acids

3.1. General Methods

3.1.1. *Paper Chromatographic Methods*

A general technique has been described by Nordmann *et al.* for the separation of nonvolatile organic acids of urine (N20, N21, N22) and blood (N19). It includes two-dimensional paper chromatography preceded by the elimination of the compounds which interfere with adequate resolution and separation of the spots.

In the case of urine, a volume of urine containing 2 mg of creatinine is passed through a column of a strongly basic anion exchange resin (Dowex 2, formate form). The effluent of the column is discarded; it contains the nonionic compounds, the cations, and some of the ampholytes. Elution of the organic acids retained on the column is then achieved with formic acid; a small part of the inorganic anions is eluted as well, but this does not disturb the subsequent chromatographic separation of the organic acids, except that of the acids which have a low R_f with the solvents used (especially citric acid). The use of a volatile acid (formic acid) for elution makes it easy to eliminate the eluting agent by concentration before spotting the residue on paper.

The first solvent used for chromatography is an alkaline one (containing ethanol, ammonia, and water), the second one an acidic one (consisting of *n*-propanol, formic acid, eucalyptol, and water).

The procedure is applied at least on duplicate samples of the urine to be studied.

The chromatograms having been dried and viewed under UV light to detect the spots which absorb UV light or which are fluorescent, acidic spots are located either with bromocresol green (L19) or with acridine (L8). One of the chromatograms is then sprayed with (or dipped in) *p*-dimethylaminobenzaldehyde in acetic anhydride (G1) (Fig. 1); the second one is chlorinated and then sprayed with a starch-iodide reagent (R11). These location techniques characterize especially aconitic and hippuric acids (first chromatogram), as well as pyrrolidonecarboxylic acid and phenylacetylglutamine (second chromatogram), which are the most useful locating compounds for the identification of the other acidic spots.

When unusual spots are found on these two standard chromatograms, it is of course necessary to run more samples of the urine through the process to provide supplementary chromatograms for other location techniques (N2) or to make specific tests on the eluate of such unknown spots.

In the case of blood plasma, the technique includes the same steps, preceded by the elimination of plasma proteins, which is done either by ultrafiltration or by the technique of Hunter (H32). Methods of deproteinization using stable acids (like trichloroacetic acid) are not suitable, since the added acid would interfere with the subsequent chromatography.

A technique which is close to that of Nordmann *et al.* has been described by Osteux and Laturaze (O5, O6) for urinary organic acids. It differs mainly in the choice of a weakly basic anion exchange resin (Amberlite IR-4B in the acetate form), the strength of the formic acid used

for elution, and the composition of the acidic solvent used for the chromatography.

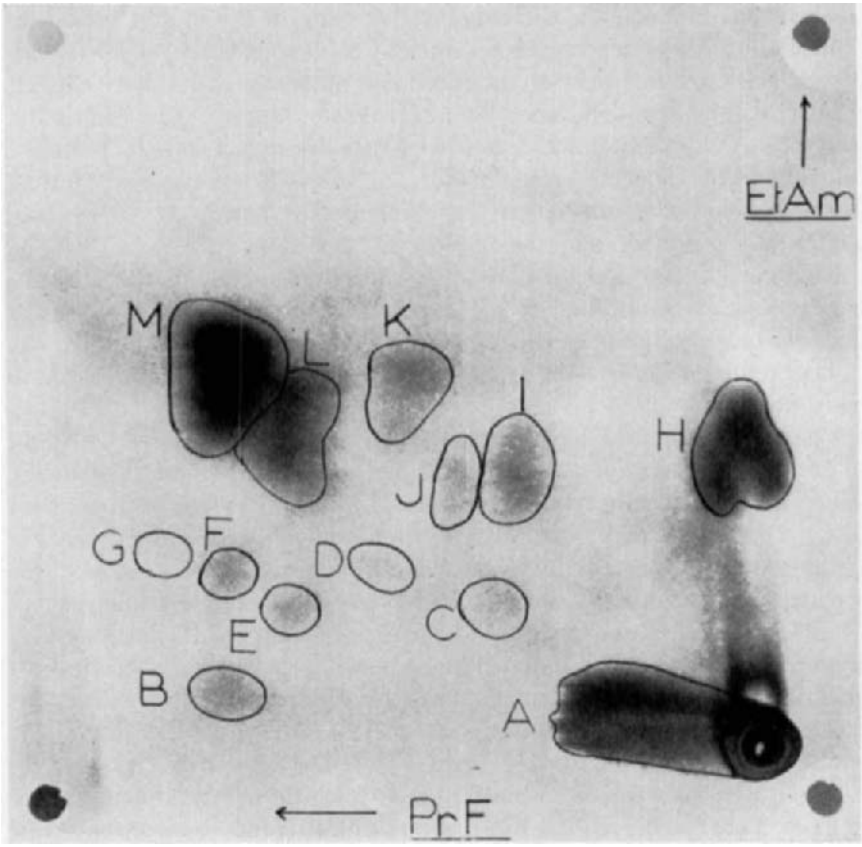


FIG. 1. Chromatogram of the organic acids of a sample of normal urine (corresponding to 2 mg of creatinine) [Nordmann *et al.* (N12, N22)]. The technique used is that summarized in Section 3.1.1. The starting point lies at the right lower corner; *EtAm*: first chromatographic separation with a solvent containing ethanol and ammonia; *PrF*: second chromatographic separation with a solvent containing *n*-propanol and formic acid. The spots have been located by successive use of acridine and *p*-dimethylaminobenzaldehyde in acetic anhydride. They correspond to the following acids: (*A*) citric; (*B*) aconitic; (*C*) malic; (*D*) α-ketoglutaric; (*E*) succinic; (*F*) glutaric (?); (*G*) unidentified acid; (*H*) hydrochloric; (*I*) pyrrolidonecarboxylic; (*J*) glycolic (?); (*K*) phenylacetylglutamine; (*L*) lactic + *m*-hydroxyhippuric; (*M*) hippuric. The location with *p*-dimethylaminobenzaldehyde in acetic anhydride gives a violet color with aconitic acid, a pink one with citric and α-ketoglutaric acids, an orange one with hippuric and *m*-hydroxyhippuric acids, and a green one with hydrochloric acid.

For the special case of the aliphatic dicarboxylic acids, solvents have been described which allow a better separation of the spots than that obtained with the solvents used in the standard technique (K3, S34).

The methods just outlined permit the characterization of organic acids in blood and urine, with the exception of volatile acids (which are totally or partly lost during the concentration of the eluate obtained from the ion exchange column) and of unstable acids, like most α-keto acids, which are also totally or partly lost. They are especially useful for the study of non-α-keto aliphatic acids and of aromatic acids conjugated with glycine or glutamine.

Special techniques, to be considered later, are more suitable for α-keto acids and for free aromatic acids.

3.1.2. *Column Chromatography*

Silica gel chromatography, first used in the field of organic acids by Isherwood (I1) during studies on fruits and later improved by different workers (K10, L1), has been used for the isolation of the acids of the tricarboxylic acid cycle and related acids from different animal tissues (D1, F14, F15, F16, G3, L7, M7, M8, M9). In this way, Frohman *et al.* (F16) have studied these acids in whole blood of rats, while Dajani and Orten (D1) have made similar studies on erythrocytes.

Meites (M16, M17) has also used silica gel chromatography for studying organic acids in normal human urine. This method has the advantage over paper chromatography that it includes volatile acids. Its resolving power is, however, much smaller, as it allows the separation of only a few peaks as compared to the large number of spots found on urinary paper chromatograms.

Silica gel chromatography has also been used for the separation of the urinary dicarboxylic acids (T19).

Busch *et al.* (B37) have determined most of the tricarboxylic acid cycle acids of rat kidney by *ion exchange chromatography* on Dowex 1. This technique has been used also in studies on liver mitochondria (A1), but has not been applied, to our knowledge, to organic acids in blood or urine.

Gas chromatography, which has been applied to the separation of the tricarboxylic acid cycle acids in the liver (F8), might prove useful in the future in the field of organic acids of blood and urine.

3.1.3. *Electrophoresis*

The separation of organic acids and especially of the aliphatic ones by high voltage paper electrophoresis has been the aim of a number of workers during the last 10 years (B11, B12, G18, G19, G20, M23, W15).

A systematic study of urinary or blood organic acids has not yet been made with this method, which can be improved through combination with chromatography (B12). Electrophoresis has, however, been used for some special cases, as for the separation of lactic and β-hydroxybutyric acids, which are difficult to separate by paper chromatography (O7).

3.2. Special Techniques for α-Keto Acids

As important losses of α-keto acids occur during *paper chromatography* of organic acids (as free acids) of blood and urine, special techniques are used for these acids; they include the preliminary transformation of the keto acids into more stable derivatives.

Since the first studies in that field by Cavallini *et al.* (C5, C6, C7, C8), most workers apply paper chromatography of the 2,4-dinitrophenylhydrazones of these acids and have gradually improved the extraction of the hydrazones of blood and urine, as well as their chromatographic separation and the elimination of artifacts (A3, B18, D15, E2, E3, I2, K1, K20, K22, M2, M29, S11a, T23, T26, Z1).

Paper chromatography of α-keto acids has also been achieved after conversion to other derivatives, especially nitroquinoxalinols through 1,2-diamino-4-nitrobenzene (H25, S27, T6).

Other workers separate by paper chromatography the amino acids obtained by catalytic hydrogenolysis of the 2,4-dinitrophenylhydrazones of the α-keto acids (H19, K19, T24).

Column chromatography has rarely been used in the special field of α-keto acids in blood and urine (D3, L10, S3), and the techniques described do not appear to be preferable to the paper chromatographic ones.

Paper electrophoresis provides a good way of separating the 2,4-dinitrophenylhydrazones (B18, N6, T2, V1, Z3) and gives results which can be compared to those obtained by paper chromatography.

3.3. Special Techniques for Aromatic Acids

Following previous paper chromatographic studies on aromatic acids in biological fluids (B25, B26, B31, B32, B33, D2, D19, L4, L5), Armstrong *et al.* (A14) have described a method for the systematic paper chromatographic analysis of phenolic acids in urine.

The urine is acidified to pH 1–2 with hydrochloric acid, saturated with sodium chloride and then extracted successively with ethyl acetate, sodium bicarbonate, and again ethyl acetate. An amount of the ethyl acetate extract thus obtained, corresponding to 1 mg of creatinine in the original urine, is submitted to two-dimensional paper chromatographic

separation with, first, an alkaline solvent (containing isopropyl alcohol, ammonia, and water) and, second, an acidic solvent (containing benzene, propionic acid, and water). Detection of the phenolic acids is achieved with UV light, diazotized sulfanilic acid, diazotized *p*-nitroaniline, *p*-dimethylaminobenzaldehyde in acetic anhydride, as well as with silver nitrate.

Ion exchange has also been used for the fractionation of aromatic compounds from urine (S24).

4. Organic Acids in Blood and Urine in Normal Subjects

4.1. Acids of the Tricarboxylic Acid Cycle

4.1.1. *Occurrence of the Acids of the Tricarboxylic Acid Cycle in Blood and Urine*

4.1.1.1. *Historical Development.* Citric acid was the first of these acids to be identified in blood and urine. A few studies were later concerned with α-ketoglutaric and succinic acids in these biological fluids, until the development of chromatographic techniques allowed the determination of all acids of the tricarboxylic acid cycle, except the unstable ones (oxalosuccinic and oxalacetic acids). Besides the chromatographic techniques, new enzymatic and fluorimetric methods have been described for some of these acids, including oxalacetic acid.

4.1.1.2. *Citric Acid.* Thunberg (T21) has summarized the steps which led to the identification of citric acid in urine. Found in lemon juice by Scheele in 1784, citric acid was considered a typical plant acid until its presence was shown in milk in 1888. Classified among the normal metabolites by Thunberg since 1910, this acid was found first in animal urine after administration of citrate, then in normal human urine by Amberg and McClure (A6). Later on, it was found also in normal human plasma by Benni *et al.* (B9).

Citric acid forms a constant spot on the chromatograms of organic acids of plasma or urine obtained by the general technique summarized earlier. This spot is, however, badly separated from the starting point, due to interference by residual quantities of phosphate and sulfate. Such chromatographic techniques are therefore not suitable for estimating the citric acid content of blood or urine.

Many chemical methods are available for the determination of this acid; most of them include the transformation of citric acid into pentabromoacetone by oxidation and bromination, followed by the colorimetric estimation of pentabromoacetone (B15, B35, C3, E7, G8, M1, M3, N3, N4, S37, T4, T5, T7, W4). All these techniques, of which a critical study

has been made by Taufel and Ruttloff (T3), do not give equally satisfactory results, especially with respect to the elimination of interfering substances (N17). Citric acid can also be determined by use of the Furth-Hermann reaction (F17) (red color with pyridine and acetic anhydride) (N7, S1); the use of this reaction is, however, not advisable for the determination of citric acid in complex media, like plasma or urine. The enzymatic determination of citrate with citrase (B29), on the contrary, gives the possibility of determining this acid specifically.

Nordmann *et al.* (N17) have recently found 1.54 ± 0.09 mg% citric acid in blood plasma from 46 normal human subjects. This plasma level does not differ significantly in males and females (R4), but depends on the age of the subject. Rechenberger and Benndorf (R4) have shown, indeed, that the plasma citrate level falls with increasing age (being, for instance, 1.70 ± 0.28 mg% between 11 and 20 years and 1.15 ± 0.24 mg% after 60 years).

The amount of citrate in the blood plasma is correlated with the amount of ionized calcium. Any increase in plasma citrate reduces the ionized calcium, which, as will be discussed later, can cause the clinical disturbances known as citrate intoxication (B36, D17, L18, W11).

In contrast to plasma, human erythrocytes have a very low citric acid content (N3).

The normal urinary excretion range of citric acid is 200–1000 mg/day, as has been established by Østberg (O12). The urinary citrate plays a fundamental role in keeping calcium in solution and so preventing the precipitation of calcium salts in the urinary tract (C9, H8, S20). This role is illustrated by the frequency of calcium lithiasis after administration of substances which, like acetazoleamide (Diamox), reduce the urinary citrate without affecting the urinary calcium (G16).

The mechanism of urinary elimination of citric acid has been studied in dogs by Herrin and Lardinois (H21, H22). Most of the filtered citric acid is reabsorbed in the tubules, the citric acid clearance in that animal being less than 3 % of the glomerular clearance.

Citrate is present in urine at birth, even before the newborn gets fed for the first time; the urinary citrate elimination is, however, relatively smaller in children than in adults (B24).

4.1.1.3. *Aconitic Acid.* Aconitic acid has been shown to be a constant constituent of urine (N20, O5). The violet color given when sprayed with *p*-dimethylaminobenzaldehyde (N15) makes its identification easy on paper chromatograms of urinary organic acids.

It is, however, found only occasionally on chromatograms of normal

human plasma sprayed with the same sensitive reagent (N19). In rat blood its concentration is less than 0.1 mg% (F16).

4.1.1.4. *Isocitric Acid.* Isocitric acid is not separated from citric acid on standard chromatograms of organic acids. Special techniques have been described for the paper chromatographic separation of these two acids (C10, P7), but they do not seem to have been applied to biological fluids.

This separation can also be achieved by chromatography on silica gel; with such a method, Frohman *et al.* (F16) have found that the concentration of blood isocitric acid is less than 0.1 mg% in the rat.

4.1.1.5. α-*Ketoglutaric Acid.* α-Ketoglutaric acid was first found in human blood and urine by Krebs in 1938 (K13). Many studies dealing with its determination have been made since, using either paper chromatography or electrophoresis as described above under the heading of special techniques for α-keto acids, or enzymatic methods with glutamic dehydrogenase (S11, S38).

α-Ketoglutaric acid being unstable in blood, most workers add the blood to the deproteinizing agent immediately after collection, so that most data are concerned with whole blood and not with plasma or serum.

The most recent figures for the normal concentration of α-ketoglutaric acid in blood lie between 0.095 and 0.16 mg% (M2).

The molar ratio of α-ketoglutaric acid to citric acid in whole blood is about 1:9.3 (G9).

The urinary elimination of α-ketoglutaric acid is higher in human females than in males (M2, Z4). The normal values found by Zelnicek (Z4) are 18.66 ± 4.41 mg/24 hours in females and 11.95 ± 2.52 mg/24 hours in males.

This elimination shows diurnal fluctuations: it is higher during the day than at night (M2). This can possibly be explained by diurnal variations in acid-base balance.

The concentration of α-ketoglutaric acid is much higher in urine than in blood (the reverse being generally true for pyruvic acid) (B18).

The maximum clearance of α-ketoglutaric acid has been found to be about 40 ml/min (that of pyruvic acid being only 7 ml/min) (B18), whereas the clearance calculated over a 24-hour period is about 8 ml/min (that of pyruvic acid being 1.5 ml/min) (Z4).

Besides the citric acid cycle, the transamination reactions play an important part in the production of urinary α-ketoglutaric acid. (C14, C15, N11).

4.1.1.6. *Succinic Acid.* Succinic acid was first detected in human blood serum by Thunberg (T20); its concentration is about 0.5–0.7 mg% (K14, T20). Its spot is generally found on chromatograms of organic acids of blood plasma (N19) (Fig. 2). It was also found in

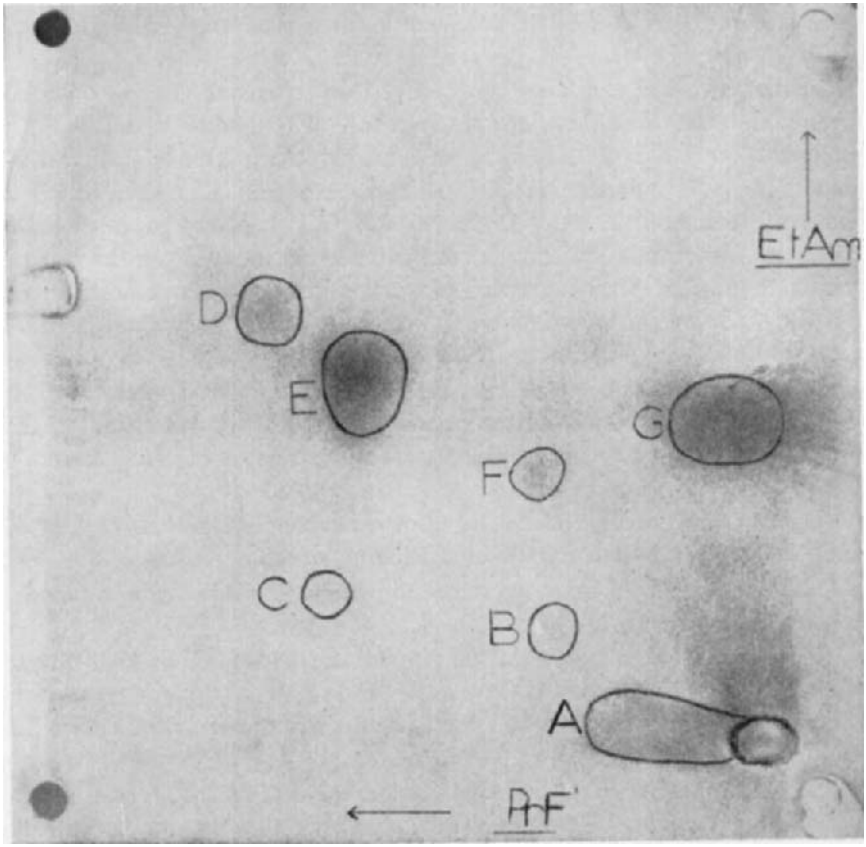


FIG. 2. Chromatogram of the organic acids of a sample of 8 ml of normal blood plasma [Nordmann *et al.* (N12, N19)]. For technical points and significance of *EtAm* and *PrF*, see the legend of Fig. 1. The spots have been located with aniline-xylose and correspond to the following acids: (*A*) citric; (*B*) malic; (*C*) succinic; (*D*) hippuric; (*E*) lactic; (*F*) pyrrolidonecarboxylic; (*G*) hydrochloric.

animal urine (K15, L6), and later in human urine (N20, O5, T16, W7, W9). Weitzel (W7) found a normal excretion of 2–12 mg/day.

4.1.1.7. *Fumaric Acid.* The concentration of fumaric acid in whole blood of normal rats is less than 0.3 mg% (M9). This acid does not appear on chromatograms of normal human plasma (N19), whereas

its presence is frequent, but not constant, on chromatograms of normal urine (N22).

4.1.1.8. *Malic Acid.* Malic acid appears regularly on chromatograms of organic acids of blood (N19) and urine (N20, O5). It can be determined quantitatively by the fluorimetric technique of Hummel (H31), who found values between 0.24 and 0.75 mg% in human whole blood. Much lower values (0.05–0.2 mg%) were found later with the same technique in dog blood plasma (V3).

Concerning the presence of malic acid in urine, the tubules seem to play a double role: they can reabsorb a part of the malate filtered through the glomeruli, and also secrete into the tubular urine malate which has been synthesized within the tubular cells (V3).

4.1.1.9. *Oxalacetic Acid.* Chromatography of the 2,4-dinitrophenylhydrazones of the keto acids shows no oxalacetic acid in blood and urine (B18, D15, E2, K1, S11a) or only traces of it (H19, V1); this fact can be explained by the low concentration and by lack of stability of this acid.

Using an enzymatic technique, Keller and Denz (K6) have recently found a level of 0.38 mg% in whole human blood.

4.1.2. *Factors Influencing the Acids of the Tricarboxylic Acid Cycle in Normal Subjects*

4.1.2.1. *Influence of age.* The influence of age on the blood citrate level has already been discussed. Excretion of acids of the citric acid cycle, as well as of other organic acids, has been studied by Zweimüller and McCance (Z6, Z7) in urine passed before and shortly after birth; the acids of the tricarboxylic acid cycle are already present in such urine samples.

4.1.2.2. *Influence of the diet.* The amount of citrate eliminated in the urine is largely dependent on the diet (B24, K24); more citrate is eliminated, for instance, when the diet is rich in carbohydrate than when it contains a high proportion of casein (S16). The type of carbohydrate is itself important, the amount of citrate eliminated being larger when the diet contains starch than when it contains glucose, galactose, or fructose, whereas sucrose has an intermediate position (M21). The influence of the diet, although obvious, is quantitatively less important in the case of urinary α-ketoglutarate (K17) or succinate (F7, T17, W7).

An interesting observation has recently been made by Fournier and Digaud (F10), who found that a much higher urinary excretion of all the acids of the tricarboxylic acid cycle occurs in rats after adminis-

tration of "structural carbohydrates" (like lactose, cellobiose, glucosamine) than after administration of carbohydrates used mainly for energy production (like starch, maltose, or sucrose). The urinary excretion of calcium is also higher after administration of "structural carbohydrates" than after administration of other carbohydrates, so that the modifications of the elimination of the acids of the tricarboxylic acid cycle in such cases could be related to the calcium elimination (F10).

4.1.2.3. *Influence of Muscular Exercise.* Muscular exercise is followed by a rise in plasma citrate and a fall in urinary citrate (K24), whereas blood α-ketoglutarate (E2) and malate (H31) are not noticeably changed.

4.1.2.4. *Influences of Alterations in Acid-Base Balance.* Alterations in acid-base balance exert a fundamental influence upon the concentrations of the acids of the tricarboxylic acid cycle in urine.

Thus, urinary citrate diminishes after administration of substances which produce an acidosis, whereas it increases after alkali administration (K24, O12, O13, S16).

The increase of urinary citrate after alkali administration provides a means of eliminating the excess of cations without parallel increase in the elimination of fixed anions; citrate would thus play for the economy of fixed anions a role which is similar to that postulated for ammonia in the case of the fixed cations (O12).

These modifications in urinary citrate determined by alterations of the acid-base balance are not parallel to the modifications of the urinary pH (B24, K24) and are not always accompanied by significant variations in plasma citrate (E8, M10, O13).

The main determinant of citrate excretion is probably the intracellular pH of the tubular cells (C11, H10, M25), as is shown by the fact that urinary citrate decreases in potassium depletion (F9), as well as after administration of maleic acid (H9) or acetazoleamide (G15, G16, H10, O11), which produces a metabolic acidosis associated with an alkaline urine.

Clark *et al.* (C11) state that two possibilities are to be considered in seeking to explain the mechanism by which the pH of the tubular cells determines the magnitude of citrate elimination: (*a*) the tubular reabsorption of citrate filtered at the glomeruli is increased by intracellular acidity, or (*b*) at normal plasma citrate levels, excreted citrate is partly or wholly derived from citrate synthesized within the tubule cells.

The second alternative seems the most probable (C11); this opinion

is reinforced by the results of Nordmann *et al.* (N11, N14) comparing the influence on citrate elimination of the administration of sodium salts of acids from the tricarboxylic acid cycle and of equivalent amounts of sodium bicarbonate.

The question arises whether or not the changes in elimination due to the variations in the acid-base balance affect citric acid specifically. Melius and Lipton (M18) thus found that, in experimental alkalosis produced in rats after injection of bicarbonate, only about 50 % of the increase of urinary organic acids is due to citrate. Evans *et al.* (E8), who did not find the same result in man after bicarbonate administration, state also that citrate represents less than 50 % of the increase of organic acids in urine produced in man by hyperventilation.

It thus appears that the elimination of acids other than citric acid is dependent upon the acid-base balance, especially that of α-ketoglutaric acid (M25).

4.1.2.5. *Influence of Hormones and Vitamins.* Hormonal factors also influence the acids of the tricarboxylic acid cycle in blood and urine.

Thus, urinary citrate, which increases after administration of estrogens, is at a maximum at about the 14th day of the menstrual cycle and at a minimum just before menstruation (S18). The variations in α-ketoglutarate elimination show similar variations during the menstrual cycle (M25).

Other hormonal influences have been studied, especially on blood or urine citrate: they include corticosteroids (H11, H16), insulin, (N5), growth hormone (H17), and parathormone (E4, L14, L20).

The influence of parathormone is only one of the numerous examples of the intercorrelations of citrate and calcium metabolisms (C1, H8, M25). Vitamin D is also correlated with citrate (F11, H8, H11, H12). The urinary citrate increases, for instance, after administration of this vitamin, which seems to affect the metabolism of citrate directly and not through prior modifications in calcium metabolism (H8). It is interesting, from that point of view, to quote De Luca *et al.* (D9, D10, D11), who have shown that vitamin D diminishes the conversion of citrate to α-ketoglutarate in rat kidney (but not liver) homogenates and mitochondria; it is possible that this vitamin is connected with the synthesis of phospho- or pyrophosphocitrate (M22).

4.2. Aliphatic Acids Not Members of the Tricarboxylic Acid Cycle

4.2.1. *Ketonic and Aldehydic Acids*

4.2.1.1. *Pyruvic Acid.* Pyruvic acid is the predominant α-keto acid of human blood, whereas α-ketoglutaric acid predominates in human urine

(B18). The mean levels of pyruvic acid in normal human whole blood found by most authors using paper chromatographic techniques are 0.35–0.92 mg% (M2). Specific enzymatic methods (H15, H23, S10, S38) have given similar results; by such a technique, Strohmeyer *et al.* (S38) found, for instance, 0.77 ± 0.19 mg%. The same reasons as those discussed in the case of α-ketoglutaric acid explain why the most reliable data deal with whole blood and not plasma.

The amount of pyruvic acid excreted in 24 hours has been found in recent works to lie between 2.5 and 11.5 mg (Z2, Z3, Z4). As stated before, the amount of pyruvic acid in urine is lower than that of α-ketoglutaric acid; Zelnicek (Z3) thus found 8.16 ± 1.55 mg/24 hours for pyruvic acid and 14.13 ± 3.20 mg/24 hours for α-ketoglutaric acid.

Studies of the renal clearance of pyruvic acid have been summarized when considering α-ketoglutaric acid.

Many factors affecting the pyruvate level in blood and urine, especially muscular exercise, have been studied; they have been reviewed previously (A2, L15, M24, P4, S21, T8). Among the newer studies concerning the effect of hormones on blood pyruvate, we shall only mention that the blood level of pyruvic acid is increased after administration of adrenal steroids, which seem to have an inhibitory effect on the utilization of pyruvate (H18).

4.2.1.2. *Glyoxylic Acid.* Trace amounts of glyoxylic acid have been detected by some workers in normal blood and/or urine (B18, H19, K1, V1, Z2), whereas others did not find it on normal chromatograms of 2,4-dinitrophenylhydrazones (D14).

We shall refer again to this important metabolite when considering the endogenous synthesis of oxalic acid.

Glyoxylic acid can also be formed from γ-hydroxyglutamic acid (D8).

4.2.1.3. *Other α-Keto Acids or Aldehydic Acids.* α-Ketoisocaproic acid, the keto acid corresponding to leucine, was found in 1955 in human blood serum by Biserte *et al.* (B19). The same authors did not find it in urine (B18), although others found traces of it in normal urine (K1, V1).

More recently, Henning and Ammon (H19) described 10 aliphatic keto and aldehydic acids in normal urine. In addition to α-ketoglutaric, oxalacetic, pyruvic, glyoxylic, and α-ketoisocaproic acids, they found hydroxypyruvic, α-keto-γ-methylthiobutyric, α-keto-β-hydroxybutyric, α-keto-β-methylvaleric and α-keto-*n*-butyric acids, that is to say, the keto acids corresponding, respectively, to serine, methionine, threonine, isoleucine, and α-amino-*n*-butyric acid. They conclude that one finds in normal human urine the keto acids corresponding to all the amino acids normally present in urine, with the exception of those correspond-

ing to cysteine-cystine, valine, lysine, and arginine. Normal urine contains, furthermore, α-keto-*n*-butyric acid, which corresponds to α-amino-*n*-butyric acid, an amino acid which is not present in normal urine.

The same acids were also found in blood serum, with the exception of α-keto-β-hydroxybutyric and of α-keto-*n*-butyric acids (H19).

De Schepper *et al.* (D15) found also α-keto-β-methylvaleric acid in normal whole blood; in contradiction to the previous results (H19) they detected also α-ketoisovaleric acid, the keto acid corresponding to valine. The concentration of this keto acid in normal whole blood of children is 0.13 ± 0.02 mg% (K1). Traces occur also in urine (K1, V1).

Succinic semialdehyde has finally been detected recently in normal human serum and urine by Van der Horst (V1).

4.2.1.4. *Acetoacetic Acid.* Whereas the results discussed previously were obtained by chromatographic techniques, acetoacetic acid cannot be determined by this means because of its instability. Besides older colorimetric or manometric methods for its determination, Walker (W1) has described a new technique, based on the color obtained with *p*-nitroaniline, which gives a normal value of 0.31 mg% acetoacetic acid in normal whole blood.

4.2.2. *Hydroxy Acids (Monocarboxylic)*

4.2.2.1. *Lactic Acid.* Lactic acid is the predominant aliphatic hydroxy acid in normal blood plasma.

It is not easily studied by paper chromatographic techniques because of its volatility and of its partial transformation into lactyllactic acid (O1).

Most investigations have employed the technique of Barker and Summerson (B1) (transformation into acetaldehyde, which is estimated colorimetrically by its reaction with *p*-hydroxydiphenyl) or a modification of the original technique (B20, H30, M6, T25). More recent studies have been made with specific enzymatic methods using lactic dehydrogenase (H27). The stabilization of lactic acid in blood samples has been studied by Long (L16).

The normal concentration of lactic acid in blood plasma found by most authors lies between 8 and 17 mg% in resting subjects (K14).

We shall not consider the factors which influence lactic acid in blood and urine, as such factors have been previously reviewed (F2, K5, P4).

4.2.2.2. β-*Hydroxybutyric Acid.* β-Hydroxybutyric acid is present in normal human plasma (at a concentration of 0.3–0.9 mg%) (K14) and in normal urine (F2).

The level of this acid increases both in blood and urine after muscular activity or during fasting (F2).

4.2.2.3. *Glycolic Acid.* Glycolic acid has been detected on chromatograms of the organic acids of normal human urine by Nordmann *et al.* (N22). It is connected to the metabolic pathway leading from glycine to oxalic acid, as will be pointed out later on.

4.2.2.4. *Glyceric Acid.* Isherwood *et al.* (I3) found glyceric acid in an amount of 2.6 mg/day in the urine of normal rats. Glyceric acid was also found in normal human urine by Osteux and Laturaze (O6).

The origin of glyceric acid is uncertain. It might be derived from 3-phospho-D-glyceraldehyde (by dehydrogenation to 3-phospho-D-glyceric acid and loss of a phosphate group) or be provided by the oxidation of glycerol (I3).

In the rat, the synthesis of D-glyceric acid seems to be connected with that of L-ascorbic acid; administration of chloretone increases the urinary elimination of both acids (cf. Article by W. E. Knox and M. N. D. Goswami, Section 3.4, this volume). D-Glyceric acid is not, however, a direct precursor of L-ascorbic acid, and Isherwood *et al.* (I3) state that the connection between these two acids may lie only in that both require some common enzyme or coenzyme system.

4.2.3. *Dicarboxylic Acids* (Fig. 3)

4.2.3.1. *Oxalic Acid.* Oxalic acid is present in normal urine and blood (F2). It cannot be determined by the standard chromatographic technique described earlier, since it gives a streak and not a well-defined spot (N21). Many colorimetric techniques, which are, however, not absolutely specific, have been described for its determination; the most recent ones have been developed by Archer *et al.* (A8) and Dempsey *et al.* (D12).

Its normal daily elimination in urine lies between 15 and 50 mg (D12), whereas its level in blood serum is about 0.2 mg% (F2).

A part of the urinary oxalate is of dietary origin, arising principally from vegetables. Ingested oxalic acid is eliminated in the urine, since it is not appreciably oxidized *in vivo* and since there is no evidence of its entering metabolic pathways rather than being excreted (C19, J4, W6). A part of the ingested oxalic acid is, however, oxidized by intestinal microorganisms (B13).

Another part of the urinary oxalate is of endogenous origin. It is a product of ascorbic acid metabolism in rats (C19) and in man (H14, L3). The metabolic pathway leading from ascorbic to oxalic acid is unknown.

Besides ascorbic acid, glycine is the most important source of oxalic acid (W5). The metabolic pathway leading from glycine to oxalic acid via glyoxylic acid (which can be readily converted to glycolic acid) and the enzyme systems involved in that pathway have been recently

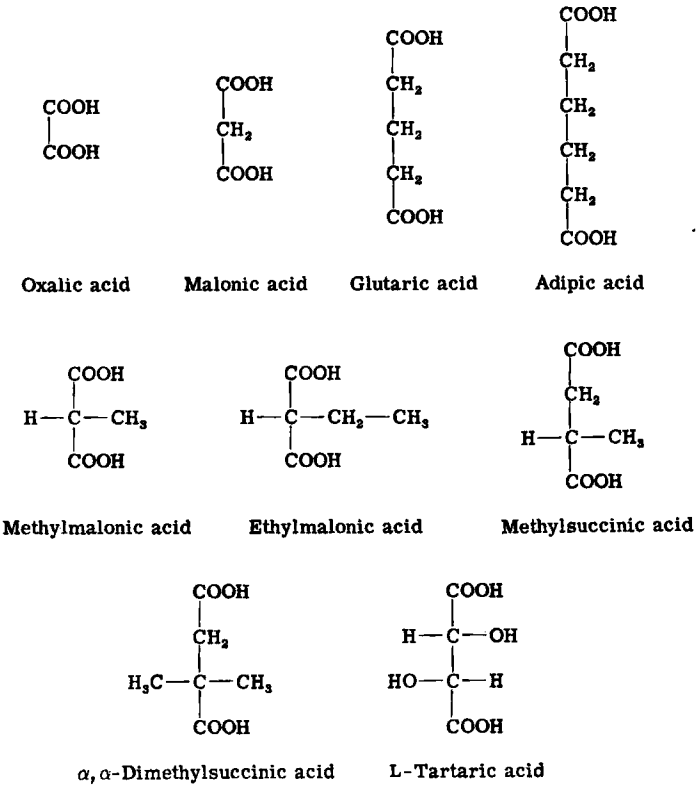


FIG. 3. Dicarboxylic acids not members of the tricarboxylic acid cycle found in normal urine.

reviewed by Elder and Wyngaarden (E1). This pathway is shown in Fig. 4.

4.2.3.2. *Malonic Acid.* Thomas and Kalbe (T11) have detected malonic acid in normal human urine.

The origin of this malonic acid is uncertain. It could be of dietary origin, since malonic acid is present in vegetables (B10). This, however, seems improbable, since malonic acid is converted to acetoacetic acid in animal tissues (N1, W12) and since only 1–2 % of administered malonic acid is found in the urine of rats (T14).

Asparagine administration to rats leads to an increase of malonic acid in urine, whereas aspartic acid has no such effect (T14). Less than 1 % of the asparagine administered, however, gives rise to urinary malonic acid. The fact that the β-carboxylic group is masked as amide seems to be responsible for the degradation of a part of the asparagine by decarboxylation and deamination at the α-carboxyl group, whereas the main part (and, in the case of aspartic acid, the totality) enters the tricarboxylic acid cycle through oxalacetic and fumaric acids (T14).

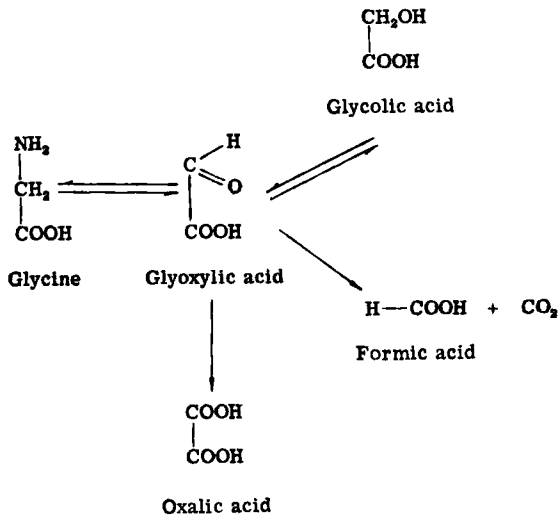


FIG. 4. Scheme showing the pathway leading from glycine to oxalic acid and the other metabolic transformations of glyoxylic acid.

Other possible sources of malonic acid are pyrimidine bases (T14), as well as malonyl-CoA formed during fatty acid synthesis.

4.2.3.3. *Glutaric Acid.* Glutaric acid has been found by paper chromatography to be a regular constituent of normal human urine (N20, O5).

Its elimination is about one-third of that of its inferior homolog, succinic acid, and can be as high as 2.5 mg/day in normal subjects (T15).

The urinary elimination of glutaric acid increases in rats after administration of L-lysine (T15), a fact which is in accordance with the known metabolic route leading from lysine to glutaric acid via pipecolic acid (R8) (Fig. 5). Another intermediate of that metabolic sequence, α-ketoadipic acid, is found in urine after administration of lysine to the rat, the amount representing about 2 % of that of the administered L-lysine

(C4). Boulanger and Osteux (B28) found, however, that in sterilized rats pipecolic acid is the only radioactive compound found in the urine after injection of labeled pipecolic acid, so that this acid does not appear to be transformed rapidly into glutaric acid.

Another possible source of glutaric acid might be tryptophan, since Gholson *et al.* (G10) have shown that administration of labeled tryptophan to rats is followed by excretion of labeled glutaric acid in urine.

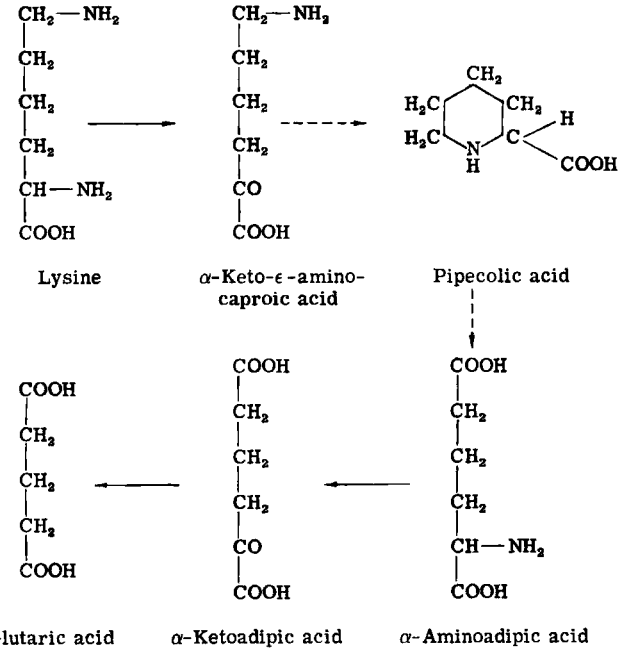


FIG. 5. Main intermediates in the pathway leading from lysine to glutaric acid. The sign - - - - → represents the stages where further intermediates have been omitted from the scheme.

The hypothetical pathway would lead from tryptophan to glutaric acid via 3-hydroxyanthranilic and *cis*-glutaconic acids.

4.2.3.4. *Adipic Acid.* Adipic acid was first shown in normal urine during paper chromatographic studies (N13); the daily normal elimination lies between 1.3 and 2.5 mg (T18) but increases after administration of ε-aminocaproic acid, a part of which would be transformed into adipic acid by oxidative deamination (T18). After administration of adipic acid, one finds a larger amount in the urine than when administering glutaric acid (W8).

4.2.3.5. *Methylmalonic Acid.* Methylmalonic acid can be separated

from its isomer, succinic acid, by means of paper chromatography with special solvents (K3) or by silica gel chromatography (T19).

It was first found in the urine of rats fed anthracene and, later, in the urine of rats on a necrogenic diet (F6), and in the urine of normal

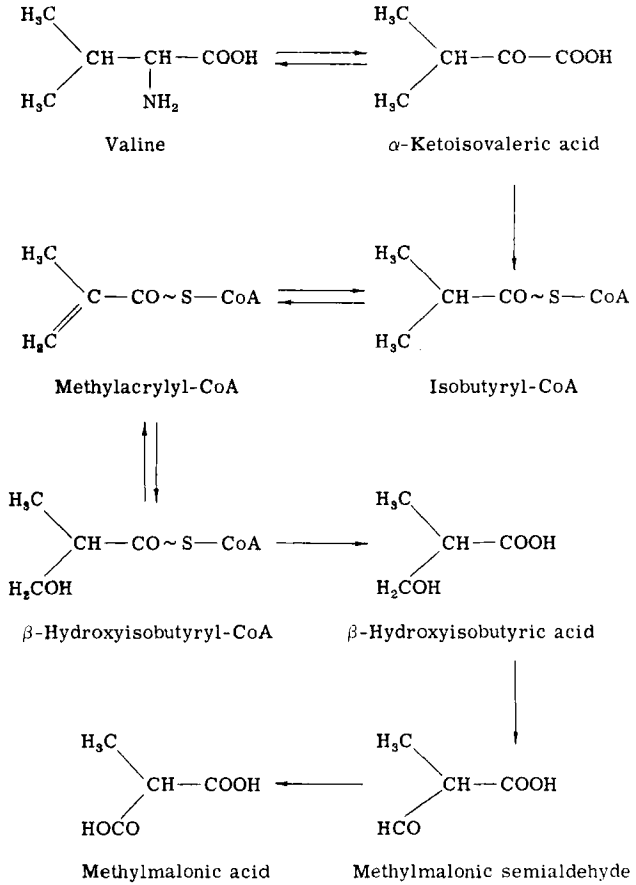


FIG. 6. Pathway leading from valine to methylmalonic acid. Methylmalonic acid could arise through direct oxidation of methylmalonic semialdehyde, as shown in the scheme, or through deacylation of methylmalonyl-CoA, which would represent an intermediate (not shown on the scheme) between methylmalonic semialdehyde and methylmalonic acid.

rats (B3), dogs, and rabbits (T19), and, finally, normal human subjects (T12).

Its elimination increases in rats when valine is administered (B2, T13), which is in accordance with the known metabolic sequence lead-

ing from valine to methylmalonic acid via isobutyryl-CoA (M15) (Fig. 6); the same increase in urinary methylmalonic acid has also been found after feeding rats with isobutyric acid (T13). The increase in urinary methylmalonic acid is accompanied by a parallel increase in urinary succinic acid, probably because of an inhibition of succinic dehydrogenase through methylmalonic acid (T13).

Another source of urinary methylmalonic acid could be methylmalonyl-CoA formed during the metabolism of propionic acid.

Administered methylmalonic acid is mostly metabolized; it has been shown that this acid is converted to methylmalonyl-CoA by liver homogenates (F5).

4.2.3.6. *Ethylmalonic Acid.* Stalder (S35) found ethylmalonic acid in normal urine of rat and man and showed that its excretion by the rat increases when isoleucine is fed. The increase is analogous to that of methylmalonic acid when valine is fed.

4.2.3.7. *Methylsuccinic Acid.* Methylsuccinic acid has been shown to be present in normal urine in about the same amount as glutaric acid (T15). It increases after administration of leucine.

Mesaconic (methylfumaric) acid, which can be formed from methylsuccinic acid, is not present in normal urine (T15).

4.2.3.8. *α,α-Dimethylsuccinic Acid.* First found, like methylmalonic acid, in the urine of rats fed a necrogenic diet (F6), α,α-dimethylsuccinic acid was identified later in the urine of normal rats (B3). Its origin is unknown.

4.2.3.9. *Tartaric Acid.* Tartaric acid is present on normal urinary chromatograms (N20, O5).

It might be of dietary origin, as it is present in many vegetables. It appears, however, that, contrary to previous results, the different isomers of tartaric acid are largely metabolized in the human body when given in small quantities (B4); the products of the enzymatic oxidation of tartaric acid have been studied by Kun and Garcia Hernandez (G4, K21).

Whether or not tartrate is formed in the course of intermediary metabolism is unknown (G4).

4.2.4. *Ascorbic, Dehydroascorbic, and Diketogulonic Acids.*

The methods of determination of these acids in blood and urine have been reviewed by Roe (R7).

Ascorbic acid can be stored in small quantities in the human body. When its concentration in the blood plasma is below 1.1–1.8 mg%, very little ascorbic acid is found in the urine. On the contrary, the urinary

elimination increases sharply when the plasma concentration is higher than this threshold value (F2).

When ascorbic acid is administered in large amounts, urine will contain, besides an increased amount of ascorbic acid, diketogulonic acid, and oxalic acid (ascorbic acid being an endogenous source of oxalic acid, as stated before) (F2).

Dehydroascorbic acid can be found on urinary chromatograms of organic acids (O5).

No diketogulonic acid is found in normal blood (M5).

4.2.5. *Glucuronic and Furan-2,5-dicarboxylic Acids*

Glucuronic acid is present in normal urine (B30, K7, N22, Z2) and blood (F2); the daily excretion varies from 65 to 670 mg (B30).

Phenolic compounds, as well as some steroid hormones and the dicarboxylic amino acids (P10), are found in urine as glucuronic acid conjugates.

Flaschenträger and Bernhard (F3) found that furan-2,5-dicarboxylic acid (Fig. 7) is eliminated in amounts varying from 3 to 4 mg per day

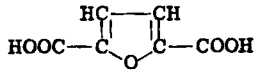


FIG. 7. Formula of furan-2,5-dicarboxylic acid.

in normal urine. Further studies (F3a, F4) showed that this acid is most probably derived from glucuronic acid by an unknown metabolic pathway, which seems to exist only in man. We mention this acid here, therefore, although it is heterocyclic.

4.3. AROMATIC ACIDS

4.3.1. *Historical Development*

The large group of aromatic acids includes mainly phenolic acids, that is to say, aromatic compounds having, besides a carboxylic group, one or more phenolic hydroxyl groups.

One must add nonphenolic aromatic acids, i.e., acids the formula of which includes an aromatic nucleus and a carboxyl group but no phenolic hydroxyl group.

These acids occur in blood and urine either free or conjugated (mainly with glycine, glutamine, glucuronic acid, or sulfuric acid).

Before the development of chromatographic techniques, the aromatic acids of biological fluids were poorly known. Their determination was made as a group by nonspecific colorimetric techniques, most of which

determine at the same time nonacidic phenolic compounds (B6, C13, D7, H28, V4, V5).

Some pathological findings were obtained with such techniques, for instance, the increase of phenolic compounds in blood during renal diseases (B5).

Besides these general colorimetric techniques, some methods had been described for the determination of certain special aromatic acids; this was the case for hippuric acid, the determination of which in urine after benzoate load has been extensively used to test the detoxication function of the liver (Q1); it is indeed known that the conjugation of aromatic acids takes place in the liver and is impaired during liver insufficiency (P2).

As in the field of aliphatic acids, considerable improvement of our knowledge about aromatic acids has been achieved through chromatographic techniques. The number of aromatic acids identified on urinary chromatograms, as well as knowledge of their metabolic precursors, increases progressively; many years will, however, still be necessary to determine the identity and the origin of all the spots detected on such chromatograms.

By applying the special technique summarized before, Armstrong *et al.* (A14) found, in 1956, 43 phenolic acids in normal human urine; about half of them were not identified. Ten of these acids are quite constantly present, seven appear on most chromatograms, whereas the remainder are found only in an occasional sample of urine, or are present in much smaller amounts.

Since that fundamental work, some of the unknown acids have been identified (A11, A16, S14), so that only one acid remains unknown in the group of those found almost constantly or frequently.

The list of acids found by Armstrong *et al.* (A14) in urine is not exhaustive. One of the solvents used for chromatography leads, indeed, to the decomposition of many easily oxidized dihydroxyphenyl compounds, so that other techniques are necessary to characterize these substances, among which would be homogentisic, homoprotocatechuic, and caffeic acids; the same holds true for *o*- and *p*-hydroxy- as well as 3-methoxy-4-hydroxy-phenylpyruvic acids (A14).

Thus, Smith *et al.* (S24) recently found more than 200 aromatic spots on chromatograms of normal urine extracts; many of them correspond, however, to phenols lacking carboxyl groups.

In order to elucidate the origin of the aromatic acids found in urine, three essential possibilities are to be considered (A14): They can be: (*1*) end products of endogenous metabolism; (*2*) acids formed through

the action of intestinal microorganisms; and (3) derivatives of food constituents.

Phenolic acids of dietary origin result essentially from such products as tea, coffee, fruits, vegetables, and flavored beverages (N10). Those acids which are end products of endogenous metabolism are, of course, particularly interesting; the study of their pathological modifications can help to detect a number of diseases.

Most of the recent studies of aromatic acids deal with urine; only little work has been done on such acids in normal and pathological blood plasma (N16, N19).

4.3.2. *Nonphenolic Aromatic Acids*

Two nonphenolic aromatic acids occur as conjugates in large amounts in normal urine: benzoic acid, as hippuric acid, and phenylacetic acid, as phenylacetylglutamine.

Hippuric acid (benzoylglycine) is one of the predominant organic acids in urine. Its elimination can be studied by chemical methods which have, however, a doubtful specificity. Better results are obtained through chromatographic techniques; hippuric acid and its ring-substituted derivatives are easily located, since they give intensely colored azlactones with *p*-dimethylaminobenzaldehyde in acetic anhydride (G1, H1).

The normal daily elimination of hippuric acid lies between 1 and 2.5 g (S36). However, not all the conjugated benzoic acid in the urine is represented by hippuric acid. Part of it is eliminated as benzoylglucuronide (Z5); doses of 10 g of sodium benzoate given *per os* are quantitatively recovered as urinary hippurate and benzoylglucuronide (S4).

Phenylacetic acid, which arises mainly through the action of intestinal microorganisms on protein (F2), is known from the work of Thierfelder and Sherwin (T10) to be conjugated in man with glutamine as phenylacetylglutamine. The only animal which also synthesizes phenylacetylglutamine is the chimpanzee (P11). The steps of this synthesis, which proceeds through phenylacetyladenylic acid and phenylacetyl-CoA, have been elucidated by Moldave and Meister (M26, M27, M28). Phenylacetylglutamine is not the only example of conjugation of an aromatic acid with glutamine; *p*-aminosalicylic acid, for instance, is conjugated with glutamine in the rat (K4).

The normal daily elimination of phenylacetylglutamine is about 250–500 mg (S36).

Phenylacetic acid is conjugated with glycine as phenaceturic acid in the dog and rabbit. This conjugate can also occur in human urine (F2),

although the bulk of phenylacetic acid is present in the form of phenylacetylglutamine, as already stated.

Phenylpyruvic acid was not found on chromatograms of the 2,4-dinitrophenylhydrazones of normal urine by some workers (B18), whereas others (H19) found it in normal urine and blood.

4.3.3. *4-Hydroxyphenyl Acids*

Among the phenolic acids found regularly in normal human urine, Armstrong *et al.* (A14) identified *p*-hydroxyhippuric, *p*-hydroxyphenyl-

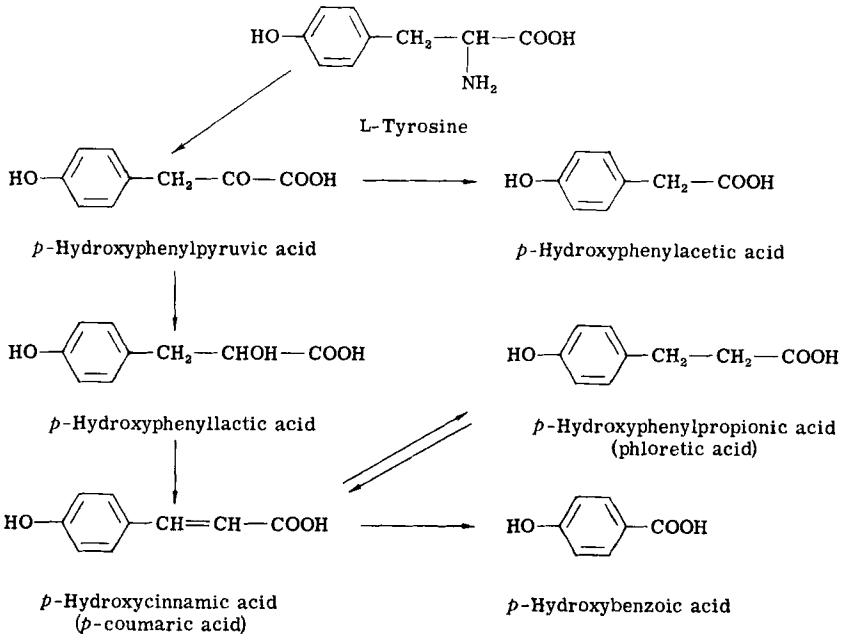


FIG. 8. Scheme proposed by Booth *et al.* (B23) for the formation of urinary 4-hydroxyphenyl acids from tyrosine.

acetic, and *p*-hydroxymandelic acids; among those frequently met are *p*-hydroxybenzoic and *p*-hydroxyphenyllactic; and finally, among the acids occasionally found are *p*-hydroxyphenylpropionic (phloretic) and *p*-hydroxycinnamic (*p*-coumaric) acids, as well as *p*-hydroxycinnamoylglycine (*p*-coumaroylglycine). The amount of *p*-hydroxyphenylacetic acid in normal human urine is 15–31 mg/day (T22). *p*-Hydroxyphenylpyruvic acid was also detected in normal human blood and urine (by chromatography of the 2,4-dinitrophenylhydrazones) (H19, W3).

The origin of the 4-hydroxyphenyl acids of urine was studied very

recently by Booth *et al.* (B23). These authors found in urine of normal rats *p*-hydroxyphenyl-pyruvic, -lactic, -propionic, -acetic, and -benzoic acids, as well as *p*-coumaric acid and *p*-coumaroylglycine, whereas *p*-coumaric and *p*-hydroxyphenylpropionic acids are reported to be absent from the urine of normal man and rabbit. All these acids are metabolites of tyrosine and could be formed according to metabolic pathways suggested in Fig. 8.

The absence of *p*-coumaric and *p*-hydroxyphenylpropionic acids from normal urine of man and rabbit would indicate that the transformation of these acids into *p*-hydroxybenzoic acids (through β-oxidation) is more rapid in these species than in the rat. The same authors found also, in the urine of rats, coumaroylglycine and the ethereal sulfate of *p*-hydroxybenzoic acid, this conjugate being the only metabolite of *p*-hydroxybenzoic acid (B21).

The formation of these phenolic acids from tyrosine does not appear to depend upon intestinal microorganisms (B21). A part of them arises from the diet, as is shown by the strong increase of *p*-hydroxybenzoic acid in urine after ingestion of coffee or bananas (S14).

4.3.4. *3-Hydroxyphenyl Acids*

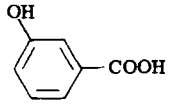
Armstrong *et al.* (A11, A14) have found the following 3-hydroxyphenyl acids in normal human urine: *m*-hydroxy-benzoic, -hippuric, -phenylacetic, -phenylpropionic, and -phenylhydracrylic (Fig. 9).

The last of these, as well as *m*-hydroxyhippuric acid, is found in almost all urine samples, whereas the presence of *m*-hydroxybenzoic and *m*-hydroxyphenylacetic acids is frequent; that of *m*-hydroxyphenylpropionic acid is much less so.

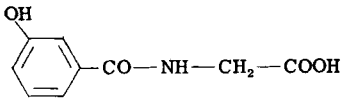
The normal daily excretion of *m*-hydroxyhippuric acid by man ranges from 2 to 150 mg, most subjects eliminating 4–6 mg (A15). The excretion of *m*-hydroxyphenylhydracrylic acid ranges from 2 to 150 mg (A11), whereas the values found for *m*-hydroxybenzoic acid are, respectively, 8.8 mg in human males and 15.0 mg in females (B31), or 10–16 mg (T22).

Although the presence of 3-hydroxyphenyl acids had been detected previously in urine, a widespread occurrence of such compounds had not been suspected before the work of Armstrong *et al.* (A12, A15). These acids probably arise from precursors found in the diet, as is shown by the influence of prolonged administration of chemically well-defined diets (A12), as well as by the big increase in the elimination of *m*-hydroxyhippuric and *m*-hydroxyphenylhydracrylic acids after ingestion of coffee (B22, S14).

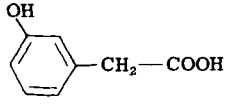
The nature of the alimentary precursors is not clearly explained, as *m*-hydroxyphenyl compounds were not previously known to exist in plant sources (A15). It seems, however, that the *m*-hydroxy-hippuric, -phenylhydracrylic, and -phenylpropionic acids arise from free or combined *m*-hydroxy-cinnamic or -phenylpropionic acids, which probably occur in plant foods (A12). The alimentary precursor does not seem to be *m*-tyrosine; the main metabolite found in urine after ingestion of



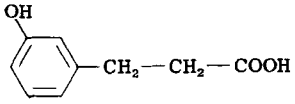
m-Hydroxybenzoic acid



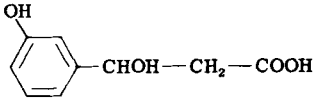
m-Hydroxyhippuric acid



m-Hydroxyphenylacetic acid



m-Hydroxyphenylpropionic acid



m-Hydroxyphenylhydracrylic acid

FIG. 9. 3-Hydroxyphenyl acids found in normal human urine.

m-tyrosine is *m*-hydroxyphenyllactic acid and not one of the 3-hydroxyphenyl acids found in normal urine (A15).

4.3.5. *2-Hydroxyphenyl Acids*

Three 2-hydroxyphenyl acids occur in normal urine (A14): *o*-hydroxyhippuric (salicyluric) acid, which is present almost constantly; *o*-hydroxyphenylacetic acid, which is found frequently in normal urine and whose daily elimination is about 1 mg (A13); and *o*-hydroxybenzoic (salicylic) acid, which is found far less frequently.

The probable precursors of these 2-hydroxyphenyl acids is *o*-tyrosine, which could be formed itself from phenylalanine (A10); phenylalanine would be transformed first into activated phenylalanine, which would give rise mainly to tyrosine, but also, by a side reaction, to *o*-tyrosine.

The pathway leading to *o*-tyrosine is of little quantitative significance in normal individuals; however, it becomes important in phenylketonuria, and we shall see later that this disease is characterized by a considerable increase in the elimination of *o*-hydroxyphenylacetic acid. This acid may be formed from *o*-tyrosine through a pathway suggested by Armstrong *et al.* (A10), which is summarized in Fig. 10.

Tashian (T1) has, however, recently reported an increase of urinary *o*-hydroxyphenylacetic acid in normal man after administration of phenylpyruvic acid. Phenylpyruvic acid might thus be a precursor of *o*-hydroxyphenylacetic acid, so that the formation of *o*-tyrosine is not

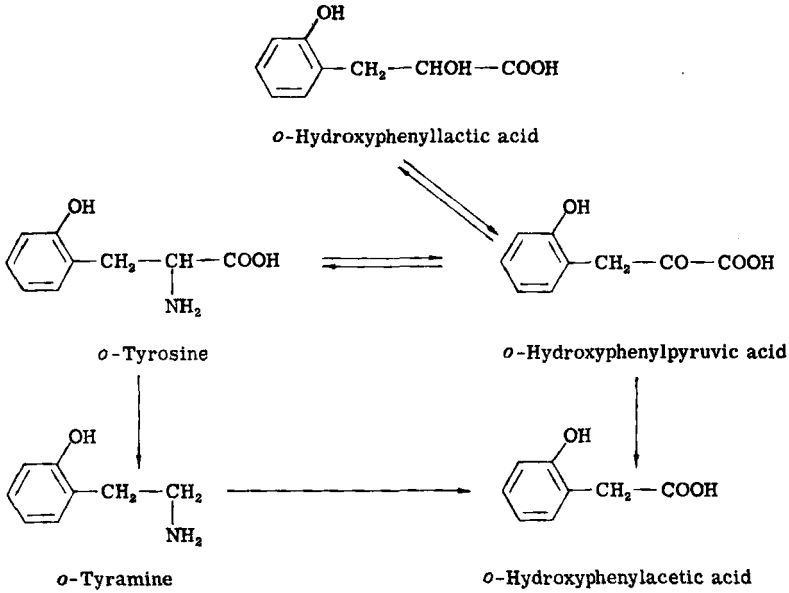


FIG. 10. Pathway suggested by Armstrong and Shaw (A10) for the formation of *o*-hydroxyphenylacetic acid from *o*-tyrosine.

necessary to account for the high excretion of *o*-hydroxyphenylacetic acid in phenylketonuria.

4.3.6. *Phenolic Acids with More than One Hydroxyl Group*

Armstrong *et al.* (A14) have identified only one dihydroxylated phenolic acid in human urine, α-resorcylic acid (3,5-dihydroxybenzoic acid), the presence of which is very irregular. It must be recalled, however, that many of these acids are unstable and could have been decomposed by the alkaline solvent used by these authors and so be lost during the chromatographic separation.

Bray *et al.* (B33) have thus detected conjugated 2,3-dihydroxybenzoic acid, 2,5-dihydroxybenzoic (gentisic) acid, and 3,4-dihydroxybenzoic (protocatechuic) acid in normal rabbit urine.

2,5-Dihydroxyphenylacetic (homogentisic) acid is absent from normal human urine, even after administration of large amounts of phenylalanine or tyrosine, whereas it appears in the urine of rats and mice after such a load (F2).

4.3.7. *3-Methoxy-4-hydroxyphenyl Acids*

The following 3-methoxy-4-hydroxyphenyl acids have been found in normal human urine (A14, A16, S14) (Fig. 11): 3-methoxy-4-hydroxyphenylacetic (homovanillic) and 3-methoxy-4-hydroxy-D-mandelic (vanillylmandelic) acids, which are almost always found; 3-methoxy-4-hydroxyphenylhydracrylic acid, found in most urine samples; 3-methoxy-4-hydroxybenzoic (vanillic), 3-methoxy-4-hydroxycinnamic (ferulic), and 3-methoxy-4-hydroxypropionic (dihydroferulic) acids, as well as vanilloylglycine and feruoylglycine, which are found less regularly.

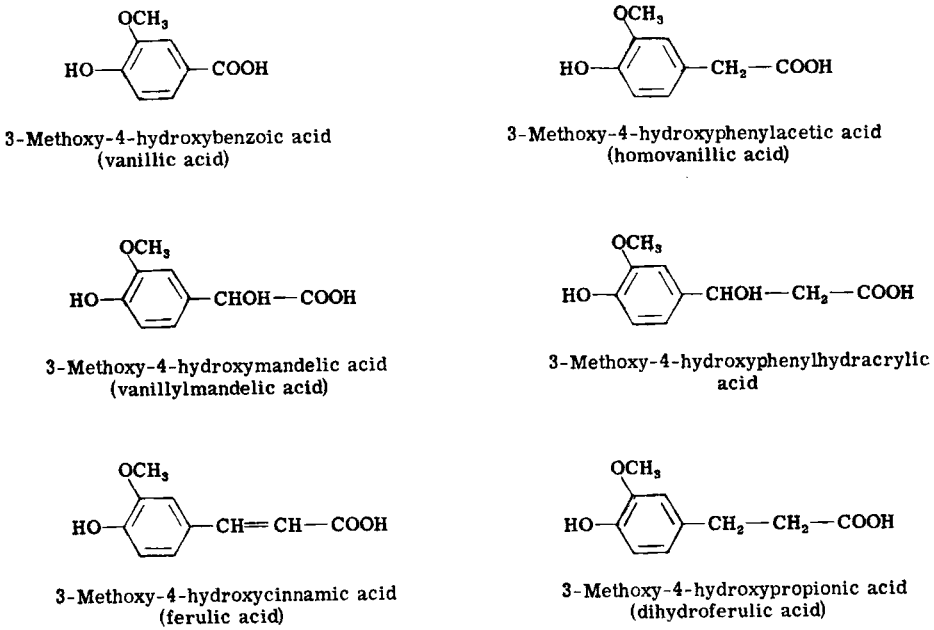


FIG. 11. Main free 3-methoxy-4-hydroxyphenyl acids present in normal human urine.

Vanillic, ferulic, and dihydroferulic acids had been found as early as 1948 in the urine of pregnant mares (L5).

The enzyme found by Axelrod (A20) to be responsible for *O*-methylation of the catechols, namely, catechol-*O*-methyltransferase, plays a fundamental role in the biological synthesis of the 3-methoxy-4-hydroxyphenyl acids.

Homovanillic acid appears to be an endogenous metabolite since Shaw *et al.* (S15) found that the amounts normally excreted (3–8 mg daily) are not changed significantly by ingestion of a well-defined

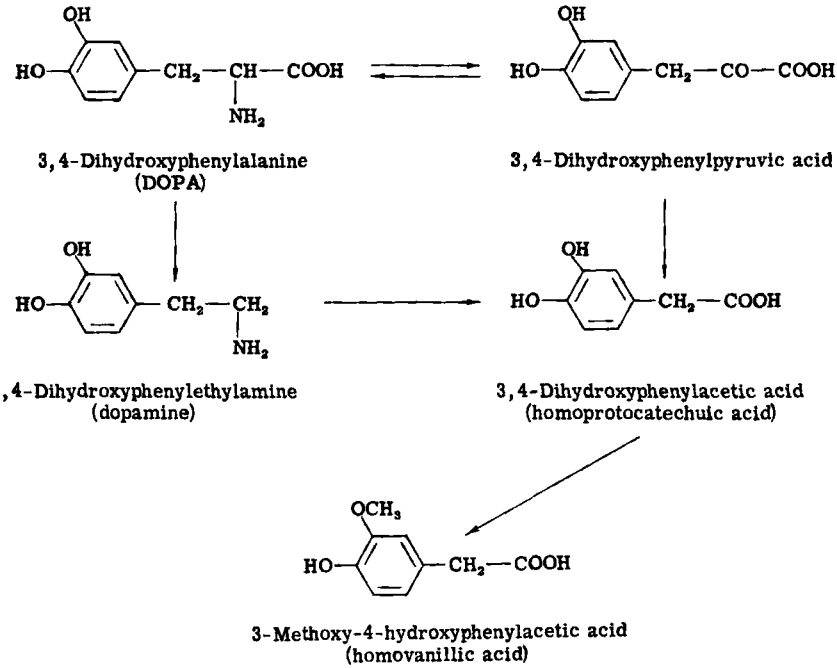


FIG. 12. Probable pathway by which homovanillic acid is formed from DOPA in higher animals [Shaw *et al.* (S15)].

laboratory diet. The same authors found that its elimination is markedly increased after the administration of 3,4-dihydroxyphenylalanine (L-DOPA) or 3,4-dihydroxyphenylacetic (homoprotocatechuic) acid. It is thus probable that homovanillic acid is a natural metabolite of endogenous L-DOPA, homoprotocatechuic acid being an intermediate, which is methylated to yield homovanillic acid, according to Fig. 12.

The elimination of homovanillic acid is also increased during stress; it therefore seems possible that an increased metabolism of 3,4-dihydroxyphenylethylamine (dopamine) is involved and that this amine may play some role in the response of the human subject to stress (R6).

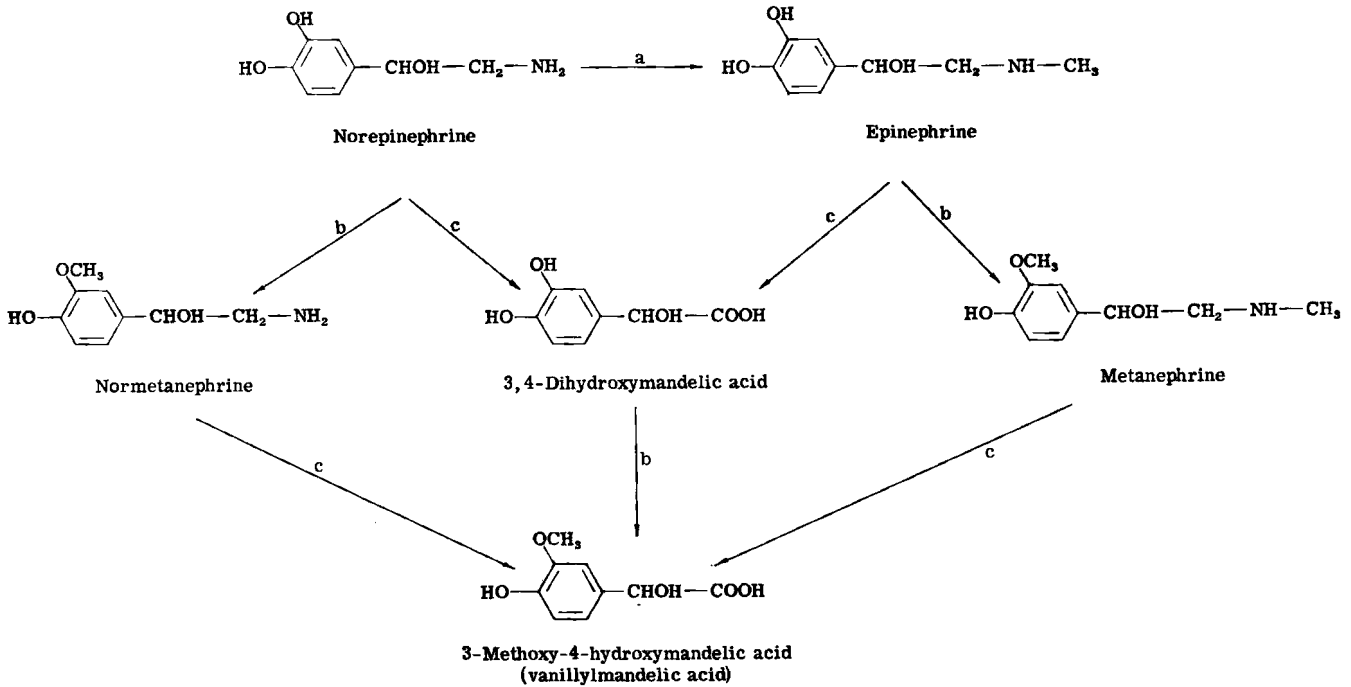


FIG. 13. Formation of vanillylmandelic acid from norepinephrine and epinephrine [adapted from Gitlow *et al.* (G12)]. (*a*) Methylation of the amino group of norepinephrine; (*b*) methylation through catechol-*O*-methyltransferase; and (*c*) monoamine oxidation + aldehyde oxidation.

Vanillylmandelic acid is of special importance, as it is a metabolite of epinephrine and norepinephrine (A16). The pathway leading from those catecholamines to vanillylmandelic acid is summarized in Fig. 13

Its normal daily elimination, studied by Sunderman *et al.* (S41) in 68 adults, ranges from 0.7 to 6.8 mg. This elimination increases during stress (R6).

The study of urinary vanillylmandelic acid is of considerable importance for the detection of pheochromocytoma, as will be pointed out later.

The other 3-methoxy-4-hydroxyphenyl acids, as well as a part of the urinary vanillylmandelic acid, arise from dietary precursors; their elimination increases markedly, for instance, after ingestion of coffee (S16).

However, Smith and Bennett (S29, S30) found an increase in urinary vanillic acid during stress and suggest that a part of this acid might be of endogenous origin and might arise through the metabolism of epinephrine or norepinephrine.

4.3.8. *3-Ethoxy-4-hydroxyphenyl Acids*

Hill *et al.* (H24) have found 3-ethoxy-4-hydroxybenzoic (ethylvanillic) acid in the urine of subjects submitted to prolonged stress or made anoxic by decompression. They suggest that this acid might be either of dietary or endogenous origin. In the latter alternative, it would be formed by biological ethylation of a catechol hydroxyl group, which would represent an alternative to methylation of such compounds. Nolan and Armstrong (N10) state, however, that in the absence of rigorous experimental proof of such a new type of metabolic reaction (biological ethylation), it seems safer to ascribe the appearance of ethylvanillic acid in urine to the intake of ethylvanillin, a component of some artificial vanilla flavorings.

5. Organic Acids in Blood and Urine in Disease

5.1. Introduction

Many data have been published about pathological modifications of a single organic acid, or a few of them, without however allowing a better understanding of the metabolic troubles involved in the disease considered, or without having a diagnostic value. Furthermore, some of these data were obtained with nonspecific techniques of doubtful reliability.

We therefore shall not try to review all the pathological findings which have been made, but shall consider only diseases for which the

study of organic acids in blood and urine has been made on a basis wide enough to help understanding or to give aid in detecting the pathological process. Metabolic diseases will be considered first, followed by diseases affecting one tissue, i.e., neoplastic diseases, as well as cardiac, liver, and renal failure.

A few facts concerning pathological modifications of citric acid in blood or urine will be discussed before considering these diseases.

Extensive work has been done on modifications of citric acid metabolism, which appear especially interesting because of the interrelationship between citrate and calcium in blood and urine, and of the numerous factors which are known to affect the citrate level in normal subjects.

A condition described as "citrate intoxication" has been found in some patients after transfusion of large amounts of citrated blood (B36, D17, L18, M12, W11). It associates a large increase in blood plasma citrate with a decrease in the ionized fraction of calcium, a decrease which may be responsible for the clinical symptoms encountered.

Considering the role of urinary citrate in relation to solubility of urinary calcium, modifications of the urinary citrate level are interesting to study in patients with urolithiasis. We recall that the association of hypocitraturia and calcium stones has been found in some varieties of renal tubular acidosis, as well as after prolonged ingestion of acetazoleamide; in both cases, hypocitraturia is associated with an often high calciuria in a neutral or alkaline urine (H10, M25).

Besides these special conditions, a reduction of urinary citrate has been found in patients with spontaneous calcium urolithiasis (K11, S7, S20), but this reduction is inconstant (B24) and may be due to bacterial destruction of urinary citrate through associated urinary infection (C16, H8). It cannot, therefore, be stated whether or not a reduction of urinary citrate plays a role in the deposition of calcium salts during spontaneous calcium urolithiasis.

The fact that the bone tissue has a very high citrate content (C12) and that many factors, such as vitamin D or parathormone, act both on calcium and citrate metabolism, explains the importance of altered citrate concentration in blood and urine during bone diseases. Such alterations will not, however, be considered here, as they have been previously reviewed elsewhere (H8, L9, N11).

In some endocrine diseases, modifications of the urinary level of citrate have been found which are easily explained by the role of the corresponding hormones on that level. Thus, the urinary citrate output is low in hypoparathyroidism, and rises after treatment with either para-

thormone or calciferol (S20). It is also low in cases of ovarian insufficiency, and increases in such patients after administration of estrogens (S19). Furthermore, the low citrate excretion found during primary aldosteronism is probably related to the associated potassium insufficiency (E8).

5.2. Metabolic Diseases

5.2.1. *Diabetes Mellitus*

We shall not discuss here either the classic increase in the blood and urine levels of ketone bodies during diabetic acidosis or the metabolic origin of such an increase; data about these subjects have been treated in recent reviews (F2).

We shall focus attention rather on studies concerning the altered blood and urine concentrations of pyruvic acid and of the acids of the tricarboxylic acid cycle, since they are capable of giving information about the still unsolved problem, whether or not there exists a definite disturbance of that cycle during diabetes mellitus.

Blood pyruvate concentration is not altered in most cases of diabetes mellitus (A5, D14, S28). Previous results, obtained by use of the colorimetric technique of Friedemann and Haugen (F12) and showing an apparent high pyruvate level during diabetic coma, are erroneous and are due to the interference of acetoacetic acid on pyruvic acid determination with that technique (L11, L12, L13).

Defective utilization of pyruvate has, however, been shown by pyruvate tolerance tests during diabetes mellitus, the abnormality not being related to coincidental factors, such as age, sex, diet, or obesity (M30). Such defective utilization of pyruvate is also shown by abnormalities in blood pyruvate following glucose administration (A5, B34, M31).

The increase of blood pyruvate following insulin administration is, however, comparable to that found in normal subjects (A5).

Data concerning blood citrate in patients with diabetes mellitus are contradictory. Thus, some authors found a slight increase in blood citrate (K18, O8), others (G9) variable values, without any correlation with the modifications of blood glucose or blood ketones. More recently, Rechenberger and Benndorf (R4), studying 54 patients at different stages of the disease, found, on the contrary, a statistically significant decrease in blood citrate; they suggest that this decrease is due to a diminished citrate synthesis related to the increased ketogenesis.

The blood citrate response to glucose administration is normal in most cases of diabetes mellitus; certain patients with such disease

show, on the contrary, abnormalities in that response which can be compared to those observed in animals having been treated with an inhibitor of the tricarboxylic acid cycle, such as malonate (P6).

Like pyruvate, α-ketoglutarate is not modified in a constant manner in the blood of such patients (D14, G9, S28).

The data summarized before, showing the coexistence of usually normal blood pyruvate and α-ketoglutarate levels with a probable decrease of blood citrate and inconstant abnormalities in the response of blood pyruvate and citrate to glucose and pyruvate administration, do not as yet allow a firm statement as to whether or not there exists a definite disturbance in one or more reactions of the tricarboxylic acid cycle during diabetes mellitus.

Other authors have studied the acids of the tricarboxylic acid cycle in the urine of patients with diabetes mellitus.

The elimination of citric acid was shown by many workers to be decreased in diabetic patients (B24, K18, O8, O12); thus, Otto (O8) found that the amount of citrate excreted in urine during diabetic coma is only 2–10 % of the normal values. It is not established whether this hypocitraturia is related to a metabolic abnormality, due to the disease itself, or to the acidosis, or even to a renal failure during coma (O8); it appears, however, that the magnitude in the decrease of urinary citrate is not related to the severity of the acidosis (O8). One must add that, if the results of Rechenberger and Benndorf (R4) concerning the decrease of blood citrate levels are confirmed, diabetic patients present a simultaneous decrease in blood and urine citrate analogous to that found by Nordmann *et al.* (N17) during acute renal insufficiency.

The reduction in urinary elimination in untreated diabetic patients affects not only citric acid. Indeed, Osteux and Laturaze (O7) have shown that the same holds true for the other acids of the tricarboxylic acid cycle; treatment with insulin re-establishes a normal elimination of these acids. Treatment with antidiabetic sulfonamides also increases the urinary citrate in such patients (O10).

5.2.2. *Oxalosis and Primary Hyperoxaluria*

Primary hyperoxaluria has been defined by Archer *et al.* (A7, A9) as a clinical entity characterized by progressive calcium oxalate urolithiasis and nephrocalcinosis beginning in early childhood. It might be associated with disseminated extrarenal calcium oxalate deposits, a condition known as oxalosis (A7); it is not known whether oxalosis always represents a stage in the natural history of primary hyperoxaluria (S9).

These patients have a urinary elimination of oxalic acid which always exceeds the normal values, ranging often from 100 to 200 mg/day and which is not related to exogenous factors (S8).

The excessive endogenous oxalate formation appears to be due to a metabolic change in glycine metabolism. Thus, Scowen *et al.* (S8) found that the administration of labeled glycine to such patients is followed by a prompt and considerable incorporation of the isotope into the urinary oxalate. The excessive glycine incorporation into urinary oxalate was confirmed recently in a patient with primary hyperoxaluria by Elder and Wyngaarden (E1), who found, on the contrary, a normal incorporation in another patient having only inconstant hyperoxaluria.

As the conversion of glycine to oxalic acid proceeds via glyoxylic acid (Fig. 4), primary hyperoxaluria may be due to an excessive conversion of glycine to glyoxylic acid or to a failure in the oxidation of glyoxylic acid.

Glyoxylic acid can be degraded normally to formic acid and CO_2, but this degradation seems inherently limited (S8). Glyoxylic acid can also transaminate with glutamic acid to form glycine and α-ketoglutaric acid, and it has been suggested by Gershoff *et al.* (G6) that an inhibition of that transamination might be responsible for the increased oxalate formation in primary hyperoxaluria.

The same authors have shown that oxalate nephrocalcinosis and hyperoxaluria can be reproduced in animals such as rats (G6) or cats (G7) by vitamin B_6 deficiency. The main difference between the experimental disease and patients with oxalosis is the absence of extrarenal deposits in the former (G7). The urinary excretion of oxalate in vitamin B_6-deficient rats can be enhanced by dietary supplementation with vitamin B_6 antagonists, such as deoxypyridoxine or isonicotinic hydrazide, or with glycine, which appears to be the endogenous source of the excessive oxalate production, as in patients with oxalosis (G6).

These findings are of primary interest, as they might relate abnormal oxalate production in humans to vitamin B_6 deficiency. It has been found, for instance, that vitamin B_6 supplements decreased oxalate excretion in human subjects receiving diets which seemed more than adequate in vitamin B_6 (G7). The role of vitamin B_6 in the production of primary hyperoxaluria and oxalosis in human subjects has, however, not been demonstrated.

It must be added that a constant increase in urinary oxalic acid is found only in patients with primary hyperoxaluria and not in all patients with urinary oxalate stones.

Thus, Hodgkinson (H26), studying urinary oxalate in patients with

nephrocalcinosis, found that only a few of his patients with calcium oxalate stones excreted increased quantities of oxalic acid and that marked hyperoxaluria appeared to be rare. Dempsey *et al.* (D12) very recently confirmed this finding and stated that, as, in the series studied, oxalate excretion was within the normal range in all but three of the 35 patients with calcium oxalate stones, the correlation between magnitude of oxalate excretion and the occurrence of oxalate stones in such patients is poor; they add that the value of restriction of oxalate intake in such conditions is, therefore, also problematic.

5.2.3. *Phenylketonuria*

The abnormalities in the urinary excretion of aromatic acids during phenylketonuria have been recently reviewed (S25) and will only be summarized here.

Besides an increase in urinary phenylalanine, phenylketonuric patients have a large excretion of phenylpyruvic and phenyllactic acids (J1), as well as of phenylacetic acid conjugated as phenylacetylglutamine (W16).

The increase in the urinary excretion of phenylpyruvic acid has been currently used as a means of detecting phenylketonuria, and a great many techniques have been described for the determination of this rather unstable acid (B14, K8, K9, K16, M19, M20, R10, S2, T9). Phenylpyruvic acid has also been detected in the blood of such patients (J2).

Phenylacetylglutamine is more stable and is readily determined by the standard chromatographic technique described earlier for urinary organic acids; the large increase of its spot is obvious during phenylketonuria (N12) on urinary chromatograms sprayed with the starch-iodide reagent after chlorination (R11). The increase in phenylacetylglutamine is related to the excessive formation of phenylacetic acid, which has been reported to be toxic to the central nervous system (S17) and which might be responsible for the mental defect.

Apart from the increased excretion of nonphenolic aromatic acids, there is a large excretion of 2-hydroxyphenyl and 4-hydroxyphenyl acids, whereas the 3-hydroxyphenyl acids present in normal urine are reduced or absent (B27).

The elevated 4-hydroxyphenyl acids are represented mainly by *p*-hydroxyphenyllactic and *p*-hydroxyphenylacetic acids; due to its instability, *p*-hydroxyphenylpyruvic acid is less regularly found (B26).

The main 2-hydroxyphenyl derivative is *o*-hydroxyphenylacetic acid, which is the predominant phenolic acid in the urine of phenylketonuric

patients. Armstrong *et al.* (A13) found that, in such patients, the amount excreted ranged from 100 to 400 mg/g creatinine, instead of less than 1 mg in normal individuals. The excretion of this acid is probably not related in any direct manner to the occurrence of the mental defect, but depends upon the phenylalanine level in the blood. After administration of phenylalanine resulting in elevated phenylalanine blood levels, normal individuals excrete *o*-hydroxyphenylacetic acid in the same manner as phenylketonuric patients (C18). The pathway leading to the formation of that acid was discussed earlier when considering 2-hydroxyphenyl acids in normal urines (Fig. 10).

The existence of a multiple specific enzymatic deficiency during phenylketonuria has been suggested by Boscott and Bickel (B26) to explain the abnormal excretion of aromatic acids other than phenylpyruvic acid, phenyllactic acid, and phenylacetylglutamine, as well as that of indolic acids. Jervis (J3) has, however, stated very recently that it is not necessary to postulate such a multiple enzymatic deficiency, since the complete biochemical urinary picture of phenylketonuria (including the presence of phenyl, hydroxyphenyl, and indolyl compounds) can be obtained temporarily in normal individuals following ingestion of large amounts of phenylalanine.

5.2.4. *Alcaptonuria*

The appearance of homogentisic acid is the classic feature of alcaptonuria, which is due to a specific defect in homogentisic acid oxidase activity (L2).

Besides chemical methods for estimation of homogentisic acid (N9), chromatographic techniques have been described for the study of the reducing substances present in the urine of such patients (F1).

5.3. Diseases Affecting One Tissue

5.3.1. *Tumors*

After having reviewed the few data published about modifications of the organic acids in patients with visceral cancers and neoplastic blood diseases, we shall focus our attention on the problem of pheochromocytoma, and add some results concerning neuroblastoma.

Contrary to previous results stating that the blood citrate level was not altered in patients with *visceral cancers* (G9, R9), a significant decrease of that level was found by Rechenberger and Benndorf (R5) in a series of 29 patients with different localizations of visceral neoplasms. These authors relate that finding to an increase in citrate catabolism.

Such an explanation could account for the finding that the blood citrate elevation following citrate injection is more evanescent in such patients than in normal subjects (K2).

In contrast to patients with visceral forms of cancer, those suffering from *chronic leukemia* or *Hodgkin's disease* have normal blood citrate levels (R5, R9).

Pheochromocytoma can best be detected by studying the urinary elimination of vanillylmandelic acid. Armstrong *et al.* (A16) first demonstrated that the elimination of this metabolite of epinephrine and norepinephrine is greatly increased in such patients. The amount found in urine ranges, generally, from 10 to 100 times that of epinephrine and norepinephrine (G13), so that the study of the increase in urinary vanillylmandelic acid appears to be a better way of detecting pheochromocytoma than the study of the increase in the catecholamines themselves. Furthermore, an abnormal elimination of vanillylmandelic acid together with a normal excretion of catecholamines can occur in patients with pheochromocytoma in the normotensive state (K12); this fact shows the superiority of the vanillylmandelic acid determination upon the determination of catecholamines for the detection of these tumors.

The urinary elimination of vanillylmandelic acid returns to normal levels in patients with pheochromocytoma following excision of the tumor; it is normal in hypertensive patients other than cases of pheochromocytoma (S41).

Various techniques, which are rather time consuming for routine use, have been described for the quantitative determination of vanillylmandelic acid in urine (G12). Rapid and more simple techniques, however, allow the semiquantitative estimation of that acid and are especially useful for screening hypertensive subjects for the presence of a pheochromocytoma. Among these rapid techniques, the one which was recently described by Gitlow and Kruk (G11) allows the use of random urine specimens, whereas the previous ones required that the patient avoid certain foods and drugs prior to the sample collection.

An interesting finding about patients with *neuroblastoma* has been obtained by von Studnitz (S39). This author found in two of six such patients an increase in the urinary metabolites of DOPA; besides dopamine and 3-methoxy-4-hydroxyphenylalanine, 3,4-dihydroxyphenylacetic (homoprotocatechuic) acid, which is not found in normal urine, occurred in the urine of these patients, whereas the elimination of homovanillic acid was increased.

5.3.2. *Cardiac Failure*

Pyruvic and α-ketoglutaric acids are increased in the blood of patients with decompensated cardiac failure; it is likely that these changes are related to the hepatic congestion, as they are not found in patients with mitral stenosis without decompensation (D14).

Some data concerning alterations in the blood concentrations of other organic acids during cardiac failure have been published, but as they concern only a few patients, they will not be reviewed here.

A recent study of blood and urine citrate during myocardial infarction has been made, however, on a large number of patients as well as on animals (P8, P9). It appears that blood citrate is elevated in most cases following myocardial infarction, whereas urinary citrate is greatly reduced.

5.3.3. *Liver Failure*

5.3.3.1. *Acids of the Tricarboxylic Acid Cycle and Related Acids.* An increase of blood *lactic acid* has been found by many authors during liver failure, in patients (B8, C2, M32, N18, S26, S31, S32) as well as in the animal (A19, N2, S6).

Very high values can be found during hepatic coma. The highest blood lactic acid concentration in a series of cases of hepatic coma studied by Carfagno *et al.* (C2) was thus 65.2 mg%, whereas Nordmann *et al.* (N18) found 195 mg% in another case characterized by a fall in the blood pH to pH 7.25 and the presence of a big increase to 32 meq/liter in the total plasma organic acids, of which the lactic acid represented about two-thirds (Fig. 14).

Pyruvic acid is also elevated in the blood in most cases of severe hepatic failure (A4, B16, C2, D5, D14, G17, M4, P1, S12, S32, S38, S40).

Thus, De Schepper (D14) found an increase in blood pyruvate in acute viral hepatitis and in different varieties of hepatic cirrhosis; the highest values were obtained during hepatic coma, whereas no elevation in blood pyruvate was observed in cases of jaundice due to extrahepatic obstruction. Other authors (A4, G17) found normal values in well-compensated cirrhosis.

The alterations of blood pyruvate levels are of limited value in assessing liver dysfunction, since they are not of regular occurrence, are nonspecific (P1), and do not always follow the clinical symptoms (S38).

An increase of *citric acid* in blood during liver failure has been found by many Scandinavian authors; these studies have been reviewed by Hagelstam (H2) and Welin (W10). The citric acid content of the plasma is elevated during hepatitis, but not in patients with extrahepatic

obstruction of the biliary ducts (H2); there is, however, no obvious correlation between the degree of damage of the liver and the increase in blood citrate (W10).

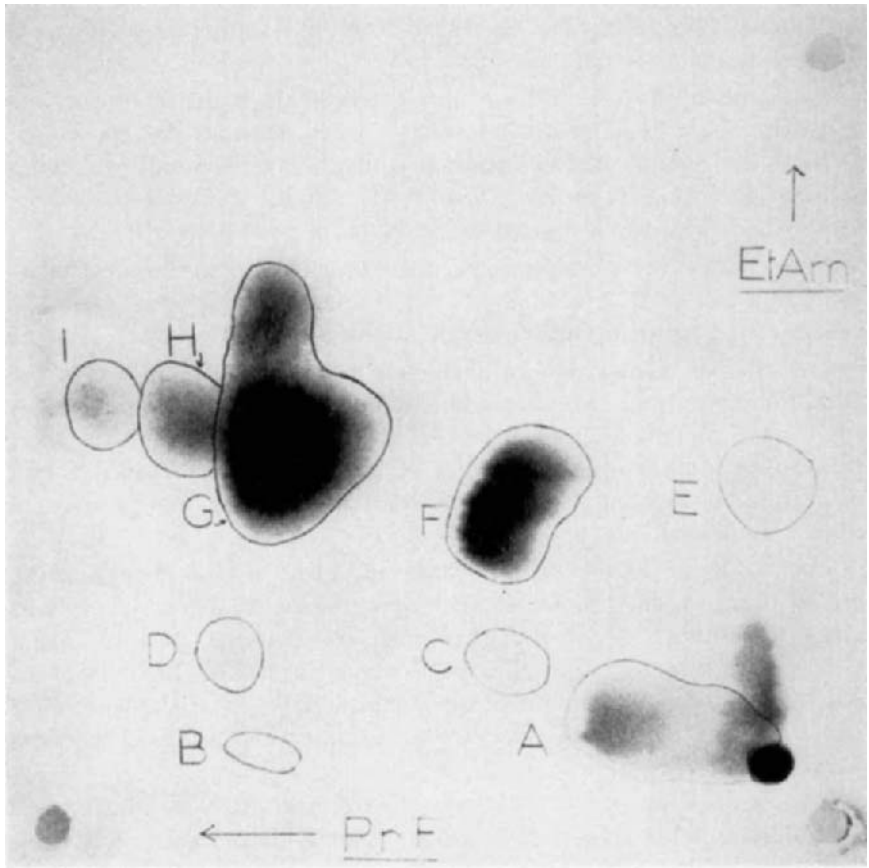


FIG. 14. Chromatogram of the organic acids of a sample of 8 ml of blood plasma during hepatic coma [Nordmann *et al.* (N12, N18)]. For technical points and significance of *EtAm* and *PrF*, see the legend of Fig. 1. The spots have been located with aniline-xylose and correspond to the following acids: (*A*) citric; (*B*) aconitic (?); (*C*) malic; (*D*) succinic; (*E*) hydrochloric; (*F*) pyrrolidonecarboxylic; (*G*) lactic; (*H*) hippuric; (*I*) nonidentified free phenolic acid. The upper appendix of spot G corresponds probably to an unknown acid, badly separated from the lactic acid spot. The lactic acid spot is strongly increased, as compared to chromatograms of normal blood plasma. The chemical determination of lactic acid in this case showed that its concentration was 195 mg%, representing about two-thirds of the total organic acids. The pyrrolidonecarboxylic acid is also strongly increased, whereas the modifications of the acids of the tricarboxylic acid cycle are quantitatively less important.

α-Ketoglutaric acid concentration is also increased in the blood during hepatic insufficiency (C2, D5, D14, G9, S12, S38, S40). The elevation of blood α-ketoglutarate is more constant in liver damage than that of pyruvate (G9, S12) and more important than that of citrate (G9). Furthermore, the changes in the blood level of α-ketoglutarate do seem to follow the clinical symptoms (S38).

Nordmann *et al.* (N11) have also detected an increase of *succinic* and *malic* acids in the blood plasma of a few cases of hepatic coma; although the number of cases studied is at present too small to reach a definite conclusion, it seems probable that all the acids of the tricarboxylic acid cycle are increased in the blood of such patients.

In the light of these findings, it is interesting to discuss the mechanism responsible for the elevation of the blood lactic and pyruvic acids as well as of the tricarboxylic acid cycle acids.

A number of workers have shown that these acids accumulate in the blood during liver failure after administration of these acids themselves or of their metabolic precursors. After a glucose load, for instance, pyruvate accumulates in the blood of such patients, the increase in pyruvate concentration being higher and more lasting than in normal subjects (A5, G5).

Other examples of such accumulation are given by the change in the rate of disappearance of lactic acid from the blood after lactate load during hepatitis (S33), as well as of citric acid after citrate load (S22), or of α-ketoglutarate after glutamate load (S11). The high levels of blood plasma citrate obtained in patients with liver damage after citrate load explain the frequency of citrate intoxication in such patients (B36, M12).

As a result of an experimental study in rats intoxicated with carbon tetrachloride, it has been suggested that those findings can be explained by a slowing down of all the reactions of the tricarboxylic acid cycle (N11).

Similar conclusions have been reached in a study of α-keto acids in the blood of carbon tetrachloride-treated rabbits; it thus appeared that the oxidative decarboxylation of pyruvate and α-ketoglutarate is impaired as a result of the liver damage (D14). The metabolic disturbances involved could lead to a decreased synthesis of adenosine triphosphate and thus determine the symptoms of hepatic coma.

As compared to the numerous studies concerning alteration of the acids of the citric acid cycle and metabolically associated acids in blood, there have been only a few investigations on changes in the urinary

excretion of these acids in liver disease (E6, H4, K18, O9, P8). The frequency of an associated impairment of kidney function makes their interpretation difficult (O9). Aconitic and succinic acids have been reported to disappear from the urine of rats during dietary liver necrosis (B3), whereas the elimination of some acids of the tricarboxylic acid cycle is increased after carbon tetrachloride intoxication (N11).

5.3.3.2. *Other Organic Acids.* Lower values of blood ketones were found after fasting in cirrhotic patients as compared with normal subjects (R2); the same finding of an impaired ketogenesis was also found in experimental liver injury (R3).

A reduction in the blood levels of α-ketoisocaproic, α-keto-β-methylvaleric, and α-ketoisovaleric acids has been reported in patients with hepatic cirrhosis, whereas no significant variation was found during acute hepatitis (D4).

In dietary liver necrosis in rats, methylmalonic acid is greatly increased in the urine, representing 70 % of total ether-soluble acids instead of 10 % as in normal animals; α,α-dimethylsuccinic acid is also increased, but to a smaller extent (B3, F6).

The pattern of aromatic acids is very much altered in both the blood plasma and the urine of patients with liver disease (A17, A18, H13, N8).

We may, finally, recall that the diminished excretion of hippuric acid following benzoate load has been considered as a test of impaired liver function (Q1).

5.3.4. *Renal Failure*

5.3.4.1. *Acids of the Tricarboxylic Acid Cycle.* Whereas earlier data dealt with only a few patients (G9, K18, O9, O12), Pierce *et al.* (P5) studied blood citrate in 23 patients with different forms of renal disease and concluded that the concentration was elevated in most cases.

Nordmann *et al.* (N17), studying a series of 66 patients and using a more specific technique, found, on the contrary, normal blood plasma citrate levels during chronic renal diseases and a significant decrease in blood citrate during acute renal failure, irrespective of the origin of the disease. The finding of a significant decrease in the citrate level of blood plasma in such conditions was confirmed by Walser (W2).

Although numerous studies have pointed out the role of the kidney in the metabolism of citric acid (E5, H3, H20, M10, N11, O2, O3, O4, S23), the nature of the factor responsible for the lowering of blood citrate during acute renal failure is still uncertain; it is interesting to note that the plasma level of calcium is lowered in the same patients (H7), so that

the decrease of both plasma citrate and calcium could be due to the same factor.

The changes in blood citrate concentration are accompanied by a diminished citrate excretion in the urine (K18, O9, R4). This decrease in urinary citrate is a very regular and early finding during renal failure, and Otto (O9) suggests that the determination of urinary citrate should be used as a routine test to assess renal function.

α-Ketoglutarate, the clearance of which is diminished during renal failure (Z4), shows no regular departure from the normal level in the blood of patients with renal failure; normal or lowered values are found in such patients (B18, G9).

5.3.4.2. *Other Organic Acids.* Pyruvic acid, the clearance of which is also reduced during renal failure (Z4), has been found to be slightly increased in the blood of a few patients with chronic renal diseases (B18).

Lactic acid is not altered in a consistent manner during acute renal failure (H5).

The changed concentrations of the acids considered up to now cannot account for the large increase of the organic acid fraction which has been found in the blood of most uremic patients (B7, H6, S13). The same holds true for glucuronic acid, which is slightly increased, and for the ketone bodies, which might be greatly increased, but only in some of the patients with acute renal failure (D13).

In order to find out which acids are actually responsible for the increase of the total organic acids in the blood plasma of uremic patients, Nordmann *et al.* (N16) made a chromatographic study on blood plasma samples from 24 patients with acute renal failure. They found that some of the acids, which are present only in trace amounts on chromatograms of normal blood plasma, are greatly increased, while many others, which are absent from normal chromatograms, appear in the plasma during acute renal failure (Fig. 15).

Thus, spots, which were tentatively identified as glutaric, adipic, and tartaric acids, are present on most chromatograms of blood plasma during acute renal failure, whereas they are absent from chromatograms of normal plasma.

Most of the abnormal spots correspond to aromatic acids; this finding can be related to the increase in blood phenols previously reported in studies using colorimetric techniques (B5, D18, H29, M14, S5). The chromatograms show that hippuric acid is the predominant aromatic acid in the blood plasma of patients with acute renal failure. Its level is often more than 25 times the normal one (N12). It is often accom-

panied by *m*-hydroxy- and *o*-hydroxy-hippuric acids. Phenylacetylglutamine is also present in considerable amounts, whereas it is absent from chromatograms of normal blood plasma (N16).

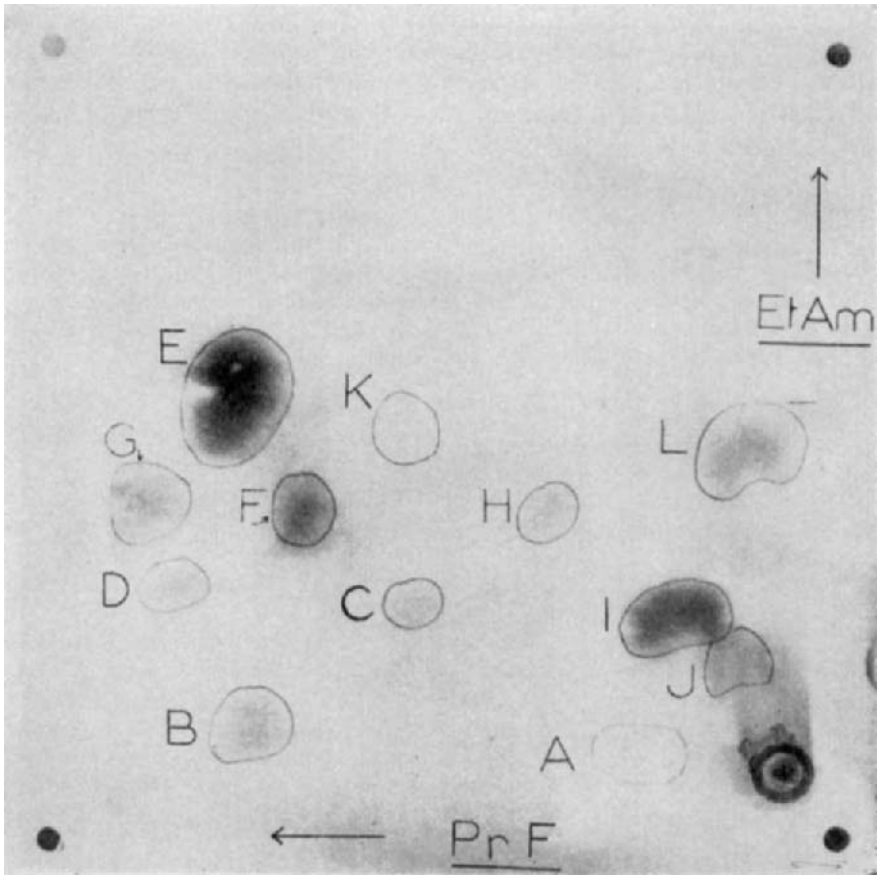


FIG. 15. Chromatogram of the organic acids of a sample of 8 ml of blood plasma from a patient suffering from acute renal failure [Nordmann *et al.* (N12, N16)]. For technical points and significance of *EtAm* and *PrF*, see the legend of Fig. 1. The spots have been located with *p*-dimethylaminobenzaldehyde in acetic anhydride and correspond to the following acids: (*A*) citric; (*B*) aconitic; (*C*) α-ketoglutaric; (*D*) unidentified acid; (*E*) hippuric; (*F*) *m*-hydroxyhippuric; (*G*) *o*-hydroxyhippuric; (*H, I, J*) nonidentified aromatic acids conjugated with glycine; (K) phenylacetylglutamine; (*L*) hydrochloric. The chromatogram differs markedly from chromatograms corresponding to the same volume of normal blood plasma. The chromatograms of normal blood plasma, located with the same reagent, show only spots corresponding to citric, α-ketoglutaric (inconstant), hippuric (in trace amount), and hydrochloric acids.

The increase of phenylacetylglutamine in plasma of patients with renal failure has been confirmed by other workers (F13), and some fractionation of the organic acids from serum was obtained by countercurrent distribution in a single uremic patient by Seligson *et al.* (S13). The results just summarized have thus given information on the nature of the organic acids which can be found in increased amounts in the plasma during renal failure. However, some of the acids present on the chromatogram of blood plasma from patients with renal failure remain unidentified.

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ASCORBIC ACID IN MAN AND ANIMALS[1]

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I. Introduction

Sometime in the evolutionary past, an antecedent of the primates suffered a mutation which inactivated the enzyme which synthesized ascorbic acid. The fresh fruits of his natural diet being full of ascorbic acid, this mutation was of little import to him or his descendants. It became critically important when man developed an agricultural society and could attempt to live on stored foods: to eke out the winter months or to support himself on prolonged military campaigns or voyages of discovery. A similar sporadic mutation occurred in the ancestors of the guinea pig, and perhaps in occasional other species. Until this century when there was a sufficiently sophisticated nutritional science to seek out and exploit this metabolic defect of the guinea pig, man's dependence upon fresh foods had been repeatedly forced upon him at great cost. Over and over again the lesson was learned, effective cures and preventatives devised, and then forgotten. The recorded instances of many of these isolated discoveries from ancient times were reviewed and some were reprinted (L22, D20, C10) on the two-hundreth anniversary of one such discovery. In view of this experience, it is extraordinary that even in 1913 Arctic and Antarctic explorers like Scott continued to die of this disease and that scurvy was a serious problem in some theaters during World War I, only because of ignorance. But even today those who do not or cannot obtain fresh foods only too easily become scorbutic.

Scurvy is now a preventable disease. In the recent past, it became possible to achieve such a practical result best by studying the principles of the problem. The nutritional science of the early twentieth century provided this basis in principle, and the nutritional problem is now essentially solved. An essential nutrient called vitamin C is ascorbic acid. We have amassed a wealth of information about its chemistry, occurrence, the requirements for it, and the deficiency state produced by its lack.

We still do not know how ascorbic acid acts in the body or how it prevents scurvy. Scurvy is clearly a molecular disease, in the sense that all its signs and symptoms should ultimately be referable to the biochemical and physiological consequences of the failure to make the ascorbic acid molecule. This is the next problem. Interest is now turning to the biochemical functions of ascorbic acid in the body and to the use of scurvy and ascorbic acid as reagents to probe deeper into other mysteries about how the body operates.

This chapter reviews the chemical, biochemical, and physiological properties of ascorbic acid and attempts to summarize the significant knowledge gained from nutritional investigations about the nature and

prevention of scurvy in animals. The presentation emphasizes the considerable hiatus in knowledge between the known behavior of ascorbic acid and the consequences of its deficiency. It is to be expected that concentration on the molecular mechanisms of biochemistry and of disease will narrow this gap. Particular emphasis has therefore been given to the most recent advances of this kind, including the mechanisms of biosynthesis of ascorbic acid, to the alteration of ascorbic acid metabolism in different physiological states, and to the specific actions of ascorbic acid in enzyme systems.

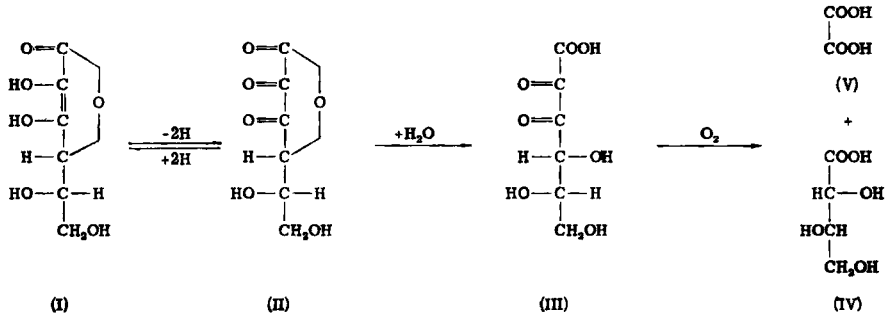
The treatment of the subject is symptomatic of the new way of thinking about ascorbic acid as a metabolite and not as a "vitamin." Since the time when the antiscorbutic factor was labeled vitamin C, our knowledge of essential metabolites has increased so enormously that even the original concept of a "vitamin" has become nebulous. A very large number of specific compounds are known which play unique roles in metabolism. If these cannot be formed by the organism, they must be supplied from the environment. The latter group includes most of the vitamins in the higher animals, although even nicotinic acid can be synthesized by most animals (B34). The metabolites which must be supplied to the organism are needed in amounts grading from the infinitely small requirements of biotin and Vitamin B_{12} to the gross requirements of the amino acids and choline. All of these substances participate in the reactions of metabolism. To preserve the idea of vitamins as something special, the group is arbitrarily divided into those present in vanishingly small amount and whose function must be primarily catalytic, and those others present in sufficiently large amount to make up a significant portion of the organism. Like the essential amino acids, they may, of course, be part of structures that are themselves catalytically active. Ascorbic acid is midway between these groups of "vitamins" and "structural" compounds. It is required in amounts greater than the B vitamins, which act as coenzymes, and in lesser amounts than the essential amino acids, which make up part of the structure of proteins. But L-ascorbic acid-1-C^{14} is not incorporated significantly into the structure of connective tissue (collagen and chondroitin), nor are there significant amounts of other chemical derivatives of it which still possess the carbon 1 of ascorbic acid (B41). Neither has a catalytic role, like that of the B vitamins as coenzymes, been recognized for ascorbic acid. Some of its unique chemical properties would seem to suit it well for such a catalytic role. But this is not ensured simply because it was labeled a vitamin many years ago. It is an essential carbohydrate, indispensable for certain species who cannot make it, whose chemical role is unclear.

The existence of several excellent reviews and treatises on ascorbic acid has made unnecessary the detailed treatment of the older references. The historical aspect of much of our present knowledge on ascorbic acid was discussed in a symposium (L15) on the bicentenary of publication of James Lind's "Treatise of the Scurvy" (L14). In the same year, two other useful reviews were published. Meikeljohn (M18) gave a critical evaluation of the effects of vitamin C on physiology, dealing as he did with different problems related to the field of medicine. More detailed information, less critically evaluated, was presented by Lloyd and Sinclair (L19). The most systematic and comprehensive appraisal of ascorbic acid in all its aspects represents the contributions by a number of workers in the field, compiled in "The Vitamins," (S14). The recent advances in knowledge of biosynthesis of ascorbic acid have been described by Burns (B38) and Burns and Ashwell (B39). There was a recent symposium on ascorbic acid with contributions from many workers (K6).

2. Chemistry of Ascorbic Acid

The chemical nature and synthesis of L-ascorbic acid and its derivatives have been authoritatively reviewed by Smith (S17). References to the early isolation studies, syntheses, and the proof of structure can be found there.

L-Ascorbic acid (L-xyloascorbic acid according to the configuration of its carbons 4 and 5) is a γ-lactone of hexonic acid (I). It behaves like a monobasic acid, a property stemming from the two enol groups. The one on carbon 3 dissociates most strongly (pK's, 4.17 and 11.57). The enol groups are also involved in the most striking property of ascorbic acid, its reversible oxidation to dehydroascorbic acid (II). The latter compound is neutral, having lost its dissociable enol groups. It readily hydrolyzes in water to the free acid, 2,3-diketo-L-gulonic acid (III). With

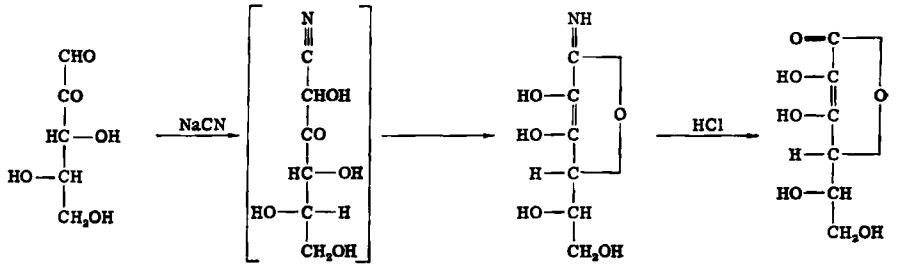


[1]

the opening of the lactone ring, the compound is no longer reducible to ascorbic acid but is readily split oxidatively to L-threonic (IV) and oxalic acids (V). This sequence of compounds represents the usual route of spontaneous decomposition of ascorbic acids (Eq. 1).

2.1. Terminology

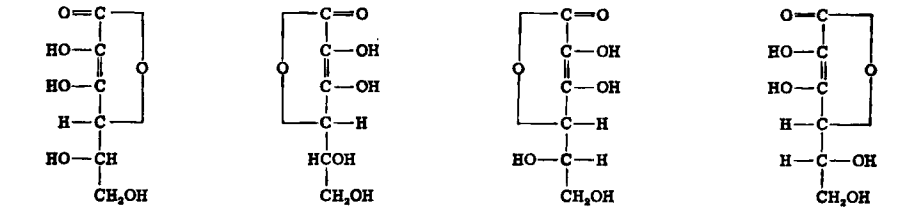
There are two asymmetric carbon atoms (4 and 5) in ascorbic acid and, therefore, four stereoisomers. These are conveniently distinguished



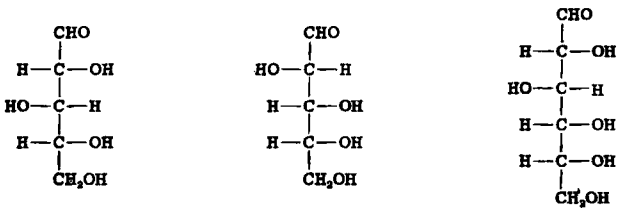
L-Xylosone (L-lyxosone) | Imino-L-ascorbic acid | L-Xyloascorbic acid

[2]

as belonging to either the xylo or arabo series (with carbons 4 and 5 having the opposite or the same configuration, respectively) and to either the D- or L-series as determined by the absolute configuration



L-Ascorbic acid (L-xyloascorbic acid, vitamin C) | D-Ascorbic acid (D-xyloascorbic acid) | L-Isoascorbic acid (L-araboascorbic acid) | D-Isoascorbic acid (D-araboascorbic acid)



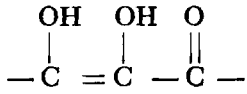
D-Xylose | D-Arabinose | D-Glucose

[3]

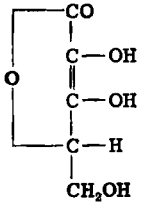
at carbon 5. (The L-form is written with the HO- group to the left of its carbon according to the Fischer convention.) The terminology is based on the starting sugar used in the synthesis by condensation of cyanide with an osone (Eq. 2). This was the reaction used for the first synthesis of a vitamin. The accessibility of the osone starting materials has greatly improved with the introduction of a copper oxidation of available sugars (S7, H3). The four isomers of ascorbic acid with some of their synonyms are illustrated along with three common sugars for comparison (for structures, see 3).

2.2. Analogs

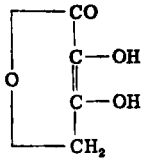
A variety of other analogs with three to eight carbon atoms have been prepared, usually by the synthetic routes given or by ones which involve condensation of a two-carbon compound (to form carbons 1 and 2) with an aldehyde or acid. The structures of the simplest of these compounds containing the enediol system



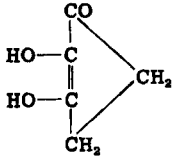
are of interest (for structures, see 4). A number of analogs with their anti-scorbutic activities in the classic bioassays are given in Table 1.



L-Erythro-ascorbic acid

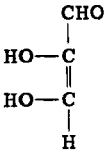


Hydroxy-tetronic acid

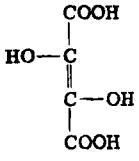


Reductinic acid

[4]



Enoltartronic dialdehyde (reductone)



Dihydroxy-fumaric acid

The remarkable structural specificity of compounds with antiscorbutic activity is clear from Table 1. Only L-xyloascorbic acid and its reversible oxidation product, dehydro-L-ascorbic acid, have full activity. Methyl 2-keto-L-gulonate, an immediate precursor of ascorbic acid in many syntheses, is also active. It is presumably converted to ascorbic acid in the animals (R3). The other compounds with partial activity have supported the generalizations that for antiscorbutic action the lactone ring must be present with carbon 4 in the D-configuration (the lactone ring must lie to the right of the carbon chain in the Fischer formulas), that both enol groups must be present, and that the carbon chain may be longer than six atoms. L-Xyloascorbic acid is the only compound with these characteristics occurring in nature.

TABLE 1

RELATIVE ANTISCORBUTIC ACTIVITIES OF ASCORBIC ACID AND ANALOGS (S16)

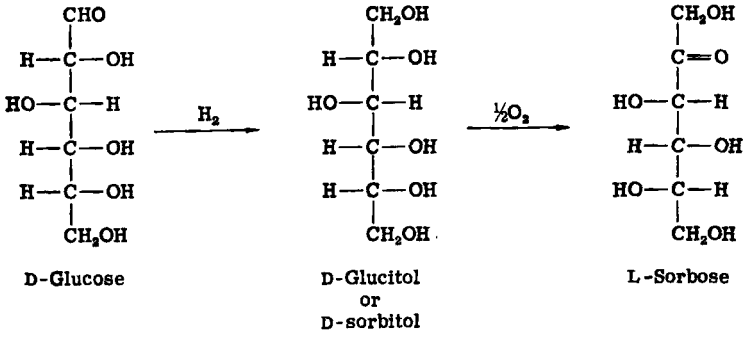
Substance	Activity	Substance	Activity
L-Xyloascorbic	100	D-Galactoascorbic	0
Dehydro-L-ascorbic	100	L-Guloascorbic	0
6-Deoxy-L-ascorbic	33	L-Alloascorbic	0
L-Rhamnoascorbic	20	L-Erythroascorbic	0
D-Araboascorbic	5	Hydroxytetronic acid	0
L-Glucoascorbic	2.5	3-Deoxy-L-gulosaccharo-	
L-Fucoascorbic	2	ascorbic acid	0
D-Glucoheptoascorbic	1	2,3-Diamino-L-ascorbic	0
2,3-Diketo-L-gulonic acid	0	2-Amino-L-ascorbic	0
D-Xyloascorbic	0	Reductinic acid	0
L-Araboascorbic	0	Reductone	0
D-Glucoascorbic	0	Dihydroxyfumaric acid	0

Antiscorbutic activity per se is more widespread than the results of Table 1 would indicate. Many of the inactive or slightly active compounds are rapidly excreted by the kidneys of the test animals and so do not remain long in the body with the conventional scheme of daily dosages. D-Ascorbic acid behaves this way and is inactive in the usual bio-assay, as predicted from its configuration. But when D-ascorbic acid is given in many small doses throughout the day, it is fully as active as L-ascorbic acid (B42). The strict chemical specificity of antiscorbutic action therefore represents the summation of properties which determine distribution and excretion by the body, as well as the chemical requirements for its particular metabolic actions. The present available data clearly show that nutritionally useful antiscorbutic compounds are severely limited, but that the chemical limitations on structure for certain metabolic actions are apparently less severe.

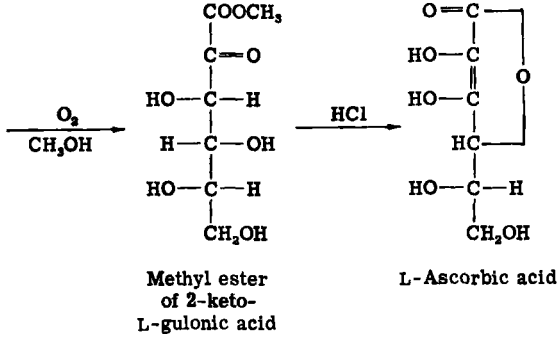
2.3. Syntheses

The two most important routes of synthesis are described:

1) The addition of cyanide to an osone was described above (Eq. 2) as the reaction by which the first vitamin was synthesized. The oxidation of aldoses by cupric salts in hot alcohol has made the osones more readily available, and the method has been extensively used for small-scale syntheses of L-ascorbic acid and its analogs. L-Ascorbic acid-1-C^{14} has been made in this way.

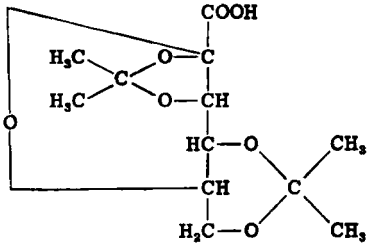


[5]

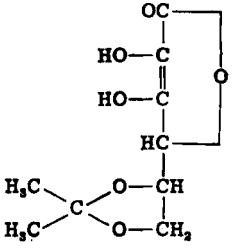


2) Large-scale commercial syntheses of L-ascorbic acid usually utilize some variant of the oxidation of L-sorbose to 2-keto-L-gulonic acid, followed by the simultaneous lactonization and isomerization of this product to L-ascorbic acid. This route of synthesis involves two changes closely analogous to those now known to occur during the biosynthesis of ascorbic acid in animals. These are inversion of the glucose molecule so that its carbon 1 becomes carbon 6 of ascorbic acid, and the final simultaneous isomerization and lactonization of the keto acid to the enol form of ascorbic acid. L-Sorbose is made commercially from D-glucose by catalytic hydrogenation to D-glucitol. Bacterial oxidation at carbon 5 forms a

keto group. When this molecule is inverted so that the keto group is on carbon 2, it is identical with L-sorbose. Further oxidation of the new carbon 1 (carbon 6 of original glucose) gives 2-keto-gulonic acid. When the methyl ester of this is heated in acid solution, the keto group enolizes, and the *cis* form of the dienol lactonizes to form L-ascorbic acid (Eq. 5). Numerous variants of these reactions have been evaluated by Smith (S17).



2,3,4,6-Diisopropylidene-2-ketogulonic acid



5,6-Isopropylidene-L-ascorbic acid

[6]

Protection of groups during the oxidation steps of the synthesis frequently is obtained by the general reaction of condensation of acetone with two *cis*-hydroxyl groups, to form the isopropylidene derivatives. These are readily hydrolyzed in water. The di- and monoderivatives of 2-ketogulonic acid and ascorbic acid have the structures shown in [6].

2.4. Properties

L-Ascorbic acid is a crystalline white solid with a specific rotation in water of + 23°, m.p. 192°C. It is insoluble in less polar solvents. These properties, plus ready oxidation, frustrated the early attempts at isolation. Dissociation of the enol on carbon 3 gives it the property of forming monobasic salts. The lead salt is relatively insoluble, and this was universally used for the first isolation step. Its oxidation-reduction potential ($E_o' = +0.051$ volts at pH 7.24 and 30°C) is higher than that of most biological compounds, and its oxidation is catalyzed by the traces of heavy metals present in most natural products. In consequence, it is a strong reducing agent, and most manipulative procedures yield a certain proportion of the labile dehydroascorbic acid unless precautions are taken to ensure a strongly reducing environment. In common with many sugars, it can be decomposed into furfural by boiling in acid. Its enediol group conjugation gives an intense absorption band ($\log \varepsilon = 3.48$) at 245 mμ in acid solution, which moves to 265 mμ in neutral solution following dissociation of the enol group on carbon 3. A minor band occurs

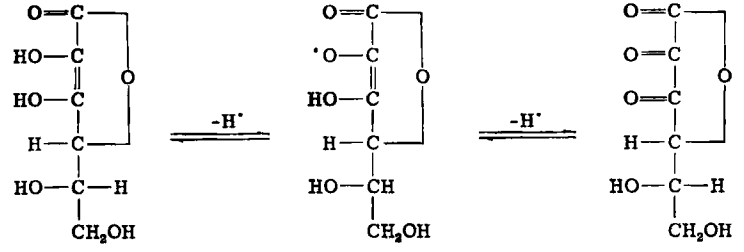
at 350–400 mμ. The absorption is lost on oxidation to dehydroascorbic acid. The lactone ring of ascorbic acid is not readily hydrolyzed, in contrast to that of dehydroascorbic acid. Hydrolysis would yield a derivative of 2-keto-L-gulonic acid, which under most conditions tends to reform ascorbic acid, as described above.

2.5. Reactions

Reactions of both ascorbic acid and dehydroascorbic acid are of interest, since the latter is readily produced in any ascorbic acid solution. The major ones are the oxidation of the enediol grouping, the reactions of the keto groups formed, and the fission of the diketo carbon chain between carbons 2 and 3.

2.5.1. *Oxidation*

The strong reducing property of ascorbic acid is the basis of most methods for its determination. It will reduce metal salts, such as copper, iron, silver, and gold; a variety of organic dyes, the most popular of which are derivatives of dichlorophenolindophenol; and oxygen itself in the presence of a suitable catalyst. It will also reduce cytochrome *c*, and this is the basis of many cytochrome oxidase assays.



Ascorbic acid — Monodehydroascorbic acid — Dehydroascorbic acid [7]

The oxidation of ascorbic acid in certain reactions has given evidence of an intermediate with the properties of a free radical which could be formed by one-electron oxidation. Thus, the rate-limiting step of ascorbic acid oxidation by Fe^{3+} and H_2O_2 was this one-electron oxidation (G12). Such a radical has now been identified in hydrogen peroxide-ascorbic acid solutions at pH 4.8 by electron paramagnetic resonance spectroscopy. The free radical, commonly referred to as monodehydroascorbic acid, decayed in about 15 minutes at this acid pH. It was also formed during the enzymatic oxidation of ascorbic acid by peroxidase (Y1). The existence of the monodehydroascorbic acid radical makes possible very

rapid one-electron step reactions during either the reduction of dehydroascorbic acid or the oxidation of ascorbic acid, as well as a source of free radicals for propagation of the reactions of other compounds. (Eq. 7).

2.5.2. *Keto Group Reactions of Dehydroascorbic Acid*

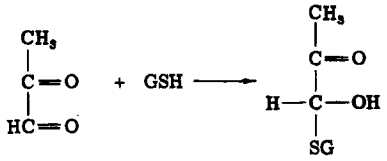
The formation of phenylhydrazones and osazones from dehydroascorbic acid is the basis of the most commonly used method of ascorbic acid analysis. The reactions are described in the section on determinations. There is no great specificity to this reaction, which is given by most compounds containing keto groups. However, most phenylhydrazones become intensely colored only in alkaline solution, while the dehydroascorbic acid type of compound is intensely colored in acid solution. Both dehydroascorbic acid and 2,3-diketogulonic acid are converted to the same osazone under the conditions used.

The reduction of the keto groups to enol groups occurs with many reducing compounds. Of particular interest are the various sulfhydryl compounds (H_2S, GSH, homocysteine)[2] used to convert dehydroascorbic acid to ascorbic acid for analysis by an oxidation method. Similar compounds can potentially reduce dehydroascorbic acid in biological systems. The redox potential of GSH is now believed to be considerably lower than was once thought (E_o' at pH 7, 30°C = —0.32) (B28), considerably below that of ascorbic acid (+0.058). This in part accounts for the inefficiency of sulfhydryl reductions of dehydroascorbic acid. Mapson (M6) estimates that the half-time of reduction of dehydroascorbic acid by GSH is about 15 minutes under physiological conditions of pH, temperature and concentrations.

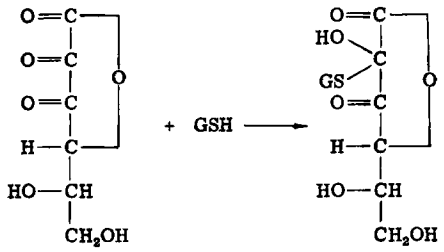
Actual compound formation also occurs between the dehydroascorbic acid and GSH reactants, though little attention has been paid to this condensation. Complexes of dehydroascorbic acid with one molecule of either GSH or thioglycolic acid were formed by Drake *et al.* (D19) in acetic acid solutions. Disappearance of iodine-titrable -SH groups and appearance of a new levorotatory material indicated the complex formation. This continued until an equilibrium was reached in about one hour at 0°C. At equilibrium more than half the dehydroascorbic acid was complexed. Remarkably little reduction of dehydroascorbic by the sulfhydryl compound occurred, since this reaction becomes significant only at neutral pH's. The complex is probably a thiosemiacetal, similar to

[2] Abbreviations used are GSH, glutathione; ACTH, adrenocorticotropic hormone; DPN and TPN, di- and triphosphopyridine nucleotides; pHPP, *p*-hydroxyphenylpyruvate; and DHA, dehydroascorbic acid.

those formed by GSH with carbonyl compounds like the active substrate of glyoxalase (Eq. 8).



Methylglyoxal — Thiosemiacetal of GSH



[8]

Dehydroascorbic acid — Thiosemiacetal of GSH

2.5.3. *Hydrolysis and Fission*

Hydrolysis of dehydroascorbic acid to 2,3-diketogulonic acid occurs readily at neutral pH. Under physiological conditions of pH and temperature, half of dehydroascorbic acid should be hydrolyzed in two minutes (B4).

Recently, the enzymatic hydrolysis of dehydroascorbic acid by cauliflower *(Brassica oleracea botrytis)* and bitter gourd *(Momordica charantia)* extracts has been reported (T5).

Very little information is available about the species which react in the further decomposition of ascorbic acid. Apparently, both dehydroascorbic acid and 2,3-diketogulonic acid can be oxidized directly, since both oxalylthreonic acid and the same two free acids have been identified. The former would result from oxidative fission of the chain while the lactone ring was intact. Even less is known of the oxidation mechanism. The oxidation occurs with iodine and with acid permanganate (H12) and also with oxygen or peroxide (R23). Recent studies on the similar decomposition of the enols of aryl pyruvates to aryl aldehydes plus oxalic acid identified these reactions as examples of the direct attack

of gaseous oxygen (P11). These studies may provide a model for the similar reaction of dehydroascorbic acid. A small fraction of ascorbic acid-1-C^{14} forms labeled urinary oxalic acid in most species (H11). There is no reason to believe that this is other than the spontaneous decomposition of ascorbic acid seen *in vitro*, though under certain conditions it may give rise to significant amounts of urinary oxalate.

2.5.4. *Para-biological Reactions*

A number of effects of ascorbic acid in biological systems would be expected from its known chemical properties. They need not represent physiological actions of the compound. The reducing actions of ascorbic acid are among the most common of these, especially its reduction of ferric to ferrous iron. Ascorbic acid is one of the most active reducing substances in increasing the absorption of organic food iron by reducing it to the better-absorbed ferrous form (M23). For the same reason, it activates several enzymes using ferrous iron as a cofactor, such as aconitase and homogentisate oxidase (D14, S28). Another reducing action of ascorbic acid which can equally well be performed by other reducing agents is its facilitation of the conversion of folic acid to citrovorum factor by liver extracts (D16).

The ready oxidation of ascorbic acid will catalyze chemical changes in a number of other substances. Thus, unsaturated fatty acids in lecithins and tissues are catalytically oxidized in the presence of ascorbic acid to a substance producing color with thiobarbiturate (B21). The product of the ascorbic acid-catalyzed oxidation is malonaldehyde, which can also inhibit L-gulonolactone oxidase, the enzyme forming ascorbic acid (C1). It has been suggested that this enzyme inhibition may occur *in vivo* in animals deficient in vitamin E, a compound believed to have antioxidant actions which would prevent the ascorbic acid-catalyzed lipid oxidation from giving rise to malonaldehyde. It is quite probable that the active intermediate in the formation of malonaldehyde is the monodehydroascorbate radical which initiates the lipid oxidation.

Other effects of ascorbic acid are probably also dependent upon the monodehydroascorbic free radical. One is the depolymerization of hyaluronic acid, first shown by Robertson *et al.* (R14). This is particularly likely, since subsequent studies have shown that the depolymerization was not prevented by catalase, as should have occurred if peroxide were the active component. There is no reason to believe that this hyaluronidase-like action is physiological. There is every reason to believe, however, that the monodehydroascorbic acid radical is formed *in vivo*. It is unlikely that all of the reactions in the body of such a reactive molecule are catalyzed by enzymes.

The most-studied reaction has been the model, hydroxylating system of ascorbic acid, chelated iron, and oxygen (U1), which forms many familiar hydroxylated ring compounds. The system presumably owes its action to the monodehydroascorbic acid and other free radicals produced in the system (G12, B30, B33). An example of this action, similar to that of the group of hydroxylating enzymes with which ascorbic acid is sometimes identified, is the hydroxylation of phenylalkyl derivatives by the model ascorbic acid system (S11). Euler and Hasselquist (E2), though attributing the action of the same system to a complex of hydroperoxide-ascorbic acid, have described its oxidation of ethanol to acetic acid, and its reduction of the viscosity of pectin, hyaluronic acid, chondroitin sulfate and mucoids.

3. Biosynthesis and Degradation of Ascorbic Acid in Animals

The most important recent addition to our knowledge of ascorbic acid is the series of stepwise enzyme reactions by which it is made in animal tissues and through which it is related to the reactions of other carbohydrates in the body. The biosynthetic pathways of ascorbic acid in plants have been partially elucidated, but less successfully, since it appears that two different pathways may operate (L21). These important advances have been the subject of a number of reviews (M7, B38, B39, K5).

Most animals do not require ascorbic acid in their diet and continue to excrete ascorbic acid while eating scorbutigenic diets. Certain drugs like chloretone (trichloro-*tert*-butyl alcohol) will stimulate the synthesis of ascorbic acid manyfold in these animals (S20). Effective use of this phenomenon has been made in the study of the synthetic pathways. The first indications of the chemical precursors of ascorbic acid came from the work of King *et al.*, who showed that D-glucose-6-C^{14} yielded L-ascorbic acid-1-C^{14} and that D-glucose-1-C^{14} yielded L-ascorbic-6-C^{14} (H14, H15). Isherwood *et al.* (I1) checked a number of possible pathways by administering proposed intermediates to rats and to cress seedlings and measuring the absolute increases in ascorbic acid production. D-Glucuronic acid and L-gulonic acid were effective intermediates that could be formed from D-glucose, and so were the two analogous derivatives of D-galactose. Thus, both the isotopic and the chemical experiments indicated that the glucose molecule was converted to ascorbic acid. In this process, we invert the formula, so that carbon 1 of glucose becomes carbon 6 of ascorbic acid. A similar inversion of the formula has already been described in the chemical synthesis of ascorbic acid from glucose via sorbose (Eq. 5).

A recent study (T1) showed that in *Penicillium notatum*, D-glucose-1-C^{14} was converted to D-araboascorbic acid with 80 % of the activity in carbon 1, showing the possibility of formation of D-araboascorbic acid from D-glucose without rupture and inversion of the carbon chain.

The possibility that the biosynthesis of ascorbic acid occurred through sorbose and 2-keto-L-gulonic acid, as in the chemical synthesis, was eliminated when the latter was not converted to ascorbic acid, and L-sorbose-6-C^{14} gave rise to ascorbic acid labeled in both carbons 1 and 6. Sorbose was evidently degraded and converted to glucose before being transformed into ascorbic acid.

With the recognition that the glucose molecule could be inverted to ascorbic acid by oxidation of carbon 6 to β-glucuronic acid, followed by reduction of carbon 1 to form the inverted L-gulonic acid, the research on this mechanism then joined the detailed enzymatic investigations of a new carbohydrate pathway. This series of reactions includes the synthesis of ascorbic acid and is now known as the glucuronic acid cycle. In contrast to the hexose monophosphate pathway which involves the oxidative loss of glucose carbon 1 through gluconic acid to form components of the pentose pool, the glucuronic acid cycle involves oxidative loss of glucose carbon 6 but supplies the same pentose pool of intermediates.

3.1. The Glucuronic Acid Cycle

The glucuronic acid cycle of reactions has a number of clinically significant aspects. Many drugs, as well as bilirubin, are excreted in conjugation with glucuronic acid made by this route. At least four hereditary diseases of metabolism result from inactivity of particular enzymes associated with this pathway. The sites affected are marked in the diagram (see 9) of the known enzymes of liver which catalyze the reactions of the glucuronic acid cycle. One of these is the hereditary inability of certain species to synthesize ascorbic acid, which predisposes them to scurvy, and others are the enzyme defects in galactosemia, congenital hyperbilirubinemia, and pentosuria (for reactions, see 9).

The reactions of the glucuronic acid cycle have been defined by studies in intact men and animals and by identification of the individual cell-free enzymes. Both glucose and galactose are interconverted and fed into the cycle as uridine diphosphoglucose (UDP-glucose). UDP-glucose can be oxidized to glucuronic acid. By a branch from the cycle the glucuronate is then transferred (glucuronyl transferase) to acceptors like bilirubin or drugs which must be so conjugated to be excreted. The hereditary absence of this reaction gives rise to the congenital hyper-

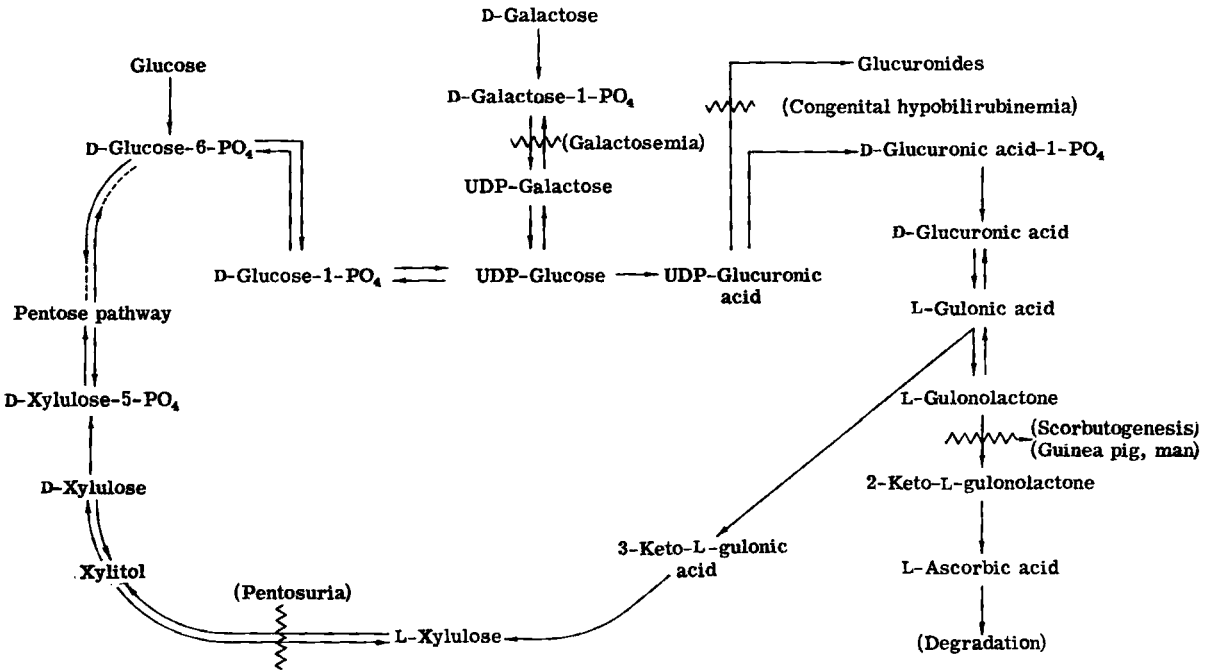
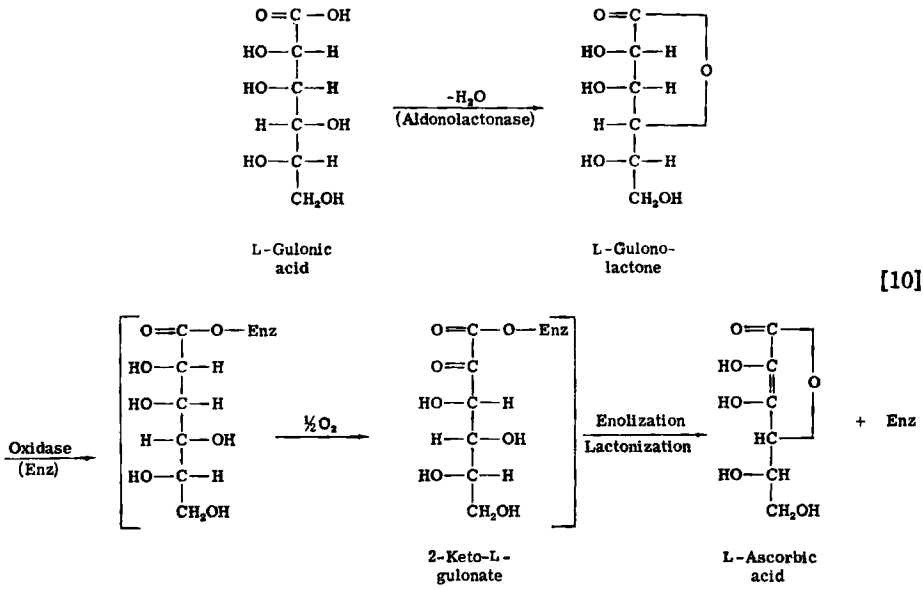


Diagram of the glucuronic acid pathway of glucose metabolism.

bilirubinemia characterized by high levels of indirectly reacting bilirubin and brain damage. D-Glucuronic acid itself can be reduced at carbon 1 and inverted to L-gulonic acid. A branching reaction at this locus leads to ascorbic acid formation in most species of animals. In the cycle proper, the carbon chain is shortened by oxidation and decarboxylation to form L-xylulose. This is the pentose excreted in gram amounts by the patients with hereditary pentosuria. Studies on these patients in whom an intermediate in the cycle of reactions was accumulated have greatly aided the understanding of this series of reactions. Normal men and animals possess the enzyme which reduces and reoxidizes L-xylulose, accomplishing a second inversion leading back to the D-series (D-xylulose). Subsequent changes are properly part of the pentose pathway, and lead to the formation, among other things, of D-ribose and re-formation of glucose.

3.2. Ascorbic Acid-Synthesizing Enzymes

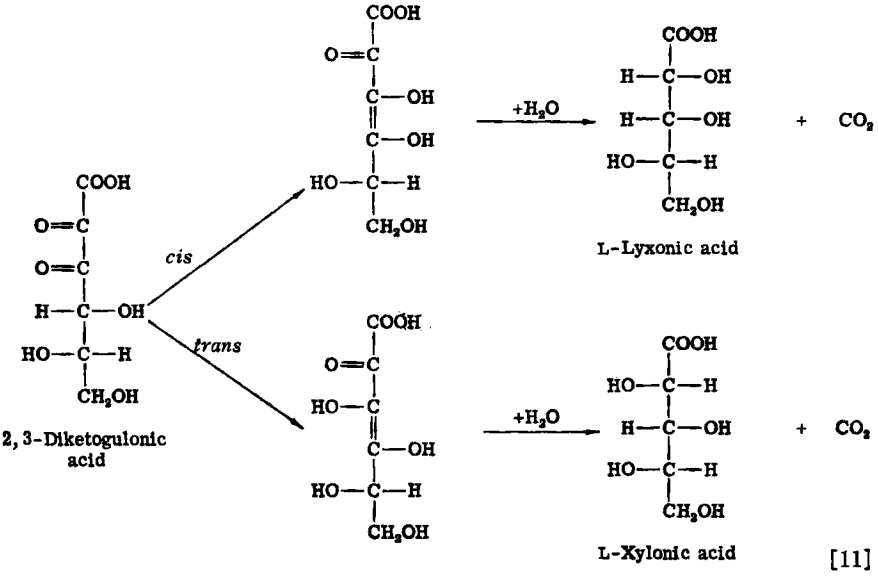
There are two enzymatic steps between the cycle intermediate L-gulonic acid and L-ascorbic acid. [A parallel reaction occurs with L-galactono-γ-lactone (I2).] The first is an aldonolactonase present in the soluble liver fraction of all species, including man and guinea pigs, which forms a lactone from L-gulonic acid and a variety of similar compounds. The second is a microsomal oxidase, possibly flavin-containing, which is absent in guinea pig, monkey, and human tissues. It specifically oxi-



dizes the lactone or ester of L-gulonic acid to 2-keto-L-gulonolactone (K6). It requires oxygen but is stimulated by the presence of H_2O_2 (F1). No additional enzyme has been identified for the subsequent enolization to ascorbic acid. These reactions are very similar to those used in the chemical synthesis of ascorbic acid by simultaneous lactonization and enolization of the ester of 2-keto-L-gulonic acid. The enzyme reactions can be portrayed in an analogous fashion. (Eq. 10).

3.3. Ascorbic Acid Degradation

The degradation of ascorbic acid in animals occurs to a very significant degree, but only a few of the intermediates are known, and there appear to be species differences. The familiar oxidation and hydrolysis of ascorbic acid to dehydroascorbic acid and then to 2,3-diketogulonic acid can be assumed. A portion must then be split oxidatively between the keto groups to account for the labeled oxalic acid formed from L-ascorbic acid-1-C^{14} (B40). Another portion enolizes and is decarboxylated by an enzyme which has been purified (S16) and which gives rise to labeled CO_2 from C-1-labeled ascorbic acid and to two pentonic acids. These arise from the decarboxylation of the *cis*- and *trans*-dienols of 2,3-diketogulonic acid (Eq. 11). 6-C^{14}-Labeled ascorbic acid also produces labeling of liver glycogen in carbons 1 and 6 of the glucose molecule. This suggests that further degradation of ascorbic acid occurs to the familiar three-carbon intermediates of glycolysis (D11).



[11]

3.4. Effect of Drugs on Ascorbic Acid Metabolism

The new knowledge of ascorbic acid biosynthesis and degradation has called attention to the variable metabolism of ascorbic acid, induced by drugs of various kinds, and to the complementary effects on the metabolism of drugs, produced by ascorbic acid. Changes of this sort may eventually explain many puzzling features of the metabolism of ascorbic acid in scorbutic animals in different physiological states.

It has long been known that drugs like aminopyrine greatly increase the excretion of L-xylulose by pentosurics (M2). Mention was made earlier of the stimulated synthesis of ascorbic acid in rats given chloretone. A similar stimulation by 3-methylcholanthrene may amount to 70-fold (B44, C15). Additional studies showed that both the body levels and rate of degradation of ascorbic acid were increased after the drugs (E3), so that greatly increased rates of synthesis must occur. It can be seen that an increase in the activity of the glucuronic acid cycle of reactions could have all of these effects. One site of action of chloretone has been localized at the reaction of UDP-glucose dehydrogenase which forms UDP-glucuronic acid. Its activity is doubled in the liver of chloretone-treated rats (C16). The change may be referable either to an increase in enzyme activity or to an increase in the amount of enzyme. There is often a lag period before the stimulated excretion, suggesting new enzyme synthesis (C16). Barbital also increases ascorbic acid excretion and likewise increases the level of UDP-glucose dehydrogenase activity. But 3-methylcholanthrene, which produces the most striking excretions of ascorbic acid, does so without altering the level of UDP-glucose dehydrogenase. Yet the stimulatory action of both drugs is blocked by ethionine administration (Fig. 1), and this inhibition is reversed by methionine, which suggests that synthesis of some additional enzyme protein is necessary for the extra ascorbic acid synthesis (T10). The site of action of 3-methylcholanthrene is of particular interest in view of its action in protecting scorbutic guinea pigs against some of the effects of scurvy.

Both synthesis and degradation (to CO_2) of ascorbic acid were increased by these drugs. The increased degradation may have occurred by mass action, or there may be increased activity of the degradative enzymes as well. If drugs produce this effect, they can raise the ascorbic acid requirement.

A similar increased degradation may be the action of the antimetabolite, ω-methylpantothenic acid (P12). The plasma ascorbic acid level fell in guinea pigs fed this compound, although the intake remained constant.

A little-known effect of scurvy is the increased sensitivity to certain drugs. Thus the rate of detoxification of acetanilide is greatly decreased in scorbutic guinea pigs (A10). Zoxazolamine, a uricosuric and skeletal muscle relaxant acting at the level of the spinal cord, can be given in doses sufficient to paralyze guinea pigs. The paralysis from a given dose lasts twice as long in scorbutic guinea pigs as in normals. In this instance, the enzyme in liver which inactivates the drug has been measured. It is reduced to one-third of the normal in the scorbutic animals. It is a reasonable conclusion that the drug acts longer because it is degraded more slowly. Chronic administration of the drug to animals on a scorbutic diet hastens the appearance of the signs of scurvy (C16). It is of

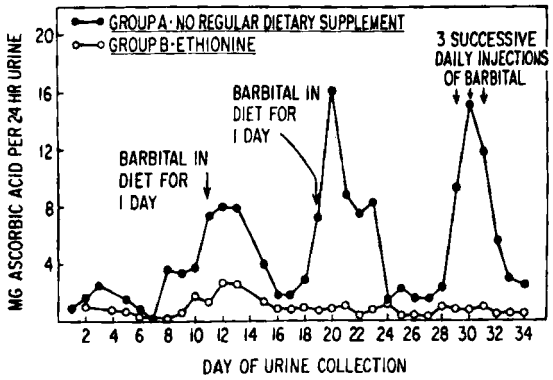


FIG. 1. Barbital stimulation of ascorbic acid excretion in the rat and its suppression by ethionine. This suggests the formation of new enzyme. From Touster *et al.* (T10).

interest that other drugs related to those which increase ascorbic acid synthesis will increase the level of enzyme which inactivates zoxazolamine (C17).

The interdependent actions of drugs and ascorbic acid, by which drugs can alter the rate of ascorbic acid formation, and ascorbic acid in turn can alter the rates of drug inactivation, may have practical applications in pharmacology. This should not be permitted to obscure the possibility that physiological compounds, like nutrilites and hormones, may also have interdependent actions with ascorbic acid according to a similar pattern. Tyrosine and growth hormones are two substances already known that could be fitted into such a pattern. Thus, hypophysectomy of rats decreased the tissue ascorbic acid by half, decreased its level in tissues, decreased its excretion, and increased the metabolic half-life of injected labeled ascorbic acid. Growth hormone reversed these changes. In doing so, its effect resembled that of chloretone and other drugs which increased ascorbic acid synthesis and excretion (S6).

Schwartz *et al.* (S13) observed that the administration of aminopterin and sulfasuxidine in the diet inhibited the biosynthesis of vitamin C in rats. The efficacy of chloretone in enhancing the urinary excretion of the vitamin could not be arrested either by aminopterin or by sulfaguanidine, although the latter compounds reduced the excretion and tissue levels of ascorbic acid (L1). It is unlikely that an antagonistic action between aminopterin and vitamin C occurs, as suggested by the following. The scurvy-like symptoms in an infant after aminopterin therapy disappeared with withdrawal of aminopterin and incidental therapy with massive ascorbic acid doses (D13).

4. Methods of Ascorbic Acid Estimation

The methods for the determination of ascorbic acid have been authoritatively reviewed from time to time (R21, A9, O2, R16, C4). It is intended here only to survey the variety of methods available and their individual advantages.

4.1. Biological Assay

The experimental investigation of scurvy began in 1912 with the recognition that the guinea pig required this vitamin (H17). An assay based on the minimum amount of the vitamin administered daily to prevent or cure scorbutic symptoms in guinea pigs (the Sherman units) was used during the isolation and early studies of ascorbic acid. The diagnosis and rating of the lesions produced depended considerably upon the experience of the investigator. This method was later replaced by more objective methods, depending upon the dental histology or upon the curative growth rate.

The derangements in dental histology produced by scurvy provide the most sensitive and specific method of bio-assay. In a standard version of this method (C20), groups of animals are fed graded levels of the test substance and of ascorbic acid for a period of 2 weeks, and then sections are made of the decalcified lower incisors. The degree to which the odontoblasts become disorganized, the scarcity and irregularity of dentine deposition, and the degree of hypercalcification of the predentine can be precisely compared with the effects of standard amounts of ascorbic acid.

The curative method involves the use of a scorbutigenic diet for ten days. On the eleventh day when weight loss would be expected to begin, groups of animals are placed on graded doses of the test sample and the ascorbic acid, and the biological value of the sample is calculated from the resultant dose response curves (H6).

The bio-assay methods were remarkably accurate in the hands of skilled

investigators, who used them to guide the isolation of ascorbic acid and to prove that antiscorbutic action was substantially limited to this one compound. The methods have since been almost entirely replaced by the more rapid and convenient chemical assays of ascorbic acid.

4.2. Physical Methods

The simplicity of the chemical methods for estimating ascorbic acid has diverted attention from the several physical methods available for measuring ascorbic acid. For certain applications these methods can be useful.

Polarography has had limited use in the estimation of ascorbic acid, since early investigations disclosed no advantage over the simpler methods of chemical titration, even as regards specificity. A recent application of this method to vegetables and fruits was reported by Krauze and Boxyk (K16). No dehydroascorbic acid was found in the samples after reduction by H_2S.

The optical rotation of ascorbic acid solutions can be used to measure relatively concentrated solutions. The observation that the optical activity of ascorbic acid increased with pH from + 21° at pH 5 to + 120° at pH 7 (N4) imparts a certain degree of specificity to this method.

The ultraviolet absorption of ascorbic acid in the region of 265 mμ has been commonly used for the estimation of ascorbic acid in simple solutions. In complex biological materials, however, other substances absorb considerably at this wavelength. A variety of ways to overcome this difficulty have been explored, such as measuring the decrease in absorption after more or less specific destruction of the ascorbic acid with cuprous ions, or irradiation, or with ascorbic acid oxidase. The effect of pH on altering the absorption band of ascorbic acid from 245 mμ in acid to 265 mμ in neutral solution has also been used to increase the specificity of the absorption measurements (D5).

4.3. Chemical Methods

The most satisfactory chemical methods for ascorbic acid analysis are based on the reduction of 2,6-dichlorophenolindophenol or on the formation of a colored dinitrophenylhydrazine derivative. A large number of other reactions, mostly with other oxidizing agents, have been used but have not found general favor (R21).

4.3.1. *Extraction and Isolation of Ascorbic Acid from Biological Samples*

A large portion of the literature on the chemical determination of ascorbic acid deals with different ways of preparing sample extracts. There is no ideal method equally applicable to the great variety of

animal and plant tissues which have been studied. The extraction should avoid the oxidation of this labile compound by the enzymes in the plant tissues or by the oxyhemoglobin and trace metals in animal tissues. It should extract the ascorbic acid completely, including any bound or sequestered by the tissue components, and it should extract a minimum of other tissue components which would interfere with the chemical assay to be used. The single extraction procedure which has found the widest application to both plant and animal tissues is one using metaphosphoric acid, which precipitates proteins, combines with trace metals, and produces an acid medium in which ascorbic acid is most stable. However, a great variety of other reagents alone or in combination has been used successfully for particular purposes (O2). Considering the lability of ascorbic acid, it is to be expected that more complete extraction and recovery of ascorbic acid in tissues by skilled analysts using the most appropriate conditions would increase the yield over earlier procedures. As the methods improved, the extra ascorbic acid not extracted by the earlier methods was considered to have been "bound." A considerable literature has accumulated about this unextracted ascorbic acid without any certain knowledge that ascorbic acid ever exists, in fact, in chemical combination in tissues.

The oxidation of ascorbic acid by oxyhemoglobin in the adrenal and the liver tissues during extraction was found to be negligible (G4). The incorporation of $SnCl_2$ into the phenylhydrazine procedure also obviates any effect of oxyhemoglobin.

The isolation of ascorbic acid from biological materials can be done on a microscale by paper chromatography. The separation of a mixture of vitamins including ascorbic acid by chromatography on a membranous gel has been described (G1). Ion exchange columns have been effectively used in small-scale isolations (K7). The simplicity of the latter isolation from green walnut hulls containing 16–20 % ascorbic acid on a dry-weight basis can be contrasted with the difficulties of the early isolation studies. The procedure consisted of aqueous extraction in SO_2 solution to avoid oxidation, absorption on an acid cycle anion exchange resin (Amberlite IR 4), elution with dilute HCl, and crystallization from the concentrated charcoal-decolorized eluate with 50 % yield. A new means of precipitating Pb ascorbate at pH 4.7 in the presence of excess Pb acetate and Cl ions was sufficiently effective to be the basis of a new analytical scheme (W13).

4.3.2. *Enzymatic Assay*

The use of ascorbic acid oxidase to convert ascorbic acid to dehydroascorbic acid is commonly used as a qualitative test in biochemical ex-

periments. The use of the enzyme for quantitative assays is handicapped by the lack of absolute specificity of the enzyme. It oxidizes a large number of analogs of ascorbic acid, but it does so at a lower rate, and it is unlikely that many of these substances are present in nature (D17).

Certain microorganisms, such as *Escherichia coli* and *Staphylococcus albus* strains will reduce dehydroascorbic acid. This reduction has been used for the estimation of ascorbic acid after its oxidation in extracts by ascorbic acid oxidase (S27) and also in the chemical reduction methods as a way of reducing dehydroascorbic acid to ascorbic acid. The reduction is not specific for L-ascorbic acid, but interfering compounds are unlikely in natural products. The use of this reducing action of bacteria has been improved by Mapson and Ingram (M8).

4.3.3. *Chromatographic Identification and Assay*

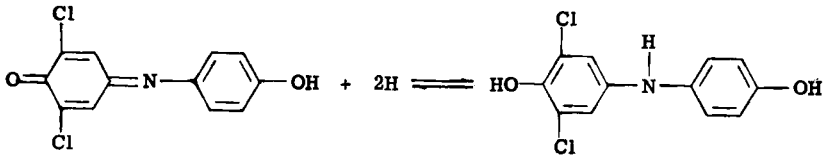
The chemical and physical methods in common use are nonspecific insofar as they determine the stereoisomers of L-ascorbic acid and other closely related enediol compounds. While these compounds are rare in natural products, there is frequently need for exact chemical identification of the substance being measured. One procedure which has been used is isotopic dilution and reisolation (D10). A more generally useful procedure for the identification is paper chromatography (M9). Subsequent quantitative analyses of the resolved spots can be carried out (C7).

The procedure of Chen *et al.* (C7) separated the ascorbic acid on paper using *n*-butanol or phenol saturated with oxalic acid to retard decomposition. A preliminary preparative separation could be used to concentrate the sample. Qualitative identification was made from the R_f of the spots located by spraying with 0.08 % 2,6-dichlorophenolindophenol, and quantitative analysis by elution with oxalic acid and titration with the same dye. Recoveries from urine and cress seedlings were 85 % or more at even the lowest concentrations tested (0.5 mg/ml), and the method could detect 1–2 μg ascorbic acid per cm^2 of paper. The method clearly separated L-ascorbic acid and D-araboascorbic acid as well as hydroxytetronic acid, reductinic acid, and reductone.

4.3.4. *Reduction of 2,6-Dichlorophenolindophenol*

Detailed analytical procedures for the several modifications of this method are given by Roe (R16) and by Chapman (C4). Dichlorophenolindophenol is blue in neutral solutions and pink in acid solutions. Its use in the estimation of ascorbic acid is based on the observation that ascorbic acid is essentially the only substance in acid extracts of tissues

which reduces the dye at pH 1–4 to the colorless leuco form (Eq. 12). The leuco form is only very slowly reoxidized by air. The main disturbance is caused by other reducing substances in the extracts. Thiosulfate, ferrous, cuprous, and stannous salts rapidly reduce indophenol so that this method cannot be used on solutions containing these substances. Phenols and sulfhydryl compounds which reduce the dye at neutral pH do so only slowly at low pH, so that corrections can be made. Another group of interfering substances are the strongly reducing compounds formed by heating sugar solutions, commonly referred to as "reductones" in connection with these analyses. These substances appear only in heat-processed food.



Oxidized form (blue or pink) Reduced or leuco form (colorless)

[12]

The reagent of choice for extracting plant and animal tissues is 5 % metaphosphoric acid solution, since this reagent fully extracts ascorbic acid, precipitates proteins, inactivates the enzymes which oxidize ascorbic acid, chelates with metal catalysts, and produces the requisite acidic solution for the analysis. Standard procedures are available for the direct titration of ascorbic acid extracts with dichlorophenolindophenol standard to a persistent, but short-lived pink endpoint and the photometric measurement of the fraction of excess dichlorophenolindophenol reduced by an aliquot of ascorbic acid. The latter procedure is not only more sensitive (2–10 μg of ascorbic acid), but also eliminates the difficulties in judging the endpoint and permits correction for substances which reduce indophenol more slowly by taking several timed readings after the primary reaction is finished. Additional modifications have been proposed to eliminate other reducing compounds by their reaction with formaldehyde, which combines slowly with ascorbic acid but very rapidly with many of the reducing substances in biological materials (M5). Such a procedure is rarely necessary in extracts of animal tissues.

More recently, p-chloromercuribenzoic acid was used to eliminate the largely sulfhydryl reducing compounds present in extracts of animal tissues (O4). Interfering substances, presumably glutathione, present in human erythrocytes were largely removed by this procedure. *p*-Chloro-

mercuribenzoic acid also removed the interfering sulfhydryl compounds in germinating soybeans (W2).

4.3.4.1. *Dehydroascorbic Acid Determination by Reduction.* A disadvantage of the dichlorophenolindophenol reduction method is that the dehydroascorbic acid can be determined only after its reduction to ascorbic acid. The reducing agent used may interfere in the subsequent reduction of the dye. Since ascorbic acid exists mainly in the reduced form in most tissues, this is not a serious disadvantage for the reduction method in the usual analysis. The reduction of dehydroascorbic acid to ascorbic acid is usually performed with H_2S by treatment for 15 min between pH 1.2 and 4.7 (R20). The completeness of the reduction was checked by the dinitrophenylhydrazine method.

A recent suggestion was the use of homocysteine at pH 7 to reduce dehydroascorbic acid (H19). Prolonged treatment of ascorbic acid solutions with sulfhydryl reagents should be avoided because of the possibility of conversion to the thiosemiacetals described earlier.

4.3.5. *Dinitrophenylhydrazine Methods*

The dinitrophenylhydrazine methods are very sensitive and remarkably specific and are the methods of choice for most analyses. Micromodifications are available for assays in microgram quantities of tissues.

The reaction depends upon the coupling of 2,4-dinitrophenylhydrazine to the keto groups of carbons 2 and 3 of diketogulonic acid to form a bis-2,4-dinitrophenylhydrazone or osazone. In strong acid solution this derivative rearranges to a stable reddish-brown product which can be measured photometrically. Ascorbic acid must first be oxidized to dehydroascorbic acid for this reaction to occur, and it is probable that dehydroascorbic acid is hydrolyzed in the strong acid medium to diketogulonic acid before the hydrazone formation takes place (P4). Other carbohydrate materials react with dinitrophenylhydrazine slowly in comparison with the oxidation products of ascorbic acid, and their osazones are unstable in the high acid concentration used. The addition of the reducing agent, thiourea or stannous chloride, further adds to the specificity of the method by avoiding interference from nonascorbic acid chromogens. Interference has been noted, however, in the analysis of heat-processed foods with a high sugar concentration, presumably because of the high concentration of "reductones," which also affect the dichlorophenolindophenol reduction assay. A particular advantage of the dinitrophenylhydrazine method is that by appropriate oxidations and reductions of the sample, each of the three compounds, ascorbic acid, dehydroascorbic acid, and diketogulonic acid, can be determined by difference. Standardized procedures for measuring each of these three compounds, for as-

corbic acid alone, and for the total of the three compounds have been given by Roe (R16).

The basic method utilizing dinitrophenylhydrazine is that described by Roe and Kuether (R 18). Norit (charcoal) is used in the trichloracetic extract to oxidize the ascorbic acid. It is applicable to both blood and urine.

4.3.5.1. *Differentiation of Ascorbic, Dehydroascorbic, and Diketogulonic Acids.* The phenylhydrazine method measures the total ascorbic acid. Reduced ascorbic, dehydroascorbic, and diketogulonic acids present in a sample have been differentiated by Roe *et al.* (R 20) with the help of H_2S, whereby dehydroascorbic acid is reduced to ascorbic acid; the second aliquot is not treated; and to a third aliquot, bromine is added to oxidize ascorbic acid to dehydroascorbic acid. Analysis by the phenylhydrazine procedure will give a measure of diketogulonic acid in the first aliquot, diketogulonic and dehydroascorbic acids in the second, and a combination of diketogulonic, dehydroascorbic, and ascorbic acids in the third. Values for the individual acids can then be calculated. To avoid any change during the course of extraction, use of reducing agents such as thiourea for plant tissues and $SnCl_2$ in metaphosphoric acid solution for animal tissues has been suggested (R19).

A study of the phenylhydrazine procedure of Roe and Kuether was undertaken by Schaffert and Kingsley (S9) to adapt the method for routine analysis of biological samples, mainly by shortening the incubation period of 3 to 6 hours outlined by the original investigators. In the modified procedure, the incubation period is reduced to 5–10 min by carrying out the operation at 100°C. Excellent accuracy was demonstrated by recovery experiments. Meyer *et al.* (M20) confirmed these results.

However, the modification of Schaffert and Kingsley has been challenged by Roe (R17), according to whom the coupling reaction at 100°C for 10 min results in "gross plus errors" due to the formation of chromogenic materials with sugars or glucuronic acid. This is also clear from other studies (L11).

4.3.5.2. *Micromodifications.* Glick *et al.* (G4) described a method for the determination of L-ascorbic, dehydro-L-ascorbic, and diketo-L-gulonic acids in microgram quantities of tissue, such as adrenal tissue, with the phenylhydrazine method. They also found slightly lower values by incubation during coupling at 100°C for 5 minutes but false, high values at 100°C for ten minutes, as compared to the values obtained at 37°C for 6 hours.

A microadaptation of the method for estimation of ascorbic acid in

0.01 ml of blood is that of Lowry *et al.* (L23). Norit treatment is avoided. Osazone formation is completed by dinitrophenylhydrazine plus a copper sulfate reagent, the copper effecting the oxidation of ascorbic acid present in the sample. The method yields slightly higher results as compared to the original method of Roe and Kuether, apparently due to exclusion of Norit, the presence of which would remove nonascorbic acid chromogen present in the sample (R16).

5. The Distribution of Ascorbic Acid in Animal Tissues

5.1. Dynamic Relations of Tissue Concentrations

The dynamic state of ascorbic acid in animal tissues has been given little attention, despite the fact that an understanding of this is essential for the interpretation of the common measurements made clinically. It is often mentioned that the concentration of ascorbic acid is unusually high in certain tissues. It is more significant that the ascorbic acid concentration of all tissues is at least several times that of the plasma. The concentration in skeletal muscle, which makes up most of the weight of the animal body, is 3 to 5 times that in the plasma, while that in most other tissues is many times higher than in plasma. Analyses for representative tissues of the normal guinea pig are given in Table 2. To these can be added the high levels recently reported for guinea pig white blood cells (A6). A very similar pattern of ascorbic acid concentrations has been found in a number of other animal species which synthesize their own ascorbic acid (L19). Somewhat arbitrarily, the tissues can be separated into three groups. A few have ascorbic acid concentrations several hundred times that of the plasma: hypophysis, adrenal, thymus, corpus

TABLE 2
Ascorbic Acid Distribution in Normal Guinea Pigs
(Given Ascorbic Acid or Cabbage)

Tissue	Concentration mg/100 g (mg%)	Reference
Plasma	0.54	(T8)
Whole blood	1.16	(K18)
Skeletal muscle	3.12	(K18)
Bone marrow	8.9	(P5)
Heart	8.98	(K18)
Kidney	11.6	(K18)
Small intestine	20.4	(P5)
Brain	22.8	(K18)
Liver	32.8	(K18)
Spleen	41.9	(K18)
Adrenal	166.0	(K18)

luteum, and retina. A large number of tissues has concentrations 50–100 times the plasma level: brain, testicle, thyroid, small intestinal mucosa, lymph glands, lungs, liver, spleen, white blood cells, pancreas, and salivary gland. A number of other tissues has only about 10 times the plasma concentration: kidney, skeletal, smooth and cardiac muscle, and erythrocytes.

The extreme concentration of ascorbic acid in the cells of various tissues suggests either a very strong active transport into these cells or some type of "binding" of the intracellular ascorbic acid. Few critical experiments have been done to investigate this problem. The problem of bound ascorbic acid is discussed later. Studies have shown that ascorbic acid added to whole blood will pass to the leucocytes preferentially (H10) and that dehydroascorbic acid was taken up rapidly by erythrocytes and reduced to ascorbic acid (L18). It is well established that the ratio of dehydroascorbic to ascorbic acid in plasma remains remarkably constant after administration of either form (L17, S1, S25). Recently, Martin (M11) has investigated the kinetics of distribution of the two forms of ascorbic acid. He called attention to the fact that dehydroascorbic acid is un-ionized and so more diffusible at body pH than the negatively charged ascorbate ion. In confirmation of this, the volume of distribution of labeled ascorbic acid in nephrectomized rats approximated the extracellular fluid volume. The volume of distribution of labeled dehydroascorbic acid (found as ascorbic acid) was much greater and must have included the intracellular volume. Similarly, the ascorbic acid concentration in brain could be increased by the systemic injection of dehydroascorbic acid, but not by ascorbic acid itself. The suggestion is that ascorbic acid enters and leaves most cells largely in the form of the neutral dehydroascorbic acid molecule. This was supported by finding a higher ratio of dehydroascorbic to ascorbic acid in the blood of the veins draining the kidney and the mesentery than in the systemic veins. Salomon (S3) earlier reported similar conclusions for the loss of adrenal ascorbic acid after ACTH. It is possible that the free diffusion of dehydroascorbic acid combined with its specific intracellular reduction to the nondiffusible ascorbic acid can account for the striking intracellular concentrations of ascorbic acid. This mechanism is also more consistent with the remarkable failure of leucocytes to lose ascorbic acid into isotonic potassium oxalate (B23) or to tungstic acid solution (B45) than is a mechanism for active transport of ascorbic acid itself into the cells. It should be emphasized at this point that no specific enzymatic reducing system for dehydroascorbic acid in animal cells is known.

Hydrocortisone slightly diminishes the diffusion of ascorbic acid from

rat liver slices *in vitro*, and it has been suggested that the hormone may activate a mechanism for concentrating ascorbic acid in the cells (V1).

There must also be a second mechanism of ascorbic acid transport through certain specialized cells, different from the diffusion of dehydroascorbic acid followed by intracellular reduction. There are no indications that dehydroascorbic acid is involved in the renal tubular absorption of ascorbic acid, and, contrary to earlier conclusions, it appears that the concentration of ascorbic acid into the aqueous humors of the eye occurs in the form of ascorbic acid itself.

5.1.1. *Transport of Ascorbic Acid by the Eye*

Ascorbic acid is present in the anterior and posterior aqueous humors of the eye of many animal species, including guinea pigs, at concentrations some 30 times that of the plasma. Local synthesis, if it occurs at all, does not account for a significant part of the concentration. Dehydroascorbic acid did not penetrate readily to the eye from plasma and when injected directly to the eye was only slowly reduced. The source is believed to be the active transport of ascorbic acid across the cells of the ciliary process in much the same way as ascorbic acid is transported by the renal tubules. Saturation of the transport mechanism occurs at levels of 0.8 mg (guinea pigs) and 2–3 mg (rabbit)/100 ml of plasma. It will be noted that similar plasma levels saturate the renal transport mechanism and give rise to the copious excretion of ascorbic acid in urine. Below these levels, the removal of ascorbic acid from blood passing through the ciliary process is thought to be substantially complete. The accumulated concentration has been used to calculate plasma flow through the ciliary process in much the same way as diodrast or *p*-aminohippurate clearances can indicate effective renal plasma flow (L3).

5.1.2. *Renal Transport of Ascorbic Acid*

The ascorbic acid in plasma exists in a free form and is completely filterable (S8) by the renal glomerulus. The ascorbic acid is reabsorbed from the glomerular filtrate by the renal tubule in amounts proportional to the concentration present up to plasma levels of 1.4 mg%. The reabsorption is never complete, and there is a constant small loss at the usual blood levels, amounting to the clearance of 1–3 ml plasma/min. High concentrations of ascorbic acid have been found during its reabsorption in the proximal tubule of the guinea pig nephron, suggesting that ascorbic acid like glucose is reabsorbed at this site. The reabsorption of ascorbic acid is unaffected when blood levels of glucose are raised to 300 mg% and also unaffected by sufficiently high levels of *p*-aminohippuric acid to saturate its secretion mechanism, which indicates that the

specific transport system for ascorbic acid is separate from those utilized by these two other compounds. High levels of these compounds retard ascorbic acid transport transiently, which would suggest that the several transport systems may compete for the same energy source although they do not occupy a common transport component. When the ascorbic acid plasma level is raised above 1.3 mg% in dog and man, the ascorbic acid load of the glomerular filtrate exceeds the maximal transport capacity of the tubule of 1.77 mg/100 ml of glomerular filtrate. The excess produces a copious excretion of ascorbic acid in the urine characteristically shown by the saturated animal. There is a delay of 20 min in the appearance of maximum excretion after sufficiently high blood levels have been reached, which has been attributed to the time necessary to saturate the renal tubule cell. It is this point which is determined in the clinical saturation tests with ascorbic acid.

The function of the specific ascorbic acid transport system in the kidney can be altered by several treatments. Estradiol decreases the reabsorptive capacity in the tubules for ascorbic acid in the dog so completely that the clearance of ascorbic acid approaches that of creatine, indicating a complete cessation of reabsorption. In consequence, there is a depletion in the plasma ascorbic acid levels, and dogs maintained chronically with estradiol treatment have shown hemorrhages, increased clotting time, and anemia possibly suggestive of scorbutic-like signs in the dog! Careful study of the effect of estradiol on the renal tubule function has indicated that the tubule can still transport ascorbic acid in the usual amounts but that it can do this only when presented with a much higher load, the excess of which is excreted. It is the efficiency or avidity of the tubule for ascorbic acid rather than its capacity to transport the compound which is decreased. It is known that estradiol increases the excretion of sodium and potassium chloride, thus, a correlation between the renal handling of these substances and ascorbic acid was established. Both sodium chloride and potassium chloride infusions decreased the maximum tubular transport capacity for ascorbic acid to half the normal values. These effects are different from those of estradiol which decreased the efficiency but did not lower the capacity. The possibility that these effects could be caused by osmotic diuresis has been eliminated (S18).

5.1.3. *The Steady State of Ascorbic Acid*

The plasma level of ascorbic acid appears to be one of the lowest concentrations existing in the body. It is effectively buffered by extremely high concentrations of ascorbic acid maintained intracellularly by some

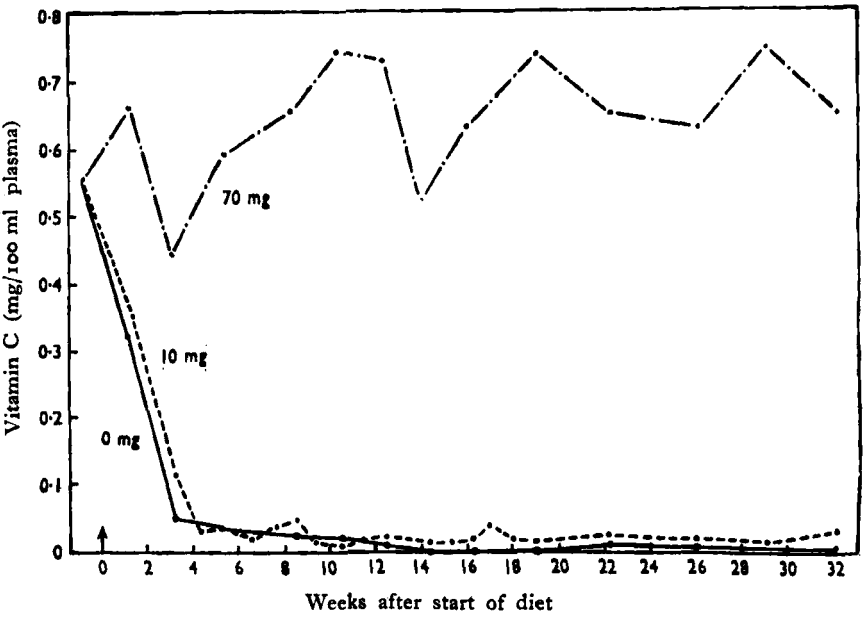


FIG. 2. Average vitamin C content of blood plasma of groups of volunteers receiving a daily supplement of 70, 10, or 0 mg vitamin C (vitamin C in basal diet about 1 mg daily). From Krebs (K17).

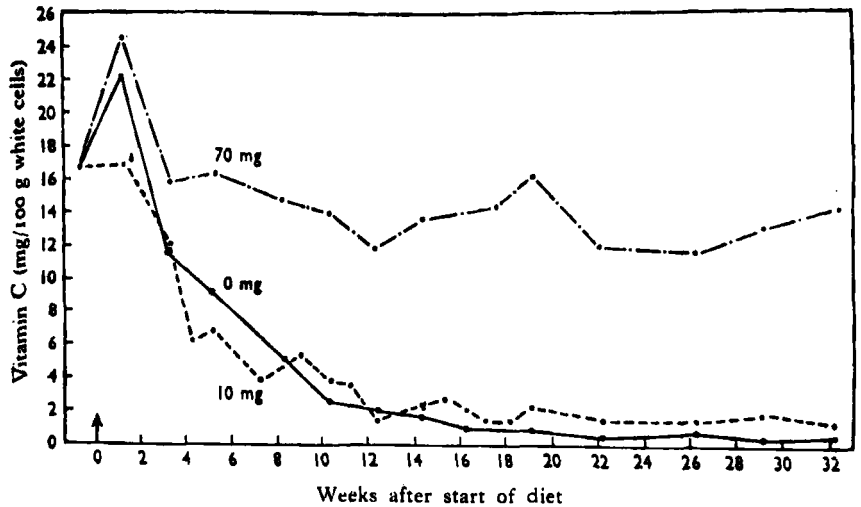


FIG. 3. Average vitamin C content of white blood cells of groups of volunteers receiving a daily supplement of 70, 10, or 0 mg vitamin C (vitamin C in basal diet about 1 mg daily). From Krebs (K17).

kind of active process in all the other tissues. Of these, the leucocyte ("buffy coat") is the most accessible for routine clinical sampling. Alterations in the intracellular concentrations occur in different physiological states, but there is no systematic knowledge of these changes. The body content of ascorbic acid, maintained by the low plasma concentration, represents a balance between the intake and the synthesis of ascorbic acid in those animals able to do this with the rates of ascorbic acid degradation and excretion. The renal excretion is small and relatively constant at the usual ascorbic acid plasma levels, but becomes very large when the "threshold" level of 1.4 mg% is exceeded. It follows from these relationships that a progressive ascorbic acid deficiency in guinea pigs or man would produce first a prompt fall of the plasma level as the intake was cut off and the small excretion persisted, and a much smaller depletion of the tissue stores commonly measured in the "buffy coat" of the blood. Only when these tissue levels are seriously depleted would the frank derangements of scurvy be expected (Figs. 2 and 3). When the curative intake of ascorbic acid was resumed, the plasma level would be expected to remain very low except during actual absorption of the vitamin until the tissue cells had taken up their required amounts. About 1 or 2 g of ascorbic acid will be absorbed in this way by the tissues of a man. Only after this will further dosage raise the plasma level sufficiently high to exceed the kidney reabsorptive capacity and cause copious ascorbic acid excretion in the urine. The amount which must be given before this occurs is the basis of the saturation test.

Some precise measurements of certain parameters of the steady state of ascorbic acid in the body have been obtained from recent studies with isotopically labeled ascorbic acid (H11, S5, S6). Thus, the total ascorbic acid in the body in equilibrium with the test dose is of the order of 10 or 20 mg/100 g body weight in the guinea pigs and about 1.5 g for a total man not saturated with ascorbic acid (H11), which is about equal to the higher figure for the guinea pig on a body weight basis. The half-life of a small dose of labeled ascorbic acid in the guinea pig is three days, while it is considerably longer in man (B41, S5). The longer persistence of ascorbic acid in man is associated with his failure to degrade significant amounts of the compound by decarboxylation and may be correlated with the much longer periods of ascorbic acid depletion necessary to produce scurvy in man (70 days) than in the guinea pig (11 days).

5.2. Alterations in Distribution

The effects of age and development on the distribution of ascorbic acid in animals is not striking. During pregnancy and during the rapid

growth of young guinea pigs (R4), more of the vitamin is retained by the animal, as would be expected from the increasing mass of tissue to be supplied. The actual content of ascorbic acid fell slightly with increasing age in the human adrenal cortex and pituitary, and, after an initial fall, remained constant after middle age in the myocardium and skeletal muscle (S10).

The localization of high concentrations of ascorbic acid about healing wounds has been commonly observed, but there have been no critical studies to associate this with the active growth processes themselves instead of with the assemblage of cell types with a normally higher ascorbic acid content. The retention of ascorbic acid and refractoriness to saturation with ascorbic acid that occurs in severe injury or illness (L26) indicates some changing pattern of distribution. The most reasonable explanation for the post-operative retention of ascorbic acid is its selective concentration in the traumatized area (B15).

Daily administration of 35 % ethanol to rats for 10 weeks (A5) decreased the ascorbic acid concentration in stomach, intestine, and liver; increased it in heart, muscle, and adrenal; and caused no change in the kidney.

A marked rise in the ascorbic acid content of the developing sea urchin egg has been correlated with the period of entomesodermal development. The vegetal larva, which has more entomesodermal tissue than the animal larva, also has a higher ascorbic acid content (B1, B2). Glucoascorbic acid, which is no longer believed to be an ascorbic acid antagonist in the usual laboratory animals, modifies the vegetal larvae in the direction of the animal larvae (G15).

The high concentration of ascorbic acid in white blood cells has naturally focused attention on this property of leukemic cells. While the ascorbic acid content of leukemic cells may be low (W1), this is not necessarily so (B13). The ascorbic acid concentrations are somewhat different in varied types of abnormal white cells and are, of course, influenced by the general condition of the patient, but in general they are not qualitatively different from normal cells.

5.3. "Bound" Ascorbic Acid in Animal Tissues

The possible existence of bound forms of ascorbic acid in animal and plant tissues forms one of the most contradictory subjects in the biochemical literature. The very high concentrations of ascorbic acid within both plant and animal cells provide reasons to look for such combined forms. The realization that most of the water-soluble vitamins function

in the body in combination with other substances (coenzymes) provides another reason. It is obvious that many of the references to bound ascorbic acid in the early literature arose from inadequacies in the chemical methods used. Analytical recoveries were often incomplete because of oxidative destruction or incomplete extraction of the ascorbic acid in the sample. When other methods were found which gave higher recoveries, the latter were credited with extracting ascorbic acid which had remained "bound" in the earlier procedures. Less and less attention has been paid to the bound ascorbic acid fraction as the more efficient analytical procedures have come into use. This is particularly true of the bound ascorbic acid in plant tissues, although the problem has never been resolved. No completely effective extraction procedure by itself will ever demonstrate a bound fraction of ascorbic acid. More than 40 studies of reported bound ascorbic acid in plant tissues have been reviewed (L25).

The possibility that some fraction of the ascorbic acid in animal tissues is bound instead of free has continued to attract interest. It can be accepted as established that there is no bound ascorbic acid in blood serum (S8). The ascorbic acid present in serum is freely dialyzable and is not increased by various hydrolytic procedures when measured by the 2,4-dinitrophenylhydrazine method. The increases after hydrolysis reported earlier may be attributed to the production of other materials which reacted with the 2,6-dichlorophenolindophenol used for titration. The possibility of bound ascorbic acid is, therefore, restricted to other tissues.

Recent interest has derived from the apparent demonstration of some ascorbic acid in liver which was not extracted by 95 % ethanol, but which could subsequently be extracted by a hot acid treatment (S31). Nearly 10 % of the total liver ascorbic acid was believed to be in this "bound" form. About 1% of the total liver ascorbic acid was actually isolated as a derivative from this fraction. These results were confirmed (H9), but still without proof that the major portion of the so-called "bound" ascorbic acid was in fact ascorbic acid. The identity of the fraction of ascorbic acid not extracted from liver by 95 % alcohol was established with guinea pigs given L-ascorbic acid-1-C^{14} (D12). The 95% ethanol-resistant fraction was chemically identified by carrier isolation as ascorbic acid. It amounted to about one-fifth of the total liver ascorbic acid in normal guinea pigs and a somewhat larger proportion in the depleted livers of scorbutic guinea pigs.

Recently, Lewis *et al.* (L11) have pointed out two defects in the

methods used in the studies of "bound" ascorbic acid. The production of nonascorbic acid chromogens in the dinitrophenylhydrazine method by the hot acid treatment of serum was demonstrated. This finding is cautionary, but not critical, for the liver studies where ascorbic acid in the "bound" fraction has been chemically identified. The second methodological deficiency is more serious: the lack of evidence that the 95% alcohol extraction procedure completely removes all of the free ascorbic acid. They suggested without experimental investigation that the gummy precipitate of liver and alcohol might retain a fraction of the free ascorbic acid. Thus, the "bound" fraction of ascorbic acid in liver of guinea pigs (L9) could equally well be nonascorbic acid chromogenic material formed by the heat treatment, free ascorbic acid sequestered in the protein precipitate, or truly bound ascorbic acid. In measurements of this fraction in liver (L9), they found its level unaltered by severe scurvy of 3 weeks duration. It must be concluded that at most only a small fraction of ascorbic acid may be bound in liver and that even this has not been conclusively demonstrated.

Somewhat similar evidence of a bound form of ascorbic acid was observed in the developing sea urchin egg after the stage of gastrulation (B1). The bound ascorbic acid was not extracted by cold water or KCl solution and only partially by hot water or cold trichloroacetic acid. But like the fractions studied in the liver of animals, it was readily extracted by 10 minutes treatment with hot trichloroacetic or metaphosphoric acids.

Ascorbic acid within the cells was localized in the cytoplasm (G3). More recent studies in rat liver and ox adrenals by the method of differential centrifugation have shown that most of the ascorbic acid was in the soluble and microsomal fractions (G2). The sum of ascorbic acid in the different fractions exceeded that of the whole tissue analyses by 40 %, suggesting to these workers the existence of bound ascorbic acid not liberated by extraction of the whole tissue. The fact of some localization in microsomes deserves confirmation.

6. Ascorbic Acid Nutrition

6.1. Absolute Requirements of Guinea Pig and Man

Estimates of ascorbic acid requirements can be small, for the amount to maintain histologically normal teeth in the guinea pig, or large, for the amount necessary to maintain blood levels equal to those of animals who synthesize their own ascorbic acid. The limits of the minimal protective dose against scurvy and the saturating dose are well known in guinea

pig and man. Numerous quantitative studies have been carried out to determine better criteria for establishing more exact requirements.

6.1.1. *Guinea Pigs*

The requirements of the guinea pigs have been set between 0.4 and 2 mg/animal/day (M3, L19). Two studies using a natural ration (C21) and a complete purified diet (C14) agreed on the larger requirement of 0.5 mg ascorbic acid/100 g body weight/day. It is significant that the total requirement is proportional to body size. Variation of the total requirement has been attributed to additional factors besides body size. Unless the ascorbic acid is given to guinea pigs daily, the requirement increases. Weekly and biweekly doses were only one-fourth and one-half as effective, respectively, as daily doses (M16). Female guinea pigs are said to be more susceptible to scurvy than males, possibly indicating a higher ascorbic acid requirement for females (B17). The ascorbic acid requirement is also increased after administration of iodinated casein, but not by thiouracil (P8, M15). Continued treatment of guinea pigs with cortisone or ACTH did not alter their ascorbic acid requirement (H7).

6.1.2. *Human Requirements*

The minimum daily requirement for protection against detectable scurvy is stated to be 10 mg or even less (K17). British publications set 30 mg as the "standard daily requirement" (B32), and opinions in the U.S.A. favor as high as 75 mg as the daily adult requirement (N3, G5). The more generous U.S.A. recommendations are based on the allowances which "permit the tissue storage within the range found in tissues of animals which synthesize, and therefore do not require, a dietary source of the vitamin, and in human tissues when there is a normal or reasonable intake of antiscorbutic food."

A critical discussion of the optimal ascorbic acid requirement of human adults has been made by Uhl (U2). When compared with the plasma levels of breast-fed infants, the higher requirements suggested by the U. S. National Research Council report appear quite reasonable. A daily ingestion of 75 mg of the vitamin brought the plasma ascorbic acid concentration of individuals to that found in breast-fed infants. This level is well below the "saturation" level at which renal excretion becomes large. Similarly high intakes are necessary in man to obtain the ascorbic acid blood levels of animals which can synthesize it, and of primates and guinea pigs eating a natural diet. Whichever requirement for man is adopted, it is clearly lower than that of the guinea pig on a body weight basis.

6.1.3. *Reasons for Species Difference in Requirements*

Unfortunately, too few quantitative studies have been done on species other than man and guinea pig to extend this comparison.

Under normal physiological conditions, the ascorbic acid concentrations in the corresponding tissues of man and guinea pigs are comparable (B22, Y2, D6). Yet their quantitative requirements of the vitamin are very different. Irrespective of the exact daily standard requirement for human (30 or 75 mg), the need for the vitamin per unit of body weight or surface area was many times less than for guinea pig (0.5 mg/100 g). Incomplete absorption of ascorbic acid from the gut by guinea pig in

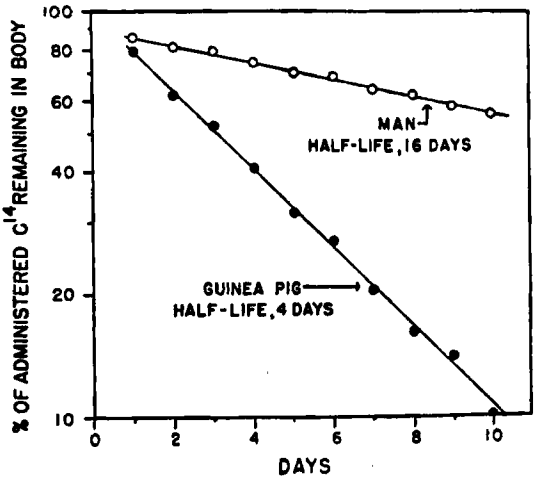


FIG. 4. Excretion of C^{14} after a dose of L-ascorbic acid-1-C^{14} to man and guinea pig. From Burns (B37).

contrast to more efficient absorption by man has been suggested as one explanation of the difference. However, the major difference must reside in the rates of metabolism as studied with labeled ascorbic acid. The guinea pig, rat, and man require daily 7, 26, and 1 mg vitamin C/kg body weight, respectively, to maintain body pools of 54, 107, and 20 mg/kg body weight (G5). The half-life of ascorbic acid is much shorter in guinea pigs (3 days) than in man (16 days) (Fig. 4), so guinea pigs require more to maintain the same pool size (B41). The degradations differ qualitatively in the two species. Guinea pigs, but not man, decarboxylate carbon 1 of ascorbic acid. Whether this is the route of the faster degradation of ascorbic acid in guinea pigs than in man is not known. The more rapid degradation of ascorbic acid in guinea pigs correlates

well with both the higher requirement and the more rapid development of scurvy in this species (2 weeks) than in man (17 weeks).

6.2. Partial Antiscorbutic Actions of Other Compounds

The possibility has been considered recently that ascorbic acid is multifunctional, and that some of its functions in the body could be performed by other compounds. Burns *et al.* (B42) administered D-ascorbic acid to scorbutic guinea pigs at sufficiently frequent intervals to maintain an adequate body concentration despite its rapid excretion. Although in the usual assays it has little or no antiscorbutic activity, given in this way D-ascorbic acid maintained the weight and prolonged the survival of the guinea pigs. It did not prevent hemorrhages. The dentine appeared to be normal, and though the predentine showed abnormalities, these were less than expected in full-blown scurvy. 3-Methylcholanthrene (F4) has a different partial action. It prevented scorbutic hemorrhages in guinea pigs. The use of this compound was suggested by its great effectiveness in increasing the synthesis and excretion of ascorbic acid when administered to rats. However, treatment of the scorbutic guinea pigs with this compound did not induce any synthesis of ascorbic acid. It will be noted later that the specific effects of ascorbic acid on isolated enzyme systems are usually shown by several congeners.

There is a qualitative difference between the action of compounds against part of the picture of scurvy, and the action against all symptoms of scurvy but with reduced potency. The small antiscorbutic activity of isoascorbic acid, which has been observed in the past, was not found when guinea pigs were first seriously depleted of ascorbic acid (R8). These authors interpreted the small antiscorbutic action of isoascorbic acid, previously observed, to its protection of residual stores of L-ascorbic acid in the body, an effect which they demonstrated. Similar protective effects by D-ascorbic acid and 3-methylcholanthrene appeared to have been eliminated in the experiments cited above. The several other compounds known to have low antiscorbutic potency deserve reinvestigation in the light of this work.

6.3. Epidemiology of Ascorbic Acid Nutrition

6.3.1. *Incidence of Scurvy*

Although scurvy is a rare disease, it is a constant threat to individuals or populations, especially during cultural changes. Adequate ascorbic acid intake is not insured without special effort in this direction. Woodruff (W12) pointed this out in commenting on the increasing incidence

of scurvy seen at Vanderbilt University Hospital during the period 1926 to 1954.

The adequacy of age-old nutritional habits and the frequent inadequacy of the usual dietary history was emphasized in two reports. Scurvy was absent and there were adequate plasma ascorbic acid levels in 62 Bantu children under 2 years of age. The poor nutritional histories of these children and the notoriously poor general nutrition of the Bantus prompted the suggestion that Bantus could synthesize ascorbic acid (A3). Scurvy has been reported, however, from an adult Bantu (G14), and it is most probable that the intake was higher than revealed by history. Scurvy is also rare among nomadic tribes in the Sahara, despite the paucity of fresh food. An outbreak of scurvy affecting 10 % of a tribe of 200 individuals occurred only when the nomads had been disturbed in their traditional living conditions (M25). As living standards are raised, children in particular become more dependent upon special ascorbic acid preparations rather than diet during the first few months of life (B16). A decline in the use of such preparations as orange juice is the major cause of infant scurvy (W3). A continuing and world-wide educational program is clearly needed to minimize the effect of present cultural changes on incidence of this severe but preventable disease.

6.3.2. *Assessment of Nutritional State*

6.3.2.1. *Plasma Ascorbic Acid Levels.* The measurement of plasma levels of ascorbic acid in populations has been used to provide more reliable data on average intakes than that obtained from the incidences of scurvy or from nutritional histories. It must be emphasized, however, that scurvy develops only in those individuals with plasma levels below those which can be accurately measured. Extensive studies have provided empirical correlations of expected plasma and white blood cell levels with different ascorbic acid intakes (L24, M24, S23). Other studies have also shown that both the ascorbic acid and the dehydroascorbic acid content of foods were equally effective in raising the plasma level of ascorbic acid (D8).

As would be expected from the very high concentrations of ascorbic acid maintained in the tissues, and as was found for the guinea pig, the intake of ascorbic acid per kilogram of body weight is more closely correlated with the plasma levels of children than the total intake of the vitamin (R15). Another study did not show this relationship (H4).

Studies of plasma levels have also demonstrated statistically some differences in distribution and possibly in metabolism of ascorbic acid of the sort which would be expected on biochemical grounds to occur in dif-

ferent physiological states. It is generally agreed that there is a sex difference in the plasma ascorbic acid levels not accounted for by the differences in intake. The level in postpubertal human females is higher than in males (R15, H4, C25). The opposite relationship occurs in rats. The ascorbic acid blood level in female rats is about one-third of the level in males (T8). It has long been suspected that ascorbic acid requirements were higher in pregnancy because of the demands of the growing fetus. An extensive study of pregnant women proved that plasma ascorbic acid levels fell during pregnancy and lactation (M12), although it cannot be concluded that this change resulted from increased utilization rather than from an altered distribution in the new physiological state. The women with the lowest levels had an increased frequency of premature births.

6.3.2.2. *Saturation and Intradermal Tests.* Ascorbic acid saturation tests, based on observations that subjects with low tissue reserves excreted less of a test dose of vitamin C in the urine than subjects with adequate stores, did not distinguish between varying degrees of deficiency at the lower levels of nutrition. Relatively high plasma levels must be attained before the effect of the renal tubular reabsorption is surpassed and significant excretion occurs (see Section 5).

Tests measuring the plasma ascorbic acid levels a few hours following the test dose were more useful for rapid evaluation of tissue storage of ascorbic acid (W7, S26, R10). The clinical usefulness of such a test was reported (D23) in a trial in which nine scorbutic patients had serum concentrations less than 0.25 mg/100 ml three hours after an oral dose of 15 mg ascorbic acid/kg body weight. Some individuals with normal intakes and low initial levels also had equally small rises. But rises to levels greater than 0.25 mg/100 ml were not consistent with scurvy.

An intradermal test measures the time required to decolorize a standard dose of 2,6-dichlorophenolindophenol injected into the skin. The accuracy and specificity of the method for diagnosis of ascorbic acid deficiency in animals (C8) and humans (C9) have been considered unsatisfactory. But in a group survey (R1) the test was reported to be useful as a guide for assessing the level of vitamin C nutrition of the population.

6.4. Relation to Other Nutrients

There are now far fewer investigations concerned with the modification of one nutritional state by another. Presumably such studies have been displaced by investigations of the biochemical mechanisms involved in the deficiencies.

6.4.1. *Iron Absorption and Metabolism*

It is well known that ferrous iron is absorbed from the intestinal tract of animals much more rapidly than ferric iron and that ascorbic acid can reduce ferric to ferrous iron. Additional proofs of the facilitation of ferric iron absorption by ascorbic acid have been reported (G11, B29, W5). The same action in a cell-free system was demonstrated with the stimulation by ascorbic acid of the incorporation of protein-bound ferric iron into protoporphyrin (L20). This enzyme reaction is known to require a reducing agent, and as these workers demonstrated, glutathione functions equally as well as ascorbic acid in this regard.

6.4.2. *Calcium*

The diminished deposition of calcium into the bone of scorbutic guinea pigs, suspected from the nature of the scorbutic lesion, was documented by isotopic calcium uptake studies (M4). Addition of ascorbic acid to a basal diet also produced a positive calcium balance in college women (L7). Somewhat similar effects have been observed in hens, although these birds do not require ascorbic acid (T7).

6.4.3. *Pantothenic Acid*

A sparing effect of large amounts of ascorbic acid fed to pantothenic acid-deficient rats has been known since the work of Daft (D2, D3), although no such effect occurs in guinea pigs (R7). It is believed that alteration of the bacterial flora of the intestine lessens the pantothenic acid deficiency under these conditions because it has been demonstrated that the antibiotic hygromycin acted in the same way as ascorbic acid, while sulfathiazole nullified the beneficial effect of ascorbic acid (B12). Pyrocatechol intensifies the beneficial action of ascorbic acid (L5).

The rat can convert glucose cycloacetoacetate to ascorbic acid, although this does not appear to be a physiological route of synthesis. The conversion is blocked by pantothenic acid deficiency (T6). This interesting compound is a cyclic ketal in which carbons 2 and 3 of acetoacetate are condensed with carbons 1 and 2 of glucose.

6.4.4. *Inositol*

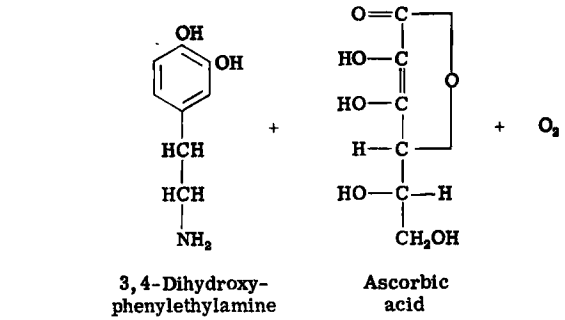
Inositol was considered at one time as a potential precursor of ascorbic acid. It does give rise to glucuronic acid (B43, C5) but not to detectable amounts of ascorbic acid. Anderson *et al.* (A2) have now reported that myoinositol has no antiscorbutic or growth-stimulating action in the guinea pig.

6.4.5. *α-Lipoic acid, Thiamine, and Riboflavin*

Partial protection from scurvy in guinea pigs provided by α-lipoic acid (R22) is probably due to the preservations of small residual amounts of ascorbic acid in the diet. Ascorbic acid has similar effects on riboflavin- (T4) and thiamine-deficient (T3) rats. The temporary improvements are the result of the reduction of thiamine disulfide to nutritionally active forms (B3).

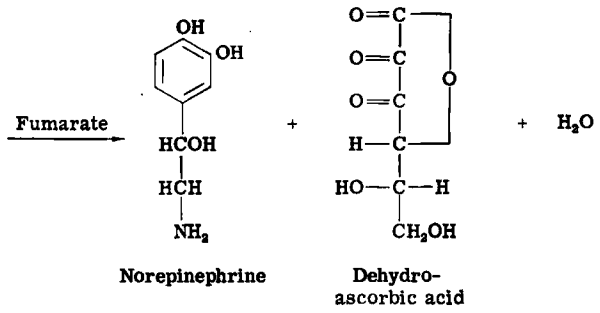
7. Actions of Ascorbic Acid on Enzymes

Ascorbic acid has an effect in many biological systems, in most of which it functions as a highly reducing chemical reagent or as a source of peroxide from its oxidation with air. Examples of these actions are the activations of aged aconitase or homogentisate oxidase preparations, in both of which it reduced Fe^{3+} to the required Fe^{++}, or the depolymerization of hyaluronic acid by monodehydroascorbic acid. The latter might be misinterpreted as an activation of hyaluronidase. Attention will be limited to effects in (7.1) defined enzyme systems with an element of specificity in the requirement for ascorbic acid, and (7.2) alterations in enzyme activities in living animals subjected to ascorbic acid deficiency.



3,4-Dihydroxyphenylethylamine — Ascorbic acid

[13]



Norepinephrine — Dehydroascorbic acid

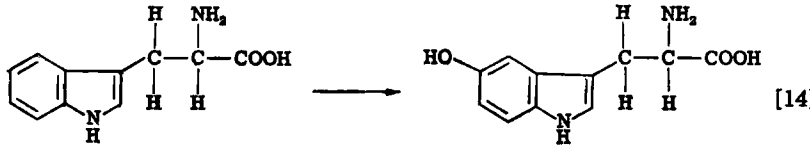
7.1. Functions in Cell-free Enzyme Systems

7.1.1. *Hydroxylation to Form Norepinephrine (Eq. 13)*

Adrenal medulla contains a particulate oxidizing enzyme concerned with the synthesis of pressor amines which catalyzes the reaction shown in Eq. 13. The enzyme was solubilized by freezing and detergent treatment and assayed by the product formed, either fluorometrically or by C^{14}. During subsequent purification, the activity increased as though a dialyzable inhibitor were being removed. The more purified preparations were almost completely dependent upon added ascorbate for activity. Maximal activity was obtained with 9×10^{-3} *M* ascorbate. The three stereoisomers of L-xyloascorbic acid were equally effective, but dihydroxyfumaric acid oxidase prevented activity, indicating that dehydroascorbic and diketogulonic acids were also ineffective. The system required oxygen and fumarate and was inhibited by KCN, DPNH, and TPNH, implicated as reducing agents in other hydroxylation reactions, had no effect in the system (L8).

7.1.2. *5-Hydroxylation of Tryptophan (Eq. 14)*

The formation of serotonin by carcinoid tissue and in smaller amounts by certain normal tissues has been demonstrated to occur from L-tryptophan by 5-hydroxylation followed by decarboxylation. The enzyme for



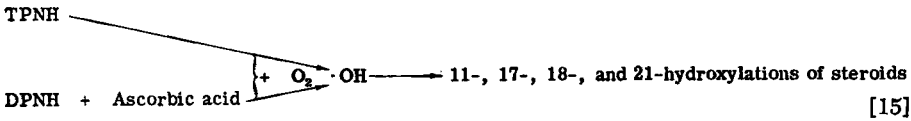
[14]

the latter reaction is well known, but, until recently, the oxidation step had not been found in cell-free preparations. The failures may in part be attributed to a soluble inhibitor of the particulate enzyme. Cooper (C19) has demonstrated the reaction in the particulate fraction of kidney and small intestine. It was not found in other tissues. C^{14}-Labeled tryptophan was used as substrate with an excess of added 5-hydroxytryptophan decarboxylase. The amount of labeled serotonin isolated with the aid of an unlabeled carrier served as the measure of the reaction. The reaction has two specific requirements: for Cu^{++} and for an ascorbic acid congener. L-Xyloascorbic acid, D-xyloascorbic acid, and araboascorbic acid were effective, and dehydroascorbic acid was even more effective than ascorbic acid itself. Strangely enough, no requirement for oxygen

has been demonstrated, since the anaerobic reaction rate equals the aerobic rate.

7.1.3. *Hydroxylation of Adrenal Steroids*

Studies of the biosynthesis of corticosteroids by adrenal glands, either perfused, minced, or in isolated mitochondria do not reveal any increased reaction with ascorbic acid, although the high ascorbic acid content of the adrenals has prompted many such experiments. Under certain conditions such an effect can be observed on the hydroxylation reactions which require molecular oxygen. An example of such a reaction is the 11-β-hydroxylase of mitochondria which converts DOCA to corticosterone, and which requires TPNH as a reducing cosubstrate. DPNH and ascorbic acid are ineffective in this reaction, but together with the microsomal preparation containing the ascorbic acid-dependent DPNH-oxidase described below, they will replace TPNH.

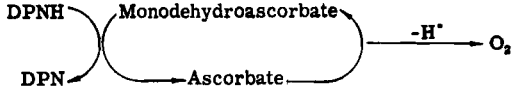


[15]

Staudinger's interpretation of this involves the central problem of such hydroxylation reactions: the generation of free radicals from O_2 as the possible hydroxylating agents (K4). The hypothesis is that the reaction of TPNH with O_2 in the mitochondrial 11-β-hydroxylase generates such free radicals, and so does the oxidation of ascorbic acid by the DPNH-oxidase-cytochrome b_5 system (Eq. 15). The monodehydroascorbic acid formed in the latter system is already well documented.

7.1.4. *Ascorbic Acid-dependent DPNH-Oxidase*

Microsomes of adrenal medullas contain an enzyme which oxidizes DPNH but not TPNH, and like the similar enzyme from plant tissues (K2, N2) utilizes oxidized ascorbic acid but not dehydroascorbic acid as the acceptor. Complexed Fe^{3+} in ferricyanide or cytochrome *c* can also serve



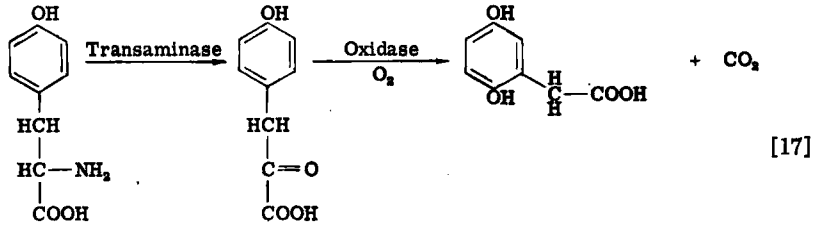
[16]

as an acceptor, so the reaction with monodehydroascorbic acid is not obligatory. The rate-limiting step is the provision of the appropriate oxidized ascorbic acid, which can be made aerobically with added ascorbic acid oxidase. The reaction presumably occurs with the monodehydroascorbic acid radical (Eq. 16).

This system cannot now be fitted into known pathways of electron transport, principally because a system that oxidizes ascorbic acid in animal tissues is unknown. The fact is clear, however, that ascorbic acid is oxidized to dehydroascorbic acid in animal tissues and that dehydroascorbic acid can be reduced. At the very least, this system emphasizes the potential efficiency of a form of ascorbic acid as an electron acceptor (K3).

7.1.5. *Tyrosine Oxidation (p-Hydroxyphenylpyruvate Oxidase) (Eq. 17)*

The interruption of tyrosine degradation at the stage of *p*-hydroxyphenylpyruvate (pHPP) in scurvy has long been considered the clearest evidence of a biochemical role for ascorbic acid. The intermediate accumulates and is excreted when doses of tyrosine are given to scorbutic man, other primates, and guinea pigs, and to premature infants (K8).



[17]

Tyrosine — *p*-Hydroxyphenylpyruvic acid — Homogentisic acid

Ascorbic acid administration prevents the excretion. The isolated pHPP oxidase of liver under certain conditions requires ascorbic acid or related compounds for its continued activity (K8). Thus, the *in vivo* and *in vitro* requirements for this metabolic reaction are similar.

It has long been doubted that this metabolic defect was a specific manifestation of scurvy. Painter and Zilva (P1) found no basal pHPP excretion by guinea pigs until unphysiologically high loading doses of tyrosine were fed. With such doses, excretion of pHPP began before the animals became scorbutic. They believed the defect occurred only when the animals were fed extra tyrosine and only if their bodies were less than saturated with ascorbic acid. But direct proof was lacking that the normal basal metabolism of tyrosine was intact in scurvy. Earlier enzyme studies were of no help, since ascorbic acid or its congeners had to be added to preparations from normal as well as from scorbutic animals to obtain activity.

Development of a new optical assay of pHPP oxidase permitted measurement of the enzyme without addition of ascorbic acid (L13). Prep-

arations from normal and scorbutic guinea pigs were then found to be equally active, supporting the theory of Painter and Zilva that the basal metabolism of tyrosine need not be defective in scurvy. The defect was produced by the dose of extra tyrosine given to manifest it. The mechanism involved the *in vivo* alteration of the activity of the two relevant

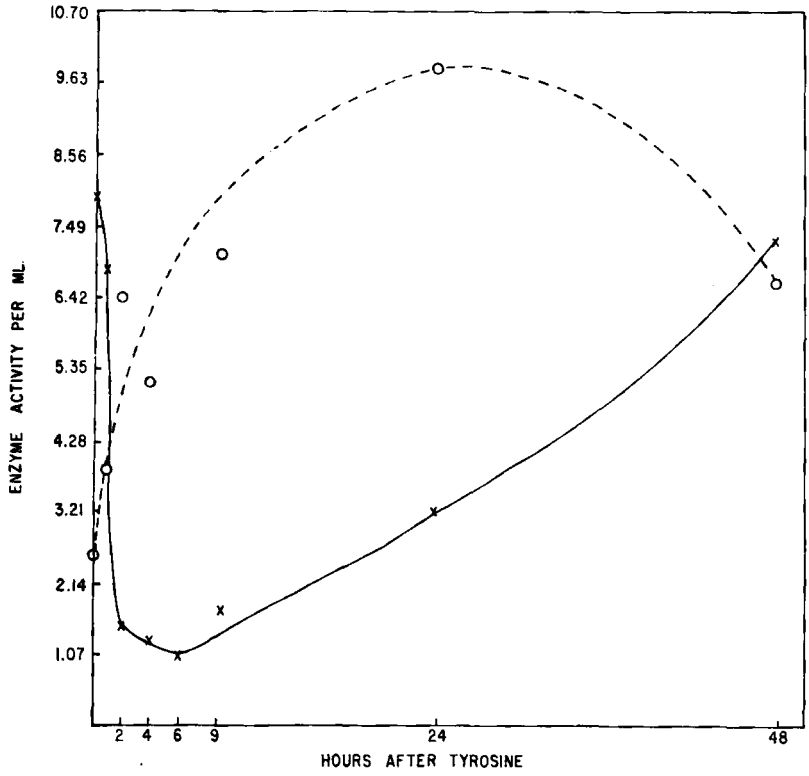


FIG. 5. Changes of liver enzyme activities after tyrosine dosage of guinea pigs; tyrosine-α-ketoglutarate transaminase (- -o- -), *p*-hydroxyphenylpyruvate oxidase (—x—). Each point is the average of homogenates from two animals. From Knox and Goswami (K11).

enzymes which formed and removed pHPP (K11). Formation of pHPP was increased by the adaptive elevation of the tyrosine transaminase activity, a change previously discovered in rats (L12). Removal of pHPP was decreased by reversible inactivation of pHPP oxidase, a common occurrence in *in vitro* systems with excess pHPP and insufficient ascorbic acid. These enzyme activity changes in guinea pig livers, following a dose of tyrosine, are illustrated in Fig. 5. Similar findings have been reported by other workers (Z2).

The metabolic defect in tyrosine metabolism is therefore induced by the extra tyrosine fed and is not a necessary part of scurvy. The fact that it can be induced in animals whose tissues are less than saturated with ascorbic acid, but not in animals saturated with ascorbic acid, supports the value of high nutritional intakes of vitamin C.

The biochemical role of ascorbic acid in maintaining the pHPP oxidase reaction *in vivo* when extra tyrosine is fed is similar to its role in the usual *in vitro* assays. The mechanism of this effect is still obscure. It is shared by the other stereoisomers of ascorbic acid and by certain other compounds such as dichlorophenolindophenol. It consists of maintaining the reaction against an inhibition which develops without ascorbic acid. It has been suggested (H1, Z1) that an inhibitor is formed from the substrate, or alternatively, that the inhibition is a reaction inactivation of the enzyme. Evidence in favor of the latter is the reversal of the inhibition by incubation of the enzyme with dichlorophenolindophenol (K11).

7.1.6. *Dehydroascorbic Acid Reductase*

$$2\ \text{GSH} + \text{Dehydroascorbic acid} \longrightarrow \text{GSSG} + \text{Ascorbic acid} \quad (18)$$

This reaction (Eq. 18) occurs spontaneously above pH 6. The similar reaction with H_2S is used in the analysis of dehydroascorbic acid by dye reduction. Such spontaneous reactions are believed to account for the dehydroascorbic acid reduction observed in animal tissue extracts, and no certain evidence of an animal tissue enzyme for this reaction has been obtained (B26, S12). Many plants contain such an enzyme, however.

$$\begin{aligned}
&\text{GSSG} + \text{TPNH} + \text{H}^+ \xrightarrow{\text{Glutathione reductase}} 2\ \text{GSH} + \text{TPN}^+ \\
&2\ \text{GSH} + \text{DHA} \xrightarrow{\text{Dehydroascorbic acid reductase}} \text{GSSG} + \text{Ascorbic acid} \\
&\text{Ascorbic acid} + \tfrac{1}{2}\ \text{O}_2 \xrightarrow{\text{Ascorbic acid oxidase}} \text{DHA} + \text{H}_2\text{O}
\end{aligned} \quad (19)$$

The plant enzyme can also utilize cysteine and thioacetic acid as reductants. The reaction goes to completion as written, and the reverse reaction has not been detected. This is one of the facts confirming the oxidation potential of GSH to be well below that of ascorbic acid (K10).

Reduction of dehydroascorbic acid is one of the steps of a potential electron transport system in plants involving ascorbic acid oxidase as the terminal oxidation reaction (Eqs. 19).

7.1.7. *Ascorbic Acid Oxidase*

$$\text{Ascorbic acid} + \tfrac{1}{2}\ O_2 \longrightarrow \text{Dehydroascorbic acid} + H_2O \quad (20)$$

The specific enzymatic catalysis of ascorbic acid oxidation is known in plants but not in animal tissues.

The aerobic oxidation of ascorbic acid by plant extracts has been attributed in the past to both traces of copper acting nonspecifically and to a specific copper-protein enzyme. The specific enzyme is now well characterized. Its action differs from that of the cupric ion–catalyzed reaction in three significant ways. Hydrogen peroxide is not formed as a product of the enzymatic reaction, while it is with cupric ions. The copper in the enzyme is about 1000 times more effective per atom as a catalyst than free copper. The enzyme exhibits a marked substrate specificity. Thus, the enzyme oxidizes L-xyloascorbic acid much more rapidly than the D-form, while cupric ions oxidize both at similar rates (D17).

The pure enzyme has been prepared from the rind of summer squash in yields of 0.3 g/ton. It has a molecular weight of 150,000 and contains 6 atoms of copper per molecule. The copper is bound very tightly in a specific way and cannot be removed by simple dialysis or resin treatment. Concentrated solutions have a blue color much more intense than ammoniacal solutions containing the same amount of copper. The blue color is bleached anaerobically by ascorbic acid and returns when oxygen is admitted. Additional evidence for the participation of the bound copper in the reduction is the fact that it can be exchanged during the reaction with labeled ionic copper added to the solution. The enzyme is inhibited strongly by cyanide, sulfide, and diethyldithiocarbamate though not by other copper-binding agents such as EDTA or amberlite resins (D9).

It is perhaps a commentary on the adequate specificity of the chemical methods of analysis of ascorbic acid that no concentrated efforts have been made to utilize the specificity of the readily available ascorbic acid oxidase in analyses.

7.2. Enzyme Activity Changes in Scorbutic Animals

In contrast to the enzymes in the previous section, with which ascorbic acid participates as a reactant, there are a number of enzymes which are found with altered activity in the tissues of scorbutic animals. Many attempts have been made to link ascorbic acid with the reactions of some of these enzymes, as a cofactor for example, but without success. Lacking any basis in biochemical principle, the relevance of the examples has often been minimized. It is probable, however, that most of

them represent instances of adaptive changes of enzyme concentration in cells, the response to the new physiological state of scurvy. Such changes occur in a wide variety of biological situations in animals. Substrate- and hormone-induced synthesis, and product repression of synthesis are the simplest examples (K12). In sum, these enzyme changes represent the metabolic adaptation of the normal animal to a new sort of scorbutic animal, and they biochemically characterize the disease.

Alkaline phosphatase of bone represents one of the best studied and most understandable instances of such enzymatic adaptation in scurvy. It can be accepted that this enzyme is produced by the functioning osteoblasts. It leaks into the blood serum, where its level is proportional to osteoblastic activity (so long as the supply of similar enzymes from liver and other tissues remains constant). The serum level is normally high in children whose bones are growing, but falls in scurvy (S19). The same occurs in guinea pigs (G8, G9). Cessation of growth in cretinism or from inanition has the same result, but the effect in scurvy is not due simply to inanition (P6). The effect is also specific for the cell type in bone. There is no significant change with scurvy in the alkaline phosphatases of intestinal mucosa, kidney, adrenal, or liver (G8). It is clear from studies of the isolated enzymes that ascorbic acid does not participate essentially in the bone phosphatase reaction. There is even a lag of a day after saturating the tissues of the scorbutic animal with ascorbic acid before the enzyme level begins to rise, referable to the time needed for enzyme synthesis. This rise can be correlated with the resumption of osteoblastic function and bone formation, not with an increase in osteoblast cell number (W9). The reappearance of alkaline phosphatase represents a biochemical alteration in the nature of the scorbutic osteoblast, closely related to its primary defect in formation of osteoid. The histochemical evidence of decreased enzyme in healing skin wounds might extend this principle to the scorbutic fibroblast (B35, D7).

The physiological significance of most of the known enzyme changes in scurvy is less clear than the example just cited, simply because the physiological consequences of alterations of the activity of most enzymes are unknown. The decrease in scurvy of the activity of the microsomal enzymes which hydroxylate and so detoxify the drugs antipyrine, acetanilide, (A10) and zoxazolamine (C16) are exceptions, since the drugs accumulate and are more toxic in the scorbutic animals unable to oxidize them. Physiological equivalents should be sought for the other known enzyme changes in scurvy. To this end the changed activities in cell-free preparations observed in scurvy are listed in Table 3. These include most

of those from the valuable compilation made in 1953 by Reid (R5), but this author should be consulted for additional early references. Significant as bases for these changes are the absence of activity changes with scurvy in aldolase of muscle, liver, and kidney (M27), alanine and aspartic transminases of liver (B36), and tryptophan pyrrolase of liver (M21). The latter enzymes undergo marked changes in some physiological states, but in scurvy they serve as indices that the relative proportion of other enzymes in cells have changed.

The decreases in cytochrome and succinic oxidases and the dehydrogenases are appropriate to the change in citrate oxidation and aconitase activity. The latter were only half reversed by *in vitro* addition of Fe^{++} and ascorbic acid, and so may well represent a quantitative decrease of enzyme amount. But some of the decrease must be attributed to iron loss, like that produced by α,α′-dipyridyl treatment of the animals, which also accounted for the loss of homogentisicase activity. These changes were the basis of the view that the primary function of ascorbic acid was to mobilize Fe^{++} in the body, and that scurvy caused inactivation of enzymes requiring Fe^{++}, including the citric acid cycle (T2). At the very least, these enzyme findings explain the increased tissue levels and urinary excretion of citric acid cycle intermediates in scurvy (B5; B9, B10), even though the amounts of these substances can be modified by insulin administration to scorbutic animals.

An increase in liver glucose-6-phosphatase like that in scurvy also occurs in starvation and may be produced by the inanition of scurvy along with the marked reduction of liver glycogen and reduced glucose tolerance commonly seen (M28, S24, B6). Inanition has not been observed to cause some of the other enzyme changes. ATPase, for example, increases in heart and skeletal muscle of fasted guinea pigs (M10) in contrast to the fall observed in the scorbutic animals (S22).

Some coenzyme measurements have been reported which should be related to the changes in enzyme activities. Scurvy decreased liver cocarboxylase concentration by half (B20) but did not alter pyridine nucleotide coenzymes (B11). Rats fed extra ascorbic acid (100 mg/day) had a decreased liver coenzyme A content of about 40 % after three days (C2).

Biochemical changes in scurvy not associated with discrete enzymes have been reported. The ratio of RNA to DNA increased in spleen and adrenals (G6). The DNA content of a fibrosarcoma was decreased by scurvy, and glucoascorbic acid given to lower the tissue ascorbic acid levels in rats decreased the DNA in rat carcinoma (S21). Dinning *et al.* (D15) expected defects in the reactions where folic acid participates,

TABLE 3

ENZYME ACTIVITY CHANGES FROM NORMAL IN SCORBUTIC GUINEA PIGS

Enzyme	Change	References
Hydrolytic enzymes		
Alkaline phosphatase	No change: intestine, kidney, adrenal, liver, and brain	(G8, H5)
	Decrease: bone and serum, marked	(G9)
ATPase	Increase: liver	(M27)
	Decrease: heart and skeletal muscle, 50 %	(S22)
Cathepsin[a]	Decrease: liver and kidney	(cited in S22, O1)
β-Glucuronidase	Decrease: liver, 90 %	(B18)
Esterase (ethyl butyrate)	Decrease: muscle	(cited in S22)
	Decrease: liver, 66 %	(H5, P3)
	Decrease: blood, marked	
Lipase	Decrease: duodenum, marked	(B25)
	Decrease: liver and kidney	(cited in S22)
Proteolytic enzyme[a]	Increase: liver, kidney, and muscle	(cited in S22)
	Increase: liver	(M13)
	Increase: blood	(P2)
Other enzymes		
Aconitase	Decrease: liver, 80 %	(T2)
Catalase	No change or Decrease: blood	(T9, T11, P2, and cited in S22)
Citrate oxidation (mitochondria)	Decrease: liver, 90 %	(T2)
Cytochrome oxidase	Decrease: heart, skeletal muscle, 45 %	(H5)
	Decrease: liver, marked	(P9)
	No change: brain	(H5)
Glucokinase[a]	Decrease: liver	(B6)

TABLE 3 (*Continued*)

Enzyme	Change	References
Other enzymes (*Continued*)		
Glucose-6-phosphatase	Increase: liver	(L2)
Homogentisicase	Decrease: liver, marked	(S29)
Hydroxylation of drugs	Decrease: liver, antipyrine, acetanilide, and zoxazolamine, 60%	(A10) (C16)
Isocitric dehydrogenase	No change: various tissues	(B11)
Lactic dehydrogenase	Decrease: liver, kidney, brain, heart, and skeletal muscle	(B11)
Malic dehydrogenase	Decrease: liver, kidney, brain, heart, and skeletal muscle	(B11)
Phosphorylase[a]	Decrease: liver, marked Decrease: kidney, slight	(M27)
Succinic dehydrogenase	Decrease: heart, skeletal muscle, 65 % Decrease: liver, kidney, brain, heart, and skeletal muscle	(H5) (B11)

[a] Identity of enzyme reactions measured is in doubt.

but found that scurvy did not alter the RNA in liver, spleen, or skeletal muscle, although the DNA of muscle was elevated, as expected from the increased number of muscle nuclei found in scurvy. There was no decrease in formate-C^{14} incorporation into purines in liver, but marked decreases into serine and methionine. These workers used a complete diet which avoided any associated deficiencies.

The increased rate of acetate-1-C^{14} incorporation into cholesterol in the adrenals of ascorbutic guinea pigs is a striking change of a similar nature (B19). The rate goes up as the scurvy progresses, and in severe scurvy is three times normal. A smaller change occurs in liver.

8. The Nature of Scurvy

Our knowledge of molecular disease mechanisms is still inadequate to explain how a specific deficiency of the unique chemical substance, ascorbic acid, produces its remarkably precise effects. A critical description of these disease effects will be given here, as a way of posing the problems to be answered by further biochemical research. It must be a future aim to correlate the molecular chemical knowledge reviewed above with the pathology and pathological physiology of scurvy. It will be well at the outset to distinguish between the specific disease of experimental scurvy and the clinical and older experimental examples which often included other sorts of disease as well as scurvy.

8.1. Experimental Scurvy in Man

Scurvy has been intentionally induced under controlled conditions in fewer than a dozen adults and in no children. These studies run from the pioneering study by Crandon on himself to the Medical Research Council study during World War II on a group of conscientious objectors (C22, R9, P10, P7, K17). The severe pathological symptoms sometimes encountered clinically were avoided, and there were no deaths. The development of scurvy was fairly uniform (K17): "no clinical signs for about 17 weeks; the first sign, after 17–21 weeks, was hyperkeratosis of the hair follicles; after 26–34 weeks perifollicular haemorrhages occurred and after 30–38 weeks swelling and haemorrhages of the gums." In the late stages, individuals also showed exacerbation of previously existing acne, joint effusions, ecchymoses of the legs, failure of healing of experimental wounds, and two suffered cardiac emergencies with electrocardiographic changes. Hemorrhage into the healed scars of experimental wounds made four months earlier were observed. It is significant that there was observed no body weight loss, anemia, increased bleeding times, increased capillary fragility, or susceptibility to infection. The

congestion and proliferation of blood vessels and eventual hemorrhage into parts, which was the basis of all of the observed changes, are compatible with a loss of structural strength of the tissue elements, especially of those intentionally wounded or normally subject to stresses and strains. The chemical observations of ascorbic acid levels during the development of scurvy were particularly important. Plasma ascorbic acid levels fell to an unmeasurable value (0.1 mg%) after 3 weeks, and the white blood cell level fell to an equally unreliable value (below 2 mg%) after 12 weeks (See Figs. 2 and 3). Twice this period elapsed before the first nonspecific signs of scurvy appeared. Individuals receiving 10 mg ascorbic acid/day were completely protected against the clinical signs of scurvy but could only be chemically differentiated from the deficient subjects by giving weight to the unreliable difference between 2 and less than 2 mg% of ascorbic acid in the white blood cells (Fig. 3). Measurable ascorbic acid levels are therefore of value in excluding scurvy, but they cannot substantiate it. Very low levels in white cells are compatible with scurvy but do not prove it.

At the conclusion of the Medical Research Council study, saturation tests were done on the subjects with long histories of accurately known ascorbic acid intakes. More than three daily test doses (10 mg/kg body weight) were necessary before a sharp rise occurred in urinary ascorbic acid excretion of subjects who had been receiving 20 mg/day or less, but again no distinction could be made in this way between the deficient and the protected individuals receiving the lower amounts.

8.2. Experimental Scurvy in Animals

The guinea pig has been the subject of most of the experimental studies of ascorbic acid deficiency. The requirement of this species for fresh foods was discovered by Holst and Frölich (H16, H17) in their attempts to reproduce the so-called "ship beri-beri" occurring in the Norwegian navy. Scurvy has also been studied in the monkey (M17). The loss of a single gene which controls the synthesis of ascorbic acid apparently occurred at some stage in evolution, so that this loss is shared by all the other surviving primates. A separate genetic mutation must be responsible for the disability in the guinea pig. Close scrutiny has revealed additional species who suffered this sporadic mutation, and study of these should greatly extend our knowledge.

8.2.1. *Ascorbic Acid Requirement in Other Species*

It will be seen that familiar animals should not be assumed always to synthesize their own ascorbic acid. Harris *et al.* (H8) could not pro-

duce experimental scurvy in rabbits by feeding a vitamin C-free diet for as many as 25 weeks. The rabbits synthesized their own vitamin C, and the ascorbic acid concentration in the rabbit organs was comparable to that in rat and chick, but greater than that in guinea pig with access to liberal amounts of vitamin C.

The marmot has been cited (M22) as another mammal which is susceptible to scurvy. It is closely related to the guinea pig. Recently, Fowler (F3) reported the occurrence of "scurvy" in a 19-month-old female elephant which responded slowly to ascorbic acid therapy alone, but markedly when vitamin C was given with vitamin D and other B vitamins.

Roy and Guha (R24) induced experimental scurvy in a bird, the red-vented bulbul (*Pycrorotus cafer*). The birds lost weight and feathers and died after 16 to 45 days on a scorbutigenic ration. The scorbutic birds recovered with administration of ascorbic acid. The susceptibility to scurvy correlated well with the lack of activity in this species of the specific liver enzyme which is indispensable for ascorbic acid biosynthesis (L-gulonolactone oxidase). This enzyme was present in the liver of bank-myna (*Acridotheres ginginmanus*) which was not susceptible to scurvy.

The Indian fruit bat is also lacking in the liver enzyme for ascorbic acid synthesis (C6), but no nutritional studies have been reported.

Ascorbic acid has been included in synthetic diets for fish (W6), but control studies revealed no requirement for ascorbic acid by rainbow trout. Halver (H2) similarly found no definite requirement for vitamin C by chinook salmon fry.

Dadd (D1) discovered that complete development of the locusts, *Schistocerca gregaria* (Forsk.) and *Locusta migratoria* L. was arrested by lack of ascorbic acid. In *Schistocerca,* reduction in growth was manifest in the third instar, leading to heavy mortality at the fourth to fifth instar; no adult *Schistocerca* were obtained without ascorbic acid. In *Locusta,* growth reduction was observed in the last nymphal instar, and mortality occurred at the final moult; the adults which emerged were short-lived and malformed. The author suggested that ascorbic acid was involved in the moulting processes in locusts. The observations may determine if this function is comparable to the elaboration of some mesenchymal tissues in animals.

The silkworm, which feeds on ascorbic acid-rich mulberry leaves, was reported to require ascorbic acid (L6).

In studying the nutritional requirements of the amoebas, Nakamura and Baker (N1) found that bacteria-free *Entamoeba histolytica* could be

maintained up to 27 days in a dialyzate of bacterial media fortified with growth factors which included vitamin C.

It is of interest that no proof of an ascorbic acid requirement by human cells in tissue culture has been presented and culture media frequently do not contain it (E1).

8.2.2. *Scurvy in Guinea Pigs*

Scurvy in guinea pigs, uncomplicated by other known dietary deficiencies, has been possible only in recent years (R6). The earlier experi-

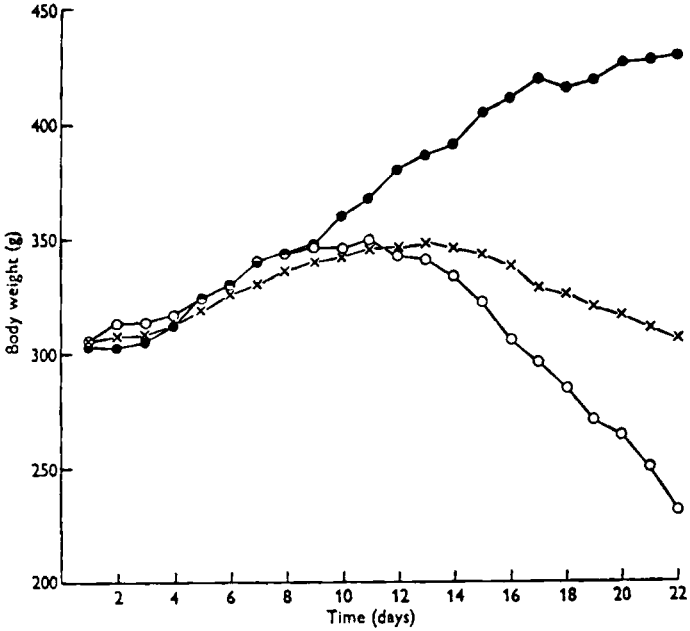


FIG. 6. Mean weight curves of six guinea pigs fed the purified diet of Reid and Briggs. Scorbutic group (o-o); 0.2 % ascorbic acid was added to the diets of the pair-fed (x-x) and control (•—•) groups. The weight loss of the pair-fed group indicates the degree of starvation which occurs in scorbutic animals. From Constable (C18).

ments produced ascorbic acid deficiency as the principal, but not necessarily the only deficiency. Debatable symptoms in the disease should therefore be neglected unless their occurrence has been confirmed by the best modern nutritional investigations.

The development of scurvy in the guinea pigs is much more rapid than in man, as would be expected from the higher requirement per unit body weight and the shorter half-life of ascorbic acid in guinea pigs.

The growth of young animals on a scorbutigenic diet continues for about 2 weeks, after which there is a rapid loss of weight until death occurs near the end of the fourth week. The characteristic symptoms during this time are severe loss of appetite, decreased activity, assumption of a typical posture—sitting hunched with a drooping head as though their faces ached—and the development of hemorrhages into almost any part of the body. The hemorrhages occur especially in those tissues subjected to stress or trauma. Beading of the rib cartilages and other evidences of interference with bone and cartilage growth are found, as well as the cessation of growth of the incisor teeth. The swollen gums and loose teeth of scurvy in primates do not occur unless life is sufficiently prolonged by a minimal ascorbic acid intake to allow these signs to develop. Typical weight curves of guinea pigs with a modern uncomplicated deficiency of ascorbic acid are shown in Fig. 6.

8.3. Clinical Scurvy

Scurvy today is a rare disease affecting two different age groups. It occurs in formula-fed infants between the ages of 6 and 18 months whose diets have not been supplemented with added sources of ascorbic acid, and in men of middle or old age who are usually bachelors or widowers and who for more than a year have neglected fresh foods. It must be emphasized that in both of these age groups scurvy is only the predominating illness, and almost always there are other deficiencies or disease states.

8.3.1. *Adults*

Adult scurvy develops exactly like that observed in the experimental human cases. Its effects are often more severe because they are more prolonged, and because the patients are debilitated by age, other diseases, and associated deficiencies. Purpura and ecchymoses develop at the sites of minor trauma on the lower extremities, and there are hemorrhages into muscles and joints. The gums become swollen, blue-red, and bleed easily, but only around teeth whose looseness provides the mechanical strain needed to initiate the lesion. Old ulcers break down, recent scars of apparently healed wounds become red and hemorrhagic and new wounds fail to heal. Death often occurs suddenly from minor precipitating reasons. The prompt administration of ascorbic acid in curative amounts can be lifesaving.

8.3.2. *Infants*

Infantile scurvy appeared late in the last century when it first became possible for infants to survive on artificial feedings. It seldom develops

before the age of 4 months. The signs differ from the disease in adults principally because of the rapid bone growth and development of other tissues taking place at this age. There is loss of appetite and listlessness for only a few days before the lesions appear. Extremely tender swellings may be felt at the ends of the long bones due to hemorrhages under the periosteum. The child cries when touched or moved. Purpura and hemorrhagic lesions around what teeth may be present are less common than in adults with scurvy, but widespread hemorrhages do develop and may cause death from involvement of vital areas if ascorbic acid treatment is delayed.

8.4. Pathological Description of Scurvy

It has been a remarkable achievement of descriptive pathology to identify the widespread effects of scurvy with the disturbance of function of four related cell types: the odontoblasts, osteoblasts, chondroblasts, and fibroblasts. Changes occur in tissues not formed primarily by one of these cell types, but these are usually minor, secondary, or unrelated changes. The central role of these special mesenchymal cells was suspected from Aschoff and Koch's (A8) finding that the lesions in human scurvy were localized in the supporting tissues. Höjer (H13) said: "All these cells seem in the absence or deficiency of antiscorbutic to be subject to atrophy and yield a product which is quantitatively as well as qualitatively inferior. If antiscorbutic is altogether lacking in the food, these cells go on living for a certain time and yield during this time products which deteriorate day by day until the activity is completely arrested and the death of the cell ensues." The classic studies of Wolbach and Howe (W11) showed that the common pathological denominator in the disturbance of these cells was the defective formation of intercellular matrix. Wolbach (W10) has given a résumé of these studies to emphasize the importance of experimental scurvy as a means of studying the intercellular substances.

Complete descriptions of the changes in the various tissues are available (F2). As a consequence of the functional defect in the mesenchymal cells, the formation by the fibroblasts of collagen ceases. This important structural protein in connective tissues makes up about one-third of the total protein of the body (N5). The formation by the osteoblasts of osteoid tissue as a base for calcification also ceases. So does the formation of dentine by the odontoblasts, and the chondroblasts in the cartilage cease to form the cartilage matrix. The greater the activity of these several cells in the organism, the more prominent are the effects when they stop. Thus, the abnormalities in the continuously growing incisor

teeth of guinea pigs provide a very sensitive index of scurvy in this species. Because of the rapidly growing long bones in infants, the effect of scurvy is outstanding on the bone and cartilage at the epiphyses. In the adult, weaknesses in the collagen-supporting tissues and the minor trauma of everyday life give rise to most of the pathological changes. This effect is seen particularly well in the failure of wounds to heal. It is clear that new formation, and not maintenance of preformed supporting structures, is most affected by scurvy. Experimental evidence that ascorbic acid is also required for the maintenance of the intracellular substances may come from the very slow reformation of these substances as they turn over normally in their dynamic state.

The defective function in four types of mesenchymal cells explains the growth of pathological lesions in scurvy so satisfactorily that there can be no doubt about the essential soundness of this generalization. The cellular defects explain not only the radiological changes in the epiphyses of long bones, but also the generalized osteoporosis. This develops when new bone formation ceases, while bone dissolution continues unabated.

There is only uncertainty as to the cause of the hemorrhages. Numerous investigations of capillaries and of the bleeding time in scurvy have been prompted by the hemorrhages. The results are remarkable only for the lack of any significant alterations uniformly observed. The possibility exists, as Wolbach (W8) assumed, that intercellular substances of the vascular endothelium are not formed. It is clear from the nature of the hemorrhages in human and experimental scurvy that a failure of support either from within or nearby the vascular tissues would permit them to occur.

The lack of specific changes in other specialized tissues of the body in scurvy reinforces the view that the disturbance in mesenchymal tissues is the primary defect. Many of the changes which have been observed are secondary to injury, to hemorrhage, and to the semistarvation of the scorbutic animal. There is waxy degeneration of skeletal but not heart muscle, degenerative changes resulting from hemorrhage in the central nervous system, and nonspecific changes in the liver. The salivary glands atrophy early in scurvy, but the closely related pancreas does not (H13). It is significant that the epithelial tissues, including skin and other glands, are little affected. The gonads are exceptions to this. Both ovary (K15) and testes (L16) degenerate at an early stage of the deficiency. With the exception of the gonads and the retina in extreme scurvy (M26), the body tissues normally possessing the highest level of ascorbic acid are not those predominantly affected in scurvy. Fascia, which can be con-

sidered representative of the connective tissues affected by scurvy, normally contains only about 10 times the plasma concentration of ascorbic acid (C23).

Chronic scurvy in the guinea pig produces articular changes resembling rheumatoid arthritis (K14, C24). The pathogenesis is unclear.

It must be remembered that the generalization given describes a histological, that is, a cellular defect and not a chemical defect. It is impossible to decide on the evidence whether the suspected biochemical defect is in the cell or in the cell products. The histological descriptions are unanimous in finding abnormalities of the mesenchymal cells themselves. They continue to proliferate during scurvy, but do not differentiate and mature in the normal way, and their arrangement in the tissues is disorganized. Although the predominant result in scurvy is the lack of the specific intercellular products of these cells, ascorbic acid could equally well cause this as a maturation factor for cell function or an essential reactant in the formation of the cell product. The many elegant chemical studies of connective tissue metabolism in scurvy are equally compatible with either view. The well-documented fall in bone and serum alkaline phosphatase that occurs in scurvy provides independent evidence of an inactivity of these cells (G8), since this enzyme level is correlated with osteoblastic function.

The skeletal and vascular form of lathyrism (odoratism) produced by feeding sweet peas (*Lathyrus odoratus*) or β-aminopropionitrile (S15) bears certain resemblances to scurvy. Only a single observation is recorded about any possible connection. The production of odoratism in rats was not modified by feeding them 12 mg ascorbic acid daily (L10).

8.5. Functional Abnormalities in Scurvy

8.5.1. *Collagen Formation*

Information on the effect of ascorbic acid on collagen biosynthesis and the nature of the constituents of the ground substances has been gathered mainly by studying scurvy repair, the formation of granulation tissues induced by injecting "carrageenin," a polysaccharide from Irish moss, and by the healing of wounded tissues.

The almost unique occurrence of hydroxyproline as a significant constituent of collagen has provided a convenient chemical measure of collagen synthesis.

Deprivation of ascorbic acid results in the inability to form collagen, as measured by hydroxyproline formation. Ascorbic acid given to scorbutic animals 10–12 days after wounding induced hydroxyproline for-

mation within 24–48 hours. Ascorbic or dehydroascorbic acids were also effective when applied locally to polyvinyl sponges implanted subcutaneously to the scorbutic guinea pigs (G7, G10). The same requirement of vitamin C for collagen formation with carrageenin granuloma of guinea pig was also shown by Robertson *et al.* (R13). They measured the conversion of C^{14}-labeled proline to hydroxyproline by similar techniques. Mitoma and Smith (M21) found both proline and hydroxyproline contents to be lower in the carrageenin granulomas of scorbutic guinea pigs than in normal guinea pigs. Since the low urinary hydroxyproline excretion was not altered by ascorbic acid deficiency, they questioned the prevalent view that an inability in the conversion of proline to hydroxyproline is the primary cause for failure of collagen synthesis in ascorbic acid deficiency [see also (A7)]. Mitoma and Smith tested an alternate hypothesis, that all protein synthesis was defective in scurvy. The adaptable liver enzyme, tryptophan pyrrolase (K9), was synthesized as usual by scorbutic animals given tryptophan. This study re-emphasized the specificity of the scorbutic lesion, recently confirmed by new histological studies (W4, A1). By both histological observation and chemical measurement, collagen synthesis fails without ascorbic acid (G13). But this is not the sole defect.

Wound repair can be taken as an expanded version of the cellular process under consideration. The normal events in repair of a wound, described by Dunphy *et al.* (D21), were accumulations of a ground substance revealed by increases in hexosamine concentration and supported histologically by increased metachromasia and colloidal iron staining of the wound. Fibroblasts increased in large numbers, and with invasion of capillaries, collagen formation proceeded. The tensile strength of the wound increased with collagen synthesis, and, simultaneously, hexosamine concentration sharply declined. By 12 days the collagen content of the wound reached a maximum value.

In contrast, studies on the effect of ascorbic acid deficiency on the sequence of wound repair in guinea pig (D21) showed an initial decrease in amount of hexosamine and absence of metachromasia. Fibroblasts were poorly oriented and immature, and no collagen formation took place. By the twelfth day, the absence of collagen and the presence of immature fibroblasts were established, and hexosamine content was found to be high instead of low as in normal repair.

It is clear that complex events occur before, but related to, collagen synthesis. These are believed to be the formation and resorption of a ground substance composed of mucopolysaccharides which is altered in scurvy. The clearest statement of the problem is still that of Meyer

(M19), who considered that the young fibroblasts secrete hyaluronic acid, and that ascorbic acid was needed perhaps as a nonenzyme catalyst for the replacement of hyaluronic acid by chondroitin sulfate and collagen.

There is definite evidence for deficient synthesis of the chondroitin sulfate fractions in the ground substance of scorbutic guinea pigs. Incorporation of radiosulfate into chondroitin sulfates of cartilage (R2) and granulation tissue (K13) was found to be reduced in scorbutic guinea pigs. There is also only little S^{35}-labeled sulfate uptake in wounds of scorbutic guinea pigs (U3). Impaired formation of chondroitin-containing mucopolysaccharides in ascorbic acid deficiency has been indicated by the report (H20) of the absence of galactosamine in scorbutic pair-fed and normal guinea pigs.

Earlier studies (K13) did not find the total hexosamine values in scorbutic granulation tissue different from those in control animals. Similar observations were made in scar tissues of scorbutic and normal guinea pigs (A1).

The hyaluronic acid concentration of the carrageenin granuloma of scorbutic guinea pigs was 5 times as high as that in ascorbic acid-supplemented animals (R12), even though the acid mucopolysaccharide concentration of the skin and cartilage was significantly lower in scorbutic than in normal guinea pigs (B27). These observations are giving some chemical significance to the historical changes seen during tissue repair (R11).

8.5.2. *Anemia*

Anemia has frequently, but not consistently, been reported in association with scurvy in clinical cases, both in adults and infants, and in experimental scurvy in guinea pigs and monkeys. The extensive literature has been cited (M18, L19, V2). The anemias were not constant in type or severity, although all were relieved when ascorbic acid was reintroduced to the diet. In contrast, no anemia or abnormality of the blood picture was ever seen in experimental human scurvy. The therapeutic effect of ascorbic acid on the anemia cannot be accepted as proof of its etiology, since associated deficiencies would also be made good with the return of normal appetite. It is well recognized that the clinical cases of scurvy usually have associated deficiencies. Often, there is iron deficiency in the milk-fed infants, and folic acid or vitamin B_{12} deficiency in the malnourished adult.

The occurrence of anemia in clinical scorbutics does not necessarily suggest that ascorbic acid deficiency causes anemia. This possibility

was underlined by the irregular occurrence of anemia in experimental scurvy. Recent careful experiments by Constable (C18) were done using guinea pigs fed a diet believed to be adequate in all known nutrient substances (R6). Neither acute nor chronic scurvy in young or old guinea pigs produced anemia. The hematocrit, hemoglobin, and red cell counts did not differ significantly from those of the pair-fed controls or the animals receiving a normal diet. There was an increase in reticulocytes, possibly in response to the blood loss by hemorrhages. There have been no similar experiments as yet in monkeys.

8.5.3. *Blood Coagulation*

The dramatic and unexplained hemorrhages of scurvy have kept attention focused on the possibilities of abnormalities of the capillary vasculature and of blood coagulation. Of these, an abnormality in blood coagulation has been most consistently found. The study of the individual components of the coagulation process has shown some abnormalities.

8.5.3.1. *Prothrombin Time.* Ascorbic acid deficiency resulted in an increased prothrombin time of the blood of guinea pigs (M14, S30). Confirmation of this finding and further proof that it was due to ascorbic acid deficiency was obtained in recent studies (B14) by using the purified diet of Reid and Briggs (R6).

8.5.3.2. *Fibrinogen.* Another abnormality of blood with the onset of scurvy is the marked increase of plasma fibrinogen concentration reported both in the guinea pig (M14) and the monkey (S2).

8.5.3.3. *Other Factors.* There is little information on the changes in other components of the blood-clotting system in scurvy. Barkham and Howard (B14) have produced evidence that plasma thromboplastic factors and the Factor VII, which are known to be involved in blood clotting (B31), are low in the blood serum and plasma of scorbutic guinea pigs. No abnormality was observed in the clot retraction of scorbutic animals. Small increases (B14) and decreases (A4) in the number of blood platelets have been reported.

8.5.4. *Blood Proteins*

Changes in the serum and plasma protein fractions in vitamin C deficiency in guinea pig have been reported in addition to the increased fibrinogen already mentioned. Howard (H18) did not find any difference in the total plasma protein concentrations or in the electrophoretic mobility on paper of plasma proteins from normal and scorbutic guinea pigs. However, in both acute and chronic scurvy, the amount of plasma albu-

min decreased and the fibrinogen increased. α-Globulin slightly increased in acute scurvy without changing in the chronic cases. γ-Globulin increased by 40 % during chronic scurvy.

Langier and Monnier (L4) reported lower total protein and albumin concentrations of the serum of guinea pigs deprived either partially or completely of vitamin C. α-Globulin increased in both partial or completely deprived groups. A smaller increase of γ-globulins occurred in animals partially deprived of vitamin C, in contrast to the decrease of the same fraction in completely deprived animals.

Banerjee and Rohatgi (B7) and D'Agostino *et al.* (D4) reported similar changes. There was a decrease in albumin fractions in the guinea pig serum as a result of scurvy. Decreases in α_2- and β_2-globulins without significant changes in β_1- and γ-globulins occurred.

8.5.5 *Adrenal Function*

The high level of ascorbic acid in the adrenal gland and the release of this ascorbic acid, together with cholesterol, under the influence of ACTH, has given rise to hypotheses that ascorbic acid is destroyed or consumed during adrenal gland activity. Some more or less reasonable corollaries of such hypotheses have been the bases of a vast amount of investigational work. Clear answers have been rare, so it is worthwhile quoting the conclusions of Kark (K1) from his studies of some of these relationships in man: "There is no evidence that the stress of cold increases significantly the ascorbic acid requirements; there is no evidence that adrenal function is curtailed as a result of ascorbic acid deficiency; and there is no evidence that ascorbic acid retention seen after operation is due to adrenal activity." Some of the opposites may be true, but there appears to be no critical relationship between ascorbic acid and adrenal gland function.

The adrenal gland does not synthesize ascorbic acid but merely concentrates it to a high level in common with a number of other tissues. The ascorbic acid lost from the adrenal under the influence of ACTH leaves as dehydroascorbic acid, which is the more diffusible, neutral form. The mechanism of the prompt oxidation under the influence of ACTH is unknown (S3, S4).

Cortisone or ACTH administration did not alter the ascorbic acid requirements of guinea pigs for prevention of scurvy (P8, H7). As would be expected, ACTH or cortisone did not diminish the antiscorbutic effectiveness of dehydroascorbic acid in guinea pigs (C13), nor did they affect the plasma levels in patients with rheumatoid arthritis (M1). Both ACTH and cortisone do, however, increase the excretion of ascorbic acid in the urine on large intakes (K1).

In contradiction to the commonly held hypothesis that ascorbic acid is necessary for adrenal gland function, the gland activity appears to be higher than normal in scorbutic animals. Serum from scorbutic guinea pigs contained high levels of an ACTH-like material when assayed by the adrenal ascorbic acid depletion method in other animals (C12). The plasma level of 17-hydroxycorticosteroids was also elevated in scurvy tenfold according to Done *et al.* (D18) and could be elevated still more than in normal animals by stressful stimuli. Increased amounts of 17-ketosteroids in the urine were reported in scorbutic guinea pigs and monkeys (B8, C11). While these studies do not preclude a role for trace amounts of ascorbic acid in the synthesis of the corticosteroids, they should disprove the long-held belief that adrenocortical function is diminished in scurvy.

8.5.6. *Aminoaciduria*

An abnormally high urinary excretion of α-amino nitrogen and glycine conjugates by vitamin C-deficient infants was reported by Jonxis and Wadman (J1). Evidence of a possible vitamin C deficiency in a premature infant resulting in hyperaminoaciduria has also been reported by Dustin and Bigwood (D22). Some of these problems have also been reviewed recently (B24). In view of the fact that an aminoaciduria occurs in all premature infants, to a lesser degree in full-term infants, and only diminishes later during childhood, it is doubtful that these studies demonstrated any additional aminoaciduria due to ascorbic acid deficiency.

Chadwick *et al.* (C3) have cited the case of an elderly scorbutic patient with low serum vitamin C level (0.4 mg/100 ml), the scorbutic condition being corrected within a month by administration of ascorbic acid accompanied with ferrous gluconate. Abnormal amounts of urinary phenylalanine, leucine, methionine, alanine, serine, threonine, glycine, and arginine were found before treatment. A normal pattern was found after six days of treatment.

8.5.7. *Drug Sensitivity*

There is now developing some evidence that the scorbutic organism is metabolically different from the normal, not only because of the deficiency, but because of various feedback mechanisms which alter the physiological state. There are, for example, the indications that the scorbutic animal has increased ACTH and corticosterone production, and there are alterations in a number of tissue enzyme activities, some of them concerned with drug detoxifications. These include the inability to

detoxify acid acetanilide (A10) and zoxazolamine (C16) at the normal rate. A dose innocuous to normal animals causes toxic effects because of the decreased activity of enzymes which hydroxylate and inactivate these drugs.

There is no explanation available for the dramatic sensitivity of scorbutic animals to coumarin. A dose of 5 mg increased the prothrombin time to 200 seconds, at which it stayed for 2 weeks in scorbutic guinea pigs (S30). The effect of even a larger dose on the clotting time of normal guinea pigs disappeared within 48 hours (O3).

Addendum

5,6-Diacetyl-L-ascorbic acid was equally as potent, and 2,3,5,6-tetracetyl-L-ascorbic acid was one-tenth as potent as L-ascorbic acid in the odontoblast assay of vitamin C activity (W. Feldheim and M. Czerny, Preparation of acetyl derivatives of L-ascorbic acid and their properties. *Biochem. Z.* **331**, 150-154, 1959).

S. Kamiya (L-ascorbic acid degradation by bacteria. I–IV. *J. Vitaminol.* **6**, 217-235, 240-245, 1960) studied the degradation of L-ascorbic acid by anaerobically adapted cells of a species of *Micrococcus*. After conversion to diketo-L-gulonic acid, pyruvic acid was formed.

More detailed accounts of particular enzyme reactions in the metabolism of ascorbic acid have appeared (K. Suzuki, Y. Mano, and N. Shimazono, Enzymatic formation of L-gulonolactone from D-glucuronolactone by rat liver microsomes. *J. Biochem. (Tokyo)* **47**, 846-849, 1960; K. Suzuki, Y. Mano, and N. Shimazono, Conversion of L-gulonolactone to L-ascorbic acid. Properties of the microsomal enzyme in rat liver. *J. Biochem. (Tokyo)* **48**, 313-315, 1960; J. D. Smiley, and G. Ashwell, Purification and properties of β-L-hydroxy acid dehydrogenase. II. Isolation of β-keto-L-gulonic acid, an intermediate in L-xylulose biosynthesis. *J. Biol. Chem.* **236**, 357-364, 1961).

Although chickens do not require ascorbic acid, its addition to a diet containing 5–10% L-tyrosine will ameliorate the low weight gain, ragged feathers, and high death rate which tyrosine feeding produces (P. E. Sanford, A. J. Wei, and R. E. Clegg, Vitamin C and tyrosine metabolism in the chick. *Poultry Sci.* **33**, 585-589, 1954).

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IMMUNOELECTROPHORESIS: METHODS, INTERPRETATION, RESULTS

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Introduction

In the early days, the fractionation of serum proteins by free electrophoresis (Tiselius method) into five components was regarded as an important scientific achievement. The numerous clinical applications and the simplification brought about by paper electrophoresis transformed it into a routine method. In the meantime, chemical techniques of protein separation (salting-out method of Derrien, ethanol fractionation by Cohn) had shown that such fractions as separated by electrophoresis were not homogeneous chemical individuals but groups of proteins which had in common an identical electrophoretic migration velocity. By using new supporting materials (starch, cellulose acetate) as well as new buffer systems, it is now possible to separate the serum proteins into 9–12 fractions. But even this progress does not satisfy the demand of hematological and genetic investigations.

In the meantime, immunochemical reactions were carried out in agar gel, and experience showed that an increased sensitivity was gained with such techniques. They allowed the identification of a single protein in far smaller microquantities than would be possible with chemical or physicochemical methods. However, the main progress was achieved when Grabar and Williams (G1, G2) combined the foregoing electrophoretic fractionation of the serum protein in agar gel with the reaction of a specific antiserum.

Within certain limits, molecular morphology also comes in, as effects are observed in the agar network, according to the size and form of the protein molecule. Thus, immunoelectrophoresis is making use of three distinct properties of the protein molecule. It follows that the analytical consequences are considerable, even if results are not strictly quantitative.

A first survey of results was given by Grabar (G3, G4), followed by reviews of Scheidegger (S1, S2) and Wunderly (W1, W2). Recently, a comprehensive book has been published, written by Grabar, Burtin, and other collaborators (G5), giving full details of results and techniques. Another book, by Heremans (H1), concentrates on the various γ-globulins in human serums, while Wieme (W3) gives an account of his studies

on agar gel electrophoresis with a special chapter on enzymoelectrophoresis. Finally, Schultze (S3, S4) has contributed valuable surveys of our present knowledge of immunity.

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PART I. Principle

1. Immunochemical Reactions in Agar Gel

As stated in the introduction, immunoelectrophoresis (abbreviated ImEl) is a two-stage method. The electrophoretic run is comparatively simple and will be dealt with in a following chapter. The immunochemical reaction in agar gel is more complex, and this makes it necessary to examine the advantages of gel diffusion methods as well as their limitations. Many investigators use the Ouchterlony method, preceding or parallel to ImEl. This method will become still more important if present attempts at quantitation gain definite shape.

The original technique (diluting method) was not particularly suitable when dealing with a substance of complex antigenic structure. In such cases, the precipitate appeared homogeneous but really consisted of various precipitates. In many cases, it was impossible with immunological techniques to find even the approximate number of antigens present. It was therefore an advance of considerable importance when Oudin (O4, O5) incorporated the antiserum in agar gel. If such a solid mixture is overlaid with the antigen solution, multiple systems form sharp bands of precipitate, the number of which is equal to or less than the number of antigen-antibody systems present. As the antibody concentration is equal throughout the gel, it cannot be assumed that "optimal proportions" are reached in every case. According to their diffusion constants, the antigens move down the narrow tube (internal diameter, 1.5–2 mm); the process is called "simple diffusion" (O6, O7).

The simultaneous movement of antigen and antibody was achieved by Ouchterlony (O1, O2) when he developed his method in 1948. It is since called the "double diffusion" method and has found wide application. As far as protein antigens are concerned, agar has generally been used as the most convenient medium. Only lately, cellulose acetate strips (supplied by Oxo Ltd., 16 Southwark Bridge Road, London) have been tried by Rhodes (R1). According to Gell (G1, G2), 0.5–0.6 % agar (New Zealand agar, Davis or Bacto Agar, Difco) is made up in phosphate buffer saline (pH 7.4) containing sodium azide (0.02 %); the completely transparent solution is filtered and poured into specially selected flat-bottomed Petri dishes to a depth of 3 mm. Two hours after the agar gel has set, cups are cut into it; if a corkborer is used, their size and distances can be varied at will; for often-repeated routine work, a template with a symmetrical arrangement of six or eight cups for various antigens, surrounding a central cup for the antiserum, is preferable. With a template,

the distance between the edges of the antiserum and antigen cups is strictly uniform. With distances of approximately 9–12 mm, results can be read after 18–24 hours. With weak or diluted antisera it is necessary to bring the reagents nearer together in order to get visible lines. The two reagents will move towards each other under steadily varying (increasing) concentration. As the mixing takes place in varying quantitative proportions, there is a fair chance of obtaining the optimal conditions for the individual antigen-antibody reaction. Optimal conditions are gained if the total amount of antigen in each cup is approximately in "equivalent proportion" to the total amount of antiserum. If two antigen-antibody systems are present whose optimal ratios are widely different, a suitable variation of the proportions will be necessary. Satisfactory antisera will contain approximately 0.25 mg precipitable nitrogen per milliliter. The limiting concentration of antibody can be ascertained by decreasing series of fourfold dilutions from 1 % to 1/1024 %. Such a control is desirable when starting ImEl with a new batch of antiserum. Reference solutions are normal serum diluted 1:20 or 1 % serum albumin. The micromethod developed by Hartmann and Toilliez (H1) necessitates 0.2 ml of these solutions per cup.

If the precipitation lines of two antigens in neighboring cups meet, forming an angle, there is reaction of *identity*. If the two lines cross and continue without any deflection, there is reaction of *nonidentity* (O3). When identification of the lines is attempted, it is advisable to keep in mind the following facts. Only substances which are antigenic can be identified. A precipitation line will form only if the antiserum contains antibody against the antigen which is being tested. The major component of a mixture may provoke less antigen-antibody reaction than a contaminant, or none at all. There is no certainty that all the antibody measured has been brought down by the protein under investigation rather than by contaminating antigen-antibody systems in the same optimal range. These limitations apply also for ImEl and imply suitable precautions.

However, the method of identification is far more specific than any purely physicochemical technique, as was shown by Wilson and Pringle (W4). The sensitivity is superior too. The photographic recording of precipitate lines in agar gel has recently been described by Hunter (H3). Glenn (G5) has developed the necessary instrumentation for direct photometry of precipitin systems in agar columns (ring test); zonal characteristics of simple and complex reactions are objectively detailed. The graphic analysis shows zone widths, densities, and slopes. Systematic observations should contribute to basic, applied, and diagnostic investiga

tions. The Serum Agar Measuring Aid, the Photronreflectometer, and the Serum Agar Measuring Integrator are available from Analytical Instruments, 238 Harmon Drive, San Antonio, Texas, U.S.A. Lately, Heremans (H2) has outlined his experience in measuring results of ring tests in a traveling microscope; he evaluates the systematic error as 0.2 mm. If the precipitation band travels along 8 mm within 72 hours, the error amounts to 2.5 %. Colloid-chemical factors limit the precision of reproduction.

Wright (W5) has developed an interesting method for quantitation by titrating antigen against antibody and following the titration on a modified Ouchterlony plate. The grade of accuracy which has been attained seems sufficient for practical purposes. Polson (P2) modified the Oudin gel precipitin technique and established an empirical relationship between the distance from the original antibody and antigen meniscuses to the precipitin bands and the antigen concentration. As the method allows accurate observations of the precipitin bands, it will be of practical value for assessing antigen concentration.

2. Colloid Chemistry of Agar Gel

Agar-agar is obtained technically as a mucilaginous substance from the cell membrane of *Gelidium corneum* and *Acanthopeltis japonica*. The quality of the product from this crude extraction differs rather widely according to the season, the kind of algae used, and the treatment of the raw material (N1). Agar is predominantly a linear polysaccharide, in which the subunits consist of 9 residues of D-galactopyranose. They are connected by 1,3-glycosidic linkages, but the exact structural formula is not known. Besides further hexoses and pentoses in small amounts, agar contains sulfate groups, and calcium and some magnesium in variable concentrations. In solution a gel reticulum is formed by secondary linkages. One molecular chain may form cohesive junction points with other molecules at several places simultaneously. The total number of such cross linkages influences the swelling, the contraction, and the aging of the gel. This last process alters the diffusion of proteins and makes it possible to compare results only if gels have a similar age (W6).

If the temperature is raised to the boiling point, these linkages are loosened and a stable lyophilic hydrosol is formed. Agar gel is thermoreversible. Polar groups like —COOH, —OH, and >C=O act as centers for hydration during the process of swelling. These centers are blocked by salt ions, so that agar swells less in salt solutions than in pure water. During the process of gelling, viscosity is sharply increased before

the gel is formed. Further properties, like diffusion, electrical conductivity, and the dielectric constant, are hardly changed during the sol/gel transformation. If gels of equal elasticity are compared, the average length of the chain molecule is greater in the pectin gel than in the agar gel. Consequently, pectin gels can be formed with as little as 0.2 % pectin in water (G8). With a well-purified agar, 0.3 % by weight is probably the limit; for handling gels experimentally, 0.4–0.5 % is preferable. In order to avoid desiccation, agar tubes (ring test) or agar Petri plates must be carefully closed. The surface of the gel is previously coated with a few drops of a 0.1 % solution of Merthiolate.

3. Electrophoretic Separation in Agar Gel

3.1. Factors Influencing Electrophoretic Fractionation

In agar gel as well as in pectin gel, both the colloid phase and the liquid phase are continuous, as the gel structure resembles that of a sponge rather than that of a honeycomb. It is therefore possible for particles carrying a charge to migrate in an agar gel under the influence of an external electrical field. The net electrostatic charge of the particle will of course depend on whether there is an excess or a deficiency of electrons. The meshwork of the agar gel serves as a carrier for the buffer solution and helps to fix the migrating substances. The latter can be ions or charged hydrophilic colloids; they can reach a molecular weight of 8,900,000 (hemocyanin) without being hindered by 0.5 % agar gel. The gel medium is considered as a two-phase system, where the agar meshwork is the stable phase and the buffer solution, the movable phase. However, the liquid phase is not entirely free to move; therefore, the laws of hydrodynamics governing laminar flow cannot be applied without correction. An evaluation of the volume of the liquid which fills the interstices of the gel is only approximate. If an external electrical field is applied, two kinds of *electrokinetic phenomena,* mainly, influence the migration of the charged particles. Directly acting upon them is a force which is proportional to the charge and to the strength of the electrical field (electrophoresis); besides, there is always the movement of the buffer solution relative to the network of the agar gel (electroosmosis). These movements bring about a streaming potential, carried by the buffer solution, and a migration potential, carried by the charged particles. The polar groups of the agar adsorb hydroxyl ions from the solution. Consequently, ions of the opposite sign will be attracted by the resulting electrical field.

It follows that the surface of the agar colloid takes on a negative charge versus the solution. The potential difference which is thus created between agar and solution is called the *electrokinetic* or *zeta-potential* (ζ). It can be evaluated by measuring electroosmosis and the streaming potential. The zeta-potential decreases exponentially with the distance; the distance at which it is reduced to $\zeta/2$ gives a measure of the thickness of the double layer, which is formed by the counter ions surrounding the particle like a cloud. This zone is called the diffuse electrical double layer and may extend over a thickness of several hundred angstroms, or even more. As Overbeek and Lijklema (O8) have outlined, electrophoresis is directly connected with the ionic part of the electrical *double layer*. Electrophoresis and electrokinetics are linked together by the tangential movement of the two phases along each other. This motion obeys the laws of viscous flow, but there is uncertainty about the viscosity coefficient η in the double layer. The short-range interactions at the phase boundary between a stable colloid (agar), a mobile colloid (protein), ions, and solution make the theoretical approach difficult. The treatment is easier with double layers at a flat interface. In agar electrophoresis where both phases, the colloid phase as well as the watery phase, contain ions, the transition layer is comparatively broad. The formula of von Smoluchowski (Eq. 1) gives the interdependence of the electroosmotic migration velocity u and the zeta-potential ζ, the dielectric constant D, and the viscosity η:

$$u_{os} = \frac{\zeta D}{4\pi\eta} \tag{1}$$

It follows that electroosmotic flow decreases with a lowered dielectric constant and with an increased viscosity of the liquid phase. This connection is more pronounced when working in agar gel than in filter paper because, in agar, electroosmosis is much stronger. We have added to 1 % agar gel 25 % by volume of 1,2-propanediol, whereupon the viscosity $\eta_{sp}^{70°}$ of the agar sol increases from 1.8 to 4.4; in the meantime, electroosmosis decreases to such an extent that the distance of the electrophoretic separation of serum proteins is reduced to less than half. If D is 80 in water, it decreases to 18 in isopropanol; therefore, the admixture of 1,2-propanediol brings about a strong reduction of D (W7). This interdependence is well contained in Eq. 2. If the constant proportion between the zeta-potential and electrophoretic mobility is represented by the factor k, the *mobility* u is

$$u = \frac{k4\pi\sigma}{D} \cdot \frac{1}{0.33 \times 10^{-8}\ (\tau/2)^{1/2}} \tag{2}$$

where σ represents the density of the charge per surface unit of the protein particle, D, the dielectric constant, and $\tau/2$, the ionic strength. In globular proteins, the average surface area per ionizable group per molecule is of the order of 100 Å^2. The ionic strength should not be reduced below 0.04 if a constant pH is to be maintained in all sections of the agar plate. If it is not attained, the fronts of the separated protein fractions become distorted. If the ionic strength of the buffer is too low, conductivity declines after some hours of the run. However, if working on a microscale, the duration of the run is too short to start this phenomenon.

If the *ionic strength* is kept low, the *heat H* which is generated by the Joule effect has no influence on the electrophoretic separation because the field strength in volts/cm can be kept low too. If the latter is 6 volts/cm and $\tau/2 = 0.05$, there is a rise of several degrees in the agar gel within 150 min; in the meantime, evaporation leads to an 18 % decrease of weight. The accompanying increase in viscosity hinders the electrophoretic separation. If the field strength is kept at 5 volts/cm or below (voltage, 85 volts), no special devices for cooling the agar plate are necessary. *Evaporation* and *conductivity* remain practically constant. In such conditions, the distance of separation depends almost solely upon time. If I represents the current intensity; V, the voltage; and R, electrical resistance; and the mechanical heat equivalent A is 4.185×10^{17} erg/cal, the heat H is given by Eq. 3.

$$H = \frac{RI^2}{A} = \frac{VI}{A} \tag{3}$$

All the factors which contribute to a good separation in unit time must be well considered because the interpretation of the final precipitate lines is much facilitated if the original distance of separation was sufficiently long.

3.2. Theoretical Base

We have outlined in the foregoing section the most important factors which contribute to electrophoretic fractionation in agar gel. It will be easier now to look at the basic relation which exists between the charge of the particle and the migration velocity (B1). The electrophoretic migration of colloidal particles follows Eq. 4:

$$u = \frac{Qd}{4\pi r^2 \eta} \tag{4}$$

where Q is the charge of a particle; d, the width of the diffuse electrical double layer; r, the radius of the particle; and η, the viscosity coefficient. Now, in agar electrophoresis the stabilizing medium is itself a colloid

and, in fact, the surface exposed by agar gel is very large in relation to the volume of the electrolyte containing the protein. As d and the zeta-potential are difficult to evaluate directly, corrections must be made when interpreting mobility measurements. If *mobility* is determined experimentally, the following formula can be applied:

$$u = \frac{sc}{tV} = \frac{\text{cm}^2}{\text{sec volts}} \tag{5}$$

where s is the distance traveled in t seconds when the electrodes are separated by c cm and the voltage is V. As has been said, the surface of the colloid agar carries a negative charge in the most convenient pH region. Consequently, there is a strong flow of electrolyte toward the cathode (electroosmotic flow u_{os}) which necessitates a correction factor. As an indicator of u_{os} the large polysaccharide molecule dextran is often used because it can easily be detected by the dye bromophenol blue. If u_{obs} is the value observed experimentally for a pure protein fraction, it must be corrected in the following way.

$$\begin{aligned} u_e &= (u_{obs} \pm u_{os}) \text{ field strength} \times \text{time} \\ &= 10^{-5} \text{ cm}^2 \text{ sec}^{-1} \text{ volts}^{-1} \end{aligned} \tag{6}$$

If the observed substance has migrated as an anion, u_{os} must be added, and it must be subtracted in the case of a cation. Details of such controls are specified under technique in Section 6.

In agar electrophoresis the pH variation is limited because the stability of agar sols is within pH 5–9. Even if correction is made for *electroosmosis*, as indicated in the above formula, u_e is sometimes (according to size and shape of the migrants) smaller than in free electrophoresis. In a dense agar gel an ion, be it amino acid or protein, does not travel the straight-line distance between the electrodes. According to the density of the agar gel mesh, an ion is forced to deviate; the resulting net increase in path length depends upon the *gel structure*. We have characterized the last-mentioned factor with colloid-chemical methods (viscosity, turbidity, gel strength, and rigidity) (W8). In loose gels the added path length is much less than in dense gels, but the fronts of separated fractions become distorted as *lateral diffusion* is considerable (W3). Amino acids, peptides, and proteins are ampholytes; therefore, acids and bases have the following effect upon them:

$$\underset{\text{Protein anion}}{NH_2\text{—R—COO}^-} \xleftarrow{OH^-} {}^+NH_3\text{—R—COO}^- \xrightarrow{H^+} \underset{\text{Protein cation}}{{}^+NH_3\text{—R—COOH}}$$

Where the ampholyte, as depicted in the center, is without net charge against the medium, the isoelectric point is reached at which the *am-*

pholyte does not migrate and solubility, viscosity, and swelling are minimal. In agar electrophoresis the pH of the buffer system is generally on the alkaline side of the isoelectric point: consequently, the ampholyte carries a negative net charge and migrates to the anode. However, if the migration velocity of a protein fraction is small, it is carried away by the electroosmotic current in the direction of the cathode. If the electrophoretic mobility and the electroosmotic flow are of equal effect on a protein, it remains stationary as a result of balanced conditions. The behavior is the same as at the isoelectric point, but only apparently so.

3.3. Comparison with Other Stabilizing Media

As early as 1923–1926, Kendall (K1) separated rare earths by electrophoresis in agar gel; in 1949 Gordon and associates (G6) showed that protein fractionation was possible in the same medium. Since then, a number of stabilizing media have been tried, and it is worthwhile to compare their merits. The ideal support would have no chemical reaction with the protein particle, nor any physical adsorption. Now, even purified agar specimens contain between 0.3 and 3.7 % sulfur as sulfate groups. It is quite possible that such acidic groups react chemically with basic side chains of proteins; they may interfere with fibrinogen or promote the resolution of normal adult hemoglobin into fractions. Physical interaction between agar gel and proteins appears to be practically nonexistent. Experience shows that several serum proteins (albumin, α_1-globulin) migrate in agar gel with the same relative velocities as in free electrophoresis. However, if agar electrophoresis is compared with the latter, there are no density gradients, no disturbances from convection currents, no necessity to work at 4°C, thus allowing a much simpler apparatus.

In paper electrophoresis, Whatman No. 1 paper absorbs approximately 0.7 μg serum albumin/cm^2, whereas the adsorption in agar gel, starch grain, cellulose acetate membranes, and in glass fiber paper is negligible. Pezold and Thomas (P1) have emphasized the fact that the tailing factor of α-glycoprotein and β-lipoprotein on filter paper is especially strong. In agar electrophoresis *evaporation* is small compared with that from filter paper; agar gel is quickly transformed into a transparent film with excellent optical properties; thus, photometry is made easy and allows the analysis of microquantities. Such are the advantages of agar gel electrophoresis (W2).

In 1957, Kohn (K2, K3, K4) demonstrated that cellulose acetate strips have excellent qualities for ImEl. Pore size is 0.5–3.0 μ, and the stained strips can be made completely transparent by immersion in white oil. They are somewhat easier to handle than agar gel, but lipoproteins do not stain well, as the background cannot be washed out adequately. Tailing is not encouraged, as the run lasts only 2 hours.

Giri (G3, G4) has carried out agar electrophoresis on cellophane and polyester films. Since 1955, Smithies (S2, S3) has worked with starch gel. As partially hydrolyzed starch is used, it is essential that conditions of hydrolysis are strictly adhered to if good reproduction of results is to be achieved. Borate buffer is used, and the duration of electrophoresis is 6 hours for a field strength of 6 volts/cm. As the pores of a starch gel act like a sieve, it is important to know the origin of the starch because the ability for fractionation is widely different in starches from different sources. Porath (P3) has described the sieve effect of dextran gels (Sephadex) with a systematically changed degree of crosslinkage. New vistas are thus opened for *gel filtration*. PVC paper (Rhovylpaper) and silica gel have also been tried as supporting media for electrophoresis, but further practical results must be awaited.

3.4. Sensitivity

In a survey of the quantitation of immunological reactions, Grabar (G7) has given the following limiting sensitivities. In qualitative specific reactions, the ring test necessitates 3–5 μg antibody nitrogen/ml; on agar gel plate (Ouchterlony technique) 5–10 μg; quantitative methods such as micro-Kjeldahl need 20 μg; turbidity (stray light) 20 μg; colorimetry (Folin reaction) 4 μg.

Rhodes (R1) has achieved a further increase in sensitivity by labeling antigen or antiserum with iodine-131; the limit is 1 μg antibody N/ml if X-ray film "Kodirex" is used for autoradiographs.

Working on a *microscale* has the great advantage that much less antiserum is used. Grabar *et al.* (G9) originally used 0.5–1.0 ml immunoserum and 0.05–0.20 ml serum per ImEl, but Scheidegger (S1) reduced these quantities to 0.05 ml and 0.001 ml. Consden and Kohn (C1) need only 1–5 μl serum, and Wieme and Rabaey (W1) have further reduced this amount, by an ultramicrotechnique, to 0.1 μl. If these minimal amounts are compared with the 0.1 ml of serum needed in the micromethods of free electrophoresis (Kern, Antweiler), the progress is seen to be considerable. It has made possible investigations on new biological objects, such as the aqueous humor and lens of the human eye, tissue fragments from biopsic material, or the hemolymph of insects.

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PART II. Technique (Practical Guide)

4. Apparatus

4.1. Macromethods

The first stage of ImEl is a simple agar electrophoresis. It leads to a somewhat better fractionation as is possible on paper. But the decisive progress is only achieved when a specific antiserum diffuses laterally into the agar gel and reacts with the homologous protein fractions. The first

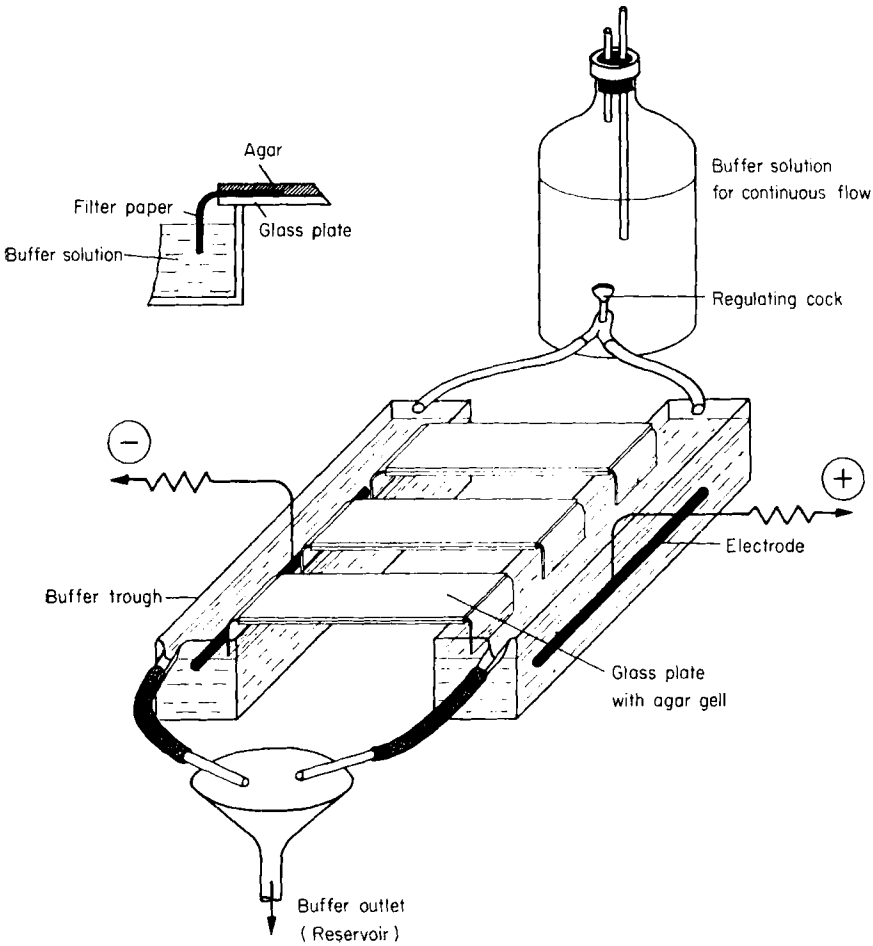

Fig. 1. Apparatus for immunoelectrophoresis in agar gel, as built in the laboratory. From Pezold and Thomas (P1).

step separates proteins physicochemically by reason of their migration velocity; whereas the second step improves antigen analysis using a serological method. As Williams and Grabar (W5) pointed out right at the beginning (1954), the essential elements of the apparatus are the same as for paper electrophoresis; they used a single strip of agar gel, 13 × 18 cm, which rested on the glass of a photographic plate. Later, investigators (G2) worked with narrower strips but used 3–5 of them in parallel position at the same time. Figure 1 shows the general layout (P1) as built with laboratory facilities. Stone and Glaubiger (S11) used agar strips, 3 × 18.5 cm; Wunderly and Bustamante (W6) placed the agar gel on the glass plates, 4 × 16 cm, of the Elphor Apparatus for scanning paper strips or dried agar films; Neuhoff (N1) worked with agar gels 4 × 40 cm in an endeavor to improve fractionation. Wicks of thick filter paper, chamois leather (W4), or plastic sponges (C1) serve to give connection with the electrode vessels. The wicks are zones of lower conductivity; it is therefore advantageous to bring the gel plate as near to the buffer surface as possible. A slow, but continuous stream of buffer solution guarantees a constant pH at the wicks. The electrodes can be of platinum wire, but a carbon rod (anode) and a thick copper wire (cathode) have given us good service too. The average field strength is 3.5 volts/cm, and the duration of the run 6–7 hours. The complete equipment can be bought at Etabliss. Jouan, 113 Boulevard Saint-Germain, Paris; or Etabliss. Chaix, 6 av. Milton, Nancy (France); or Model E-200-2 "Reco," a multipurpose apparatus, from Research Equipment Corp., Oakland 20, Cal. (U.S.A.). The macrotechnique, though less often used, is indicated for the measurement of physicochemical characteristics like migration velocity, diffusion constant of immunochemical reactants, and zeta-potential.

4.2. Micro- and Ultramicromethods

In order to economize antiserum, Scheidegger (S2) has developed a micromethod, which has come to be used in many places. The agar gel is formed by pouring 2 ml of a hot 2 % agar gel (barbital buffer of pH 8.2 and $\tau/2 = 0.05$) on microscope slides 76 × 26 mm. The thickness of the gel is approx. 1 mm. With a field strength of 6 volts/cm, the run is completed in 45 min. With the collaboration of J. J. Scheidegger, the first complete equipment for microscale has been developed; it is commercially available from S. Quadri, Bleulerstrasse 5, Zürich 8, Switzerland, or from National Instrument Laboratories, Inc., 828 Evarts Street, Washington 18, D. C., U.S.A. In one cell of Plexiglas 8 runs can be made simultaneously, with 0.001 ml serum each. There is a constant-

voltage power supply; an agar cutter for cutting slits and holes; slide frames; a washing bath for 10 slide frames; a staining unit; and a viewer for observation of finished patterns (precipitates).

For ultramicroelectrophoresis, Wieme and Rabaey (W2) also use slides 76 × 26 mm or even cover glasses but form an agar layer 1.5–2 mm

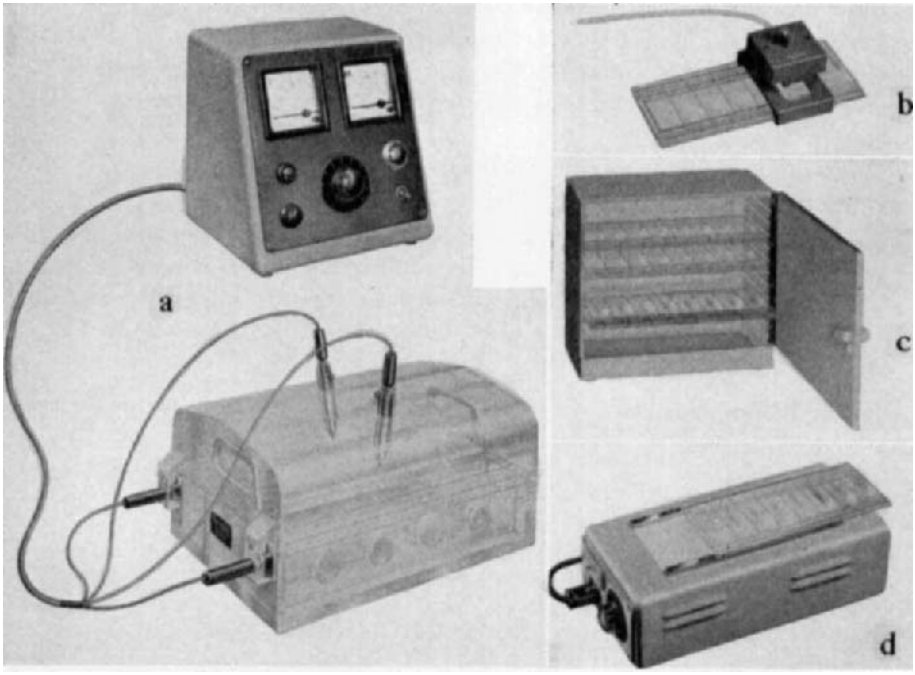

FIG. 2. Agafor 1 equipment. (*a*) Cell for making 8 microelectrophoresis runs simultaneously; (b) cutter with template; (c) cabinet for development of precipitates in damp atmosphere; (d) viewer with glass diffusing screen.

thick. As they work with a field strength of 8–12 volts/cm, the duration is reduced to 10–15 min. Cooling is obtained by placing the glass slide on a copper plate which rests on ice cubes (W4).

5. Preparation of Gel and Buffer

Most applications of ImEl have been realized with commercial forms of agar. "Difco Bacto Agar" from Difco Laboratories, Detroit, Mich., U.S.A. is very useful; other products of Difco—"Special Agar Noble" and "Difco Purified Agar"—come close to maximum purification. The product of Merck A. G., Darmstadt, Germany (Agar-agar, pulv. subst.)

is not easily soluble, as solubility tends to decrease with progressive purification.

Grabar and Williams (G5) have developed the following procedure for purification of the commercial product. Agar is dissolved by boiling in distilled water; when a 6 % sol is attained, it is poured into wide trays; after setting, the gel is cut into small blocks; these are extracted for 2 days in distilled water, which is renewed 5 times. The gel is now perfectly white and is kept in stoppered sterile containers. In order to eliminate organic substances, Wieme (W4) extracts the gel with 50 % ethanol in distilled water. An exacting method of purification (B1) uses electrodialysis.

All these techniques tend to lower the ionality of the agar gel; the rigidity is thereby increased, whereas the intensity of the electroosmotic flow is decreased. It follows that results with ImEl are comparable only if identical conditions have prevailed during the foregoing purification. The following stock buffer solutions have an ionic strength of $\tau/2 = 0.1$ (W4) when the specified quantities of constituents are made up to 1 liter with distilled water (plus 0.5 g sodium azide).

Barbital buffer, pH 8.4	
Sodium barbital	17.0 g
N Hydrochloric acid	23.5 ml
TRA buffer, pH 7.6	
Triethanolamine (hydrochloride)	11.9 g
N Sodium hydroxide	26.0 ml
Borate buffer, pH 9.0	
Boric acid	11.1 g
N Sodium hydroxide	93.0 ml
Phosphate buffer, pH 7.5	
Secondary sodium phosphate ($Na_2HPO_4 \cdot 2\ H_2O$)	6.8 g
Primary potassium phosphate (KH_2PO_4)	0.64 g

For preparing the gel the buffer solution is brought to the desired ionality and placed in a boiling water bath. After some minutes, agar is added and the boiling is continued until the solution is visually completely homogeneous. In the meantime, the glass plates (support) are immersed in 96 % (v/v) ethanol and warmed on a hot plate; then a few drops of agar sol are spread over them. When these drops have dried and with the warm glass in an exactly horizontal position, the hot agar sol is poured slowly upon it from a pipet. The sol must spread out evenly to the edges of the glass. If Elphor glasses (W6) are used, 11 ml

of agar sol is necessary; for the microscale (S2), only 2 ml. It is necessary to leave the gel two hours for setting. Then one or several cuts are made into the agar by pressing into it razor blades which are fixed on both sides of a microscope slide and are thus a distance of approximately 1 mm apart. The parallel cuts (Fig. 2d) in the center of the gel have a length of approximately 4 cm. At a distance of 3–4 mm, small holes are made (e.g., with a corkborer), and their content of agar gel is sucked away with a pipet. Often, these cuts and holes are all made with a metal template (Fig. 2b), thus assuring identical distances and a better reproducibility of results. Now the agar plates [Fig. 3(*i*)] are laid parallel on the support, and the wicks g are applied. The buffer *d* has been filled in beforehand. The plastic box *a* is covered by *b* and the current is

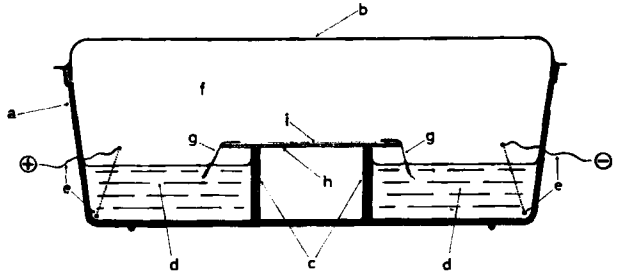

FIG. 3. Apparatus for immunoelectrophoresis in agar gel as built by Heremans (H1). In a plastic box *a* is the buffer *d* on both sides of the support *c*. Upon the latter are the slides with agar gel *i* and the wicks *g*. The electrodes *e* are of platinum wire.

switched on to the electrodes *e*. Twenty minutes later, the proper conditions of conductivity have been stabilized. The box is opened and a small drop of the antigen (serum, protein solution, etc.) is placed in the small holes using a curved micropipet.

6. Controls, Run, Diffusion of Antiserum, and Drying

In order to gain *visual control* during the electrophoretic migration, we have found the following technique satisfactory (W8). One indicator consists of a 35 % sol of dextran (mol. wt. 150,000) colored a deep blue with bromophenol blue. A small drop is placed on the agar near the serum sample. Part of it will dye the serum albumin exclusively, since the serum globulins are only colored after denaturation. Thus, the albumin front will take on a pure bluish color and demonstrate anionic progress.

The other indicator has the same dextran as colloid base, but is colored

brown with *o*-nitraniline; it moves towards the cathode (see arrow 1 in Fig. 4) because *o*-nitraniline at pH 7–9 bears no charge and is thus an indicator for the electroosmotic flow. The diffusion of the highly viscous indicators is slow, so that they can easily be traced even after several hours.

Now the plastic cell is closed to reduce evaporation. The electric current is switched on again for the run. An individual voltmeter and milliammeter are useful.

When separation has proceeded long enough, the current is stopped and the agar is lifted out of the longitudinal cuts. Into these narrow channels antiserum is added from a micropipet connected to rubber tubing. Care must be taken that it is distributed evenly along the whole

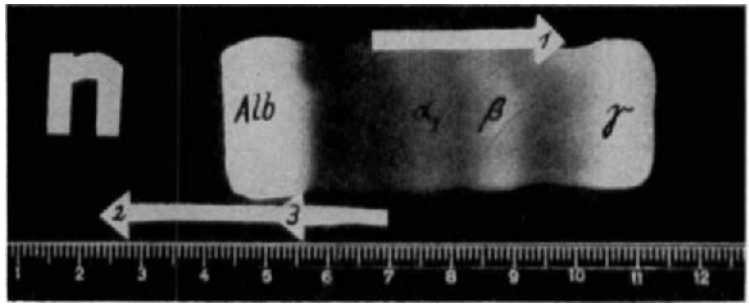

FIG. 4. Controls of the agar electrophoresis of a normal serum. Arrow 1 shows the control of electroosmosis with *o*-nitraniline. Arrow 2 gives the position of bromophenol blue (free dyestuff) and Arrow 3 the albumin front stained blue. From Wunderly (W8).

length of the groove. Horse antihuman serum from "Serpasteur" (36 rue du Dr. Roux, Paris 11e, France) should be diluted 1:1 with 0.86 % NaCl, whereas rabbit antiserum should be used undiluted. The plates are now kept for diffusion in a moist chamber; it is practical to lay them evenly on a 4-cm thick layer of foam rubber which is saturated with distilled water. *Temperature* may be anywhere between 16° and 22°C. but *must not vary*. A few drops of a 0.5 % (v/v) solution of Merthiolate prevents fungus growth. In work on a microscale the precipitation lines are complete after 24 hours; with the macromethod it needs 4–5 days. In order to elute all the proteins which have not reacted, the plates are bathed for 3 days in 0.86 % (w/v) NaCl, which is renewed twice daily. Then the salts are eliminated by bathing twice in distilled water for 2 hours. Now the agar layer is evenly covered with a strip of well-moistened filter paper, an infrared lamp is fixed approximately 20 cm above the

covered plate and an electric fan helps a rapid evaporation. After some 30 min, the paper strips roll up and thus indicate the end of the process. The slides are washed under running water. The agar layer has been transformed into a perfectly transparent, thin film. Drying can also be done in a thermostat at 37°C overnight.

7. Staining

7.1. Proteins

Amido black 10B (Bayer), or naphthalene black 12B.200 (ICI), or Pontacyl blue black SX (Du Pont), or Buffalo black (National Aniline) are most often used for routine processes. One-half gram of one of these dyes is dissolved in a solution of 5.0 g mercuric chloride and 5.0 ml acetic acid in 100.0 ml distilled water (W4). The solution is filtered before use. The staining of the slides with the agar film is done in the ordinary tray used for microscopy and takes 5 min. For differentiation, the slides are rinsed in a 2 % solution of acetic acid in distilled water or in a water solution of 50 % methanol and 10 % acetic acid. The bath is renewed until the background is colorless. Often, new lines of precipitate show up at this stage. The slides are finally washed in water and dried within a few minutes.

For staining with nigrosine (water soluble, Geigy), ponceau red, or azocarmine, Uriel (U1) adds one gram of dye to a mixture of 450 ml acetic acid, 450 ml 0.1*M* sodium acetate, and 100 ml glycerol. The last-named substance makes the agar film supple and facilitates detaching it from the glass slide; it is recommended in all cases where films are to be stored. Staining takes 3–4 hours and is followed by two rinses in a water solution containing 2 % acetic acid and 10–15 % glycerol.

Gorringe (G4) stains with lissamine green (BDH) using a solution containing 0.30 g dye in a mixture of 15.0 ml acetic acid and 100.0 ml distilled water. This stain can also be applied to films which have been previously stained with Sudan black, thus achieving a simultaneous coloration of proteins and lipids (W4).

7.2. Lipids and Lipoproteins

If precipitation lines contain a lipoid component, they can be stained with a combination of oil red and Sudan black (U1), first using a solution of 1 g of oil red O or scarlet red R (Geigy) or Sudan IV dissolved in one liter of 60 % ethanol during 24 hours at 37°C. The solution is filtered after cooling and kept in dark-colored bottles. The agar films remain 16 hours in this solution; then they are soaked for 2 hours in a

saturated solution of Sudan black prepared in the same way, and alkalinized before use with 0.2 ml of 25 % (w/v) aqueous NaOH per 100 ml of dye solution. Differentiation is achieved by washing in 50 % ethanol and must be carefully watched.

For further staining methods after simple agar electrophoresis (not ImEl), the reader is referred to the complete survey given by Uriel (U1).

8. Photography

Photographing of the stained and dried agar films of the macrotechnique is quite possible by contact printing.

1. The glass with the agar film under it is laid on a sheet of orthochromatic paper (Agfa: Brovira, hard, white, glossy).
2. The agar plate is exposed for 2–4 sec at a distance of 24 cm from a small Phillips 15-watt lamp.
3. The paper is moistened and developed in Eukopin for 2–3 minutes.
4. It is washed, and fixed with "Kodak acid quick-fixing salt" for 10 minutes.
5. It is washed in running water for one hour.

In this way, a negative of the stained agar film is obtained. Areas of special interest may be enlarged. If the micromethod has been employed, enlargement is necessary. This is easily effected with an instrument for enlargement of microfilm, using Ilford Multigrade paper and a dark-yellow filter. An enlargement of approximately 100-fold is attained, and the lines of specific precipitates can easily be traced with a pencil along the image projected on white paper. In order to detect abnormalities, the lines of a normal serum are also projected upon the diagram (H2). Such a straightforward comparison of results gained under exactly identical conditions shows additional lines or missing ones (defect dysproteinemia) in a dependable way.

A more elaborate technique for photographic recording of agar plates has been developed by Hunter (H3). With the use of two 500-watt spotlights, he succeeds in accentuating the faintest lines to the greatest possible degree against a dark background. High contrast Ilford "Ortholine" G.5.51 film is recommended.

9. Interpretation

Parallel with the technical improvement and analytical refinement of measurements grows the difficulty of the interpretation of results. Whereas the identification of the 4–5 peaks achieved with free electrophoresis of a normal serum was comparatively simple, the more complex

patterns of immunoelectrophoresis depend upon a more complicated theoretical base. Their comparison is often questionable, and a strict standardization both of the technique and of the antisera used is obviously required. Any laboratory taking up ImEl should carefully identify as a first step the lines of precipitation obtained with a normal serum (W7). Only when repeated tests show satisfactory and *strictly comparable results* should further problems be tackled. At this stage the comparative projecting method (compare Part II, Section 8) is recommended; it is of great help for clinical routine work and even more helpful if new problems are approached.

9.1. Methods of Identification

In some cases antigens can be identified with chemical procedures. Lipoid components are recognized by staining with the combination of Sudan black and oil red O (compare Section 7.2). For differentiation of the precipitation lines of α_2- and β-lipoproteins, the latter can be precipitated beforehand by heparin in the presence of Ca^{++} according to Burstein and Samaille (B2) or eliminated by filtration through an agar gel (W4). The complexing action of haptoglobin can be used by adding hemoglobin to the antigenic mixture; as the haptoglobin-hemoglobin complex has a slower migration velocity, its formation can easily be localized (W4). Finally, radiochemistry offers further possibilities of identification. Scheidegger and Buzzi (S3) made use of normal gammaglobulin labeled with iodine-131; Heremans (H1) used the same label with albumin and studied the formation of complexes with paraproteins. When using labeled fractions of serum proteins, a careful preliminary study has to prove whether the protein molecules have come unaltered through the processes of precipitation (for purification) and labeling. The formation of dubious artifacts must be excluded because even slight chemical alterations can lead to a changed migration velocity and consequently to a misleading location.

Most important are the immunological means for identification of unknown antigens. As a first test of identity or nonidentity, the *Ouchterlony test* (see Section 1) will often be used. Sensitivity can be increased by labeling standard antigens or antiserum with iodine-131 (R2).

A similar reaction can be achieved with the technique of ImEl. The unknown antigen is placed in the middle of the agar gel, test antigens (albumin, γ-globulin, etc.) are placed along the channel to the left and to the right at a distance of 1 cm. If there is identity, the precipitation lines will join (W9).

If the channel with the antiserum is kept rather short and the unknown

antigen is run on both sides of it, the lines of homologous precipitation will meet above the end of the channel. This effect is easily reached with serum albumin.

Identification may be helped by changing the precipitating capacity of the antiserum. If the latter is absorbed with pure fractions of plasma proteins until no precipitation lines are formed in the Ouchterlony test, the pattern of ImEl will lack the corresponding lines. If the unknown antigen mixture contains fractions of tissues or tumors, the antiserum has to be absorbed first with pooled lyophilized human plasma. Another technique absorbs the antiserum specifically with a considerable excess of the unknown antigen; a control ImEl shows the *effect of absorption*, thereby revealing the active antigen. Heremans (H1) has used this

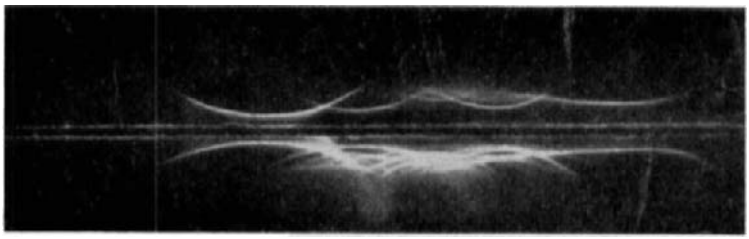

FIG. 5. ImEl of the 5 standard protein fractions (indicator lines), on top; and ImEl of a normal human serum, below. From Wunderly (W8).

method in order to prove the antigenic similarity between certain paraproteins and the β_{2A}-globulin.

As a good antiserum reveals up to 10 precipitation lines in the region of the α_2-globulin, it is important to use some pure *test proteins* in a parallel run as standard for location (marker substances) (Fig. 5). We have proposed (W9) the following mixture of three serum protein fractions: serum albumin (fast anodic); α_2-macroglobulin (remaining at the origin); γ-globulin (cationic). Clear lines (indicator lines) are obtained regularly, and the whole range of migration is well covered. Lines of prealbumin are situated nearer the anode in an advanced position. Wieme (W1, W4) proposes albumin and transferrin as marker proteins. If isolated protein fractions are used for absorption or indicator lines, a high purity is of the greatest importance. Schultze (S5) has demonstrated the influence of microheterogeneities by small admixtures of accompanying substances. For this purpose, α-seromucin, α-glycoprotein, α_2-macroglobulin, β-iron-bound proteins, and fibrinogen were isolated and purified (Fig. 6) with the latest methods of protein chemistry (S4, S5). They form the specific reactants with which unknown mixtures

of antigens or the antigenic structure of a protein can be analyzed. The narrow selectivity of their action is based on a strong individual specificity. The latter is determined by certain peptide groupings, in contrast to blood-group substances where the determining factors are localized in polysaccharide components.

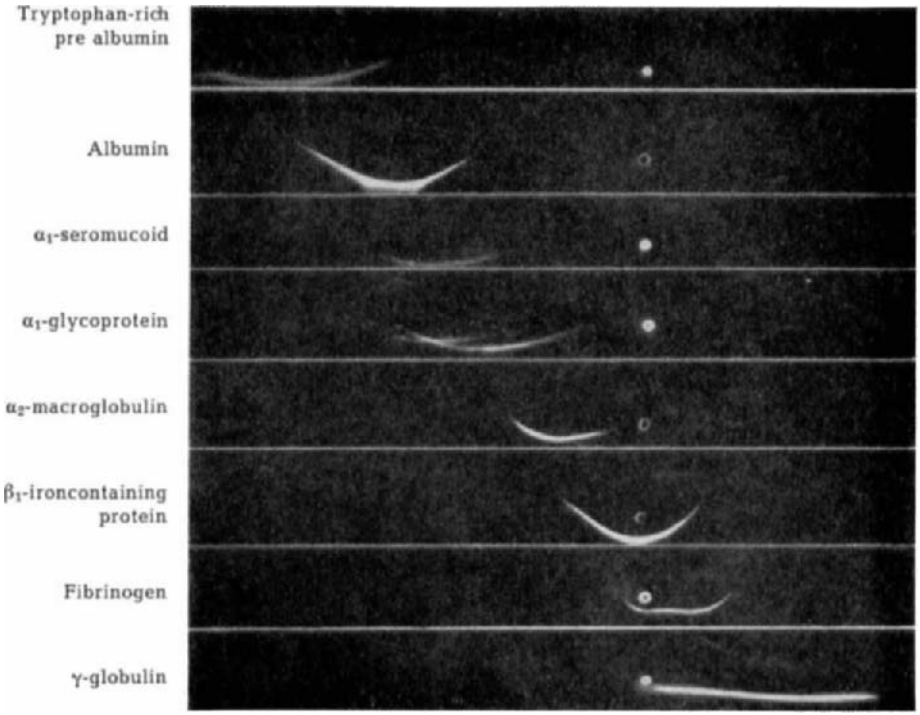

FIG. 6. Immunoelectrophoresis of isolated human plasma protein fractions. From Schultze and Schwick (S4).

9.2. DIFFERENT ANTISERA

After this description of specifically active antigens as tools for identification, we must now give an account of different antisera (G5). The shortest way for identification of an unknown antigen with ImEl makes use of a strictly monovalent, homologous antiserum. Such reagents will produce results as definite as the ion reactions of classic analytical chemistry. But if it is easy to obtain pure inorganic reagents, it needs much experience and time to obtain monovalent antisera (S6). Recent investigations on the antigenicity of proteins have revealed the following facts (A1, K1, S10). It needs a certain chain length of the polypeptide chain to induce antigenic action in the animal or human organism. The

sequence of amino acids which form the polypeptide chain determines the primary structure of the protein molecule. It influences also the folding of the polypeptide chains, and it is supposed that this effect has a direct bearing upon specificity (S7, S8). Certain groupings of relatively few amino acids are thus responsible for antigenic action, but their success depends on their power to change the template at the site of protein synthesis. This change will be all the more successful if the grouping of amino acids, at least in certain parts of the chain, is radically different from the physiological products of the template. Some examples will demonstrate this preliminary condition. It is quite possible to obtain antisera against albumin, siderophilin, ceruloplasmin, or fibrinogen of a sufficiently high specificity to give no cross reactions. But it seems hardly possible to differentiate immunologically the normal hemoglobin-A from Hb-S or from Hb-C. The difference of one amino acid in a total of 300 is apparently not enough to induce a marked change of the template. These two examples indicate extremes in antigenicity; between them every variation is possible. Further, the same protein antigen acts very differently from one animal species to another; certain animals form anti-γ-globulin sera, other animals only anti-α-globulin, anti-β-globulin, and antialbumin sera. Apart from these species differences there remains always the uncertainty from animal to animal. It is therefore of the utmost importance to examine the antisera by as many techniques as possible. Hereby, a *"basic pattern"* is gained, which can serve as reference standard as long as the same batch of antiserum is in use. As antisera are *not* stable over prolonged periods, it has often proved difficult to obtain reproducible results. Deep freezing of antisera and subsequent thawing cannot be repeated very often, as the titer will diminish. The importance of working on a *microscale* is stressed, as it enables a long series of ImEl to be performed with one batch of antiserum.

Personal experience has proved the excellent quality of the horse immune serum against normal human serum from "Serpasteur" (36 rue du Dr. Roux, Paris 15e). For injection, 200 ml of a Red Cross plasma pool is mixed with 200 ml of Bayol-F and 70 ml of Arlacel (W5); 9 subcutaneous injections of doses of 2.5 ml are given over a 40-day period. After an 80-day interval, a second series of 7 injections is administered. Serum from the withdrawn blood is sterilized by filtration and sealed in ampoules with Merthiolate at 1:10,000. The same laboratory has produced a horse antiserum against human serum albumin (W5) by a series of 39 intramuscular injections of a 5 % solution of Fraction V over a period of 4½ months.

Immune rabbit serum against normal human serum was prepared by four intravenous injections per week for 5 weeks. To this end, normal serum was diluted to 1 % for injection. Finally one rabbit was chosen out of 20 for its response to the largest number of different human serum antigens. Another rabbit formed no anti-γ-globulin but strong anti-β_2-globulin (W5). Heremans (H1) has obtained antisera from rabbits and hens by giving a first intramuscular injection of 10–100 mg antigen suspended in the adjuvant of Freund; after several weeks of rest, more injections are given without adjuvant. None of these antisera was strictly monovalent. Even if formed with very pure preparations of γ-globulin, there was some reaction with β_{2A}- and β_{2M}-globulins. It is well known that both these fractions are absent from the blood serum of newly born infants; therefore, such sera can well serve for absorbing. With a similar technique, Heremans (H1) has also absorbed polyvalent antisera with γ-globulin, β_{2A}-globulin, β_{2M}-globulin, and albumin. Finally, the antiserum "CRPA" (Schieffelin, New York), specifically reacting with the C-reactive protein, deserves to be mentioned.

9.3. Artifacts and Different Forms of Precipitate Lines

Unwanted artifacts can arise from technical irregularities (heterogeneities of the agar gel), contaminating antigens or from chemical interaction of the agar gel with the migrating protein. Heremans (H1) has stated that antisera from the hen have a tendency to precipitate spontaneously. Lysozyme of egg white, certain lipoproteins, and cryoglobulins also react. It is a common experience of clinical routine work that sera of a strong dysproteinemia often have a tendency to precipitate within agar gel or to migrate as a striated band (W4); such difficulties can originate in a sieve effect or in a chemical action or in both. Asymmetrical macromolecules like fibrinogen (length 700Å) show it strongly. In such cases, ImEl becomes impossible because there is always the possibility that a small percentage is still migrating and thus forming extra gradients or unidentified precipitating lines.

It has been observed (H1) that, when bathing the agar gels in saline, parts of the albumin are swept on to the cationic side and form new lines of precipitate. Finally, old sera, where enzymatic action has set in, must be avoided, as proteolytic degradation of proteins leads to interactions and lowers the general dispersion of the serum. If all such cases had been carefully discarded, many a doubtful line would not have been discussed in the literature!

Now, if one of the reactants is in an excessive concentration, such a disproportion can give rise in ImEl to the forming of double lines (Fig.

7, *a* and *a'*) which have the tendency to cross each other. It is equally possible that in such a case no line of precipitate is formed at all or that it is formed initially, only to become dissolved later (U1). Distinct and stable lines are formed only if both reactants are in optimal proportion. If they are not, adequate dilutions are indicated. The result is often a broad line (Fig. 7, *b*) such as is formed by serum albumin normally. Fractions with identical electrophoretic migration velocity will form two distinct lines (Fig. 7, *c*), unless the concentrations of the fractions are very different. In such cases, distinct lines will form only if the diffusion constants are sufficiently different; a limit of the method is evidently set because even then a differentiation of the lines will show only if the

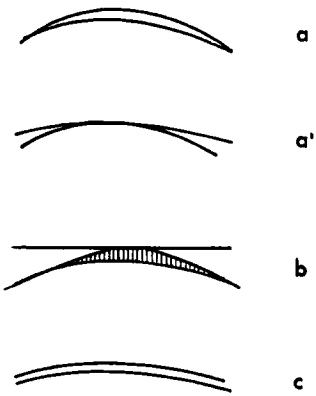

FIG. 7. Double lines crossing (*a* and *a'*); a broad line resulting from a relatively high antigen concentration (*b*); double lines deriving from two different antigens (*c*).

fraction with the slower diffusion is not far more concentrated (W3). As a rule, such antigens diffuse very nearly up to the channel of the antiserum (S1). It is safe to repeat the ImEl with slightly changed concentration of the antigen.

9.4. Quantitative Aspects

The photometry of agar films after simple agar electrophoresis allows a good quantitation because agar film is optically a homogeneous, highly transparent medium. An objective interpretation of precipitate patterns in agar diffusion plates is rendered possible along the lines outlined by Jennings (J1), Polson (P2), Feinberg (F1), and Augustin (A2). The preparative possibilities of zone electrophoresis in agar gel have been assayed by Wieme (W4) and Reuter (R1). A survey of these techniques has been given by Smith (S10) and by Kunkel and Trautman (K2). As

direct elution or pressure are not applicable, freezing out of the agar block has been tried with a certain measure of success. The structure of the agar gel breaks down at —20°C, and on thawing the protein sol can be separated by filtration through nylon gauze. Around 50 % of the protein can thus be regained. However, it must be concluded that gel techniques offer much better chances for analytical methods of a non-preparative character.

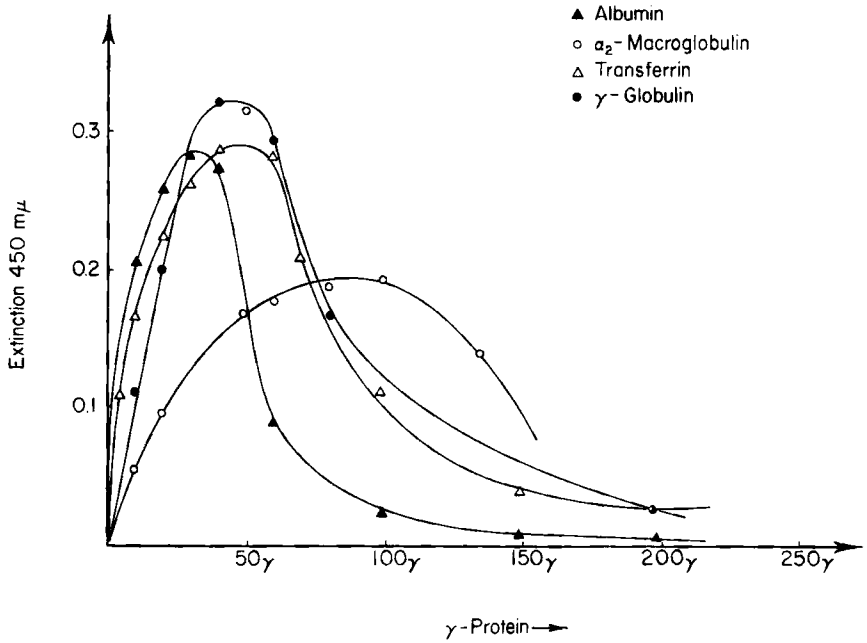

FIG. 8. Reference curves for the quantitative evaluation of albumin, α_2-macroglobulin, transferrin, and γ-globulin. From Schultze and Schwick (S4).

Recently, Schultze and Schwick (S8) have given details of an immunological method by which it is possible to estimate quantitatively precipitates formed in optimal proportions. The reactants are protein fractions of controlled purity as antigens and monovalent rabbit antisera. The reaction product of several different proportions was measured at the wavelength of 450 mμ in a spectrophotometer (Zeiss Opton). Thus, *reference curves* were gained, and their peak values gave a standard extinction for each of the nine fractions (Fig. 8).

The high sensitivity of this quantitative method is well illustrated by the following limits of observation: system prealbumin/antiprealbumin, 0–4.5 μg; albumin/antialbumin, 0–25 μg; ceruloplasmin/anticerulo-

plasmin, 0–4.5 μg; α_2-macroglobulin/anti-α_2-macroglobulin, 0–25 μg; α_2-lipoprotein/anti-α_2-lipoprotein, 0–4.0 μg; β-lipoprotein/anti-β-lipoprotein, 0–45 μg; β_1-metal-binding protein/anti-β_1-metal-binding protein, 0–35 μg; fibrinogen/antifibrinogen, 0–30 μg; and γ-globulin/anti-γ-globulin, 0–40 μg. The limiting quantities of these fractions which can be measured in diluted plasma or sera are stated in Table 1.

TABLE 1
THE SMALLEST QUANTITIES WHICH CAN BE MEASURED IN DILUTED PLASMA OR SERA

Protein fraction	Dilution of plasma or serum	Measurable protein quantity μg
Prealbumin	1:10	1.0–4
Albumin	1:400	0.5–14
Ceruloplasmin	1:30	0.5–4
α_2-Macroglobulin	1:50	0.6–12
α_2-Lipoprotein	1:30	0.5–4
β-Lipoprotein	1:30	5.0–42
β_1-Metal-binding protein	1:30	0.5–20
γ-Globulin	1:100	0.5–19
Fibrinogen	1:20	1.0–33

Here then, is a micromethod, less time-consuming than nitrogen estimation, well suited to supplement ImEl with quantitative data. It will gain broad application once univalent antisera are available on a commercial basis (S9).

Another possibility offered by ImEl is the measurement of relative mobilities by comparison with pure test fractions (U1). Interglobulin ratios can be established. If measures are taken to keep the agar gel perfectly homogeneous during the whole run, mobilities will almost equal those obtained by moving boundary electrophoresis. The results of Williams and Grabar (W5) are quoted in Table 2.

TABLE 2
THE RELATIVE MOBILITIES OF SERUM PROTEIN FRACTIONS

Protein fraction	Mobility; moving boundary	ImEl
		(cm^2/volt sec) × 10^{-5}
Prealbumin	—	7.2–7.8
Albumin	5.9–6.1	6.0
α_1-Globulin	5.3	5.2–5.3
α_2-Globulin	4.0–4.2	4.0–4.5
β_1-Globulin	3.0–3.2	3.0–3.2
β_2-Globulin	—	2.1–2.4
γ-Globulin	0.7–0.9	0.7–1.0

Based upon such data, direct analysis of some fractions is possible when the components can be identified by their mobility alone. However, such an application is very limited in practice, as many subfractions have almost equal mobilities. In order to measure *relative* mobilities, it is practical to use pure serum albumin as reference. If the mobility of the latter is u_{alb} and the mobility of the unknown protein u_x, the quotient u_x/u_{alb} is independent of the carrier medium if the migration velocity does not change during the run. In Table 3, relative mobilities

TABLE 3
THE RELATIVE MOBILITIES WITH ALBUMIN AS REFERENCE

Protein fraction	Mobility		
	Uriel (U1)	Wieme (W4)	Moving boundary electrophoresis
	(cm^2 $volt^{-1}$ sec^{-1} × 10^{-5})		
Prealbumin	1.15	1.19	—
Albumin-*x*	0.97	0.98	1
α_1-Globulin	0.86	0.86	0.85
α_2-Globulin	0.84	0.67	0.68
β_1-Globulin	0.51	0.47	0.48
β_2-Globulin	0.37	0.40	—
γ-Globulin	0.15	0.11	0.16

in agar gel are quoted, as measured by Uriel (U1) and Wieme (W4) with albumin as reference.

For quantitative evaluation of the stained fractions in the agar film, the Zeiss Extinktionsschreiber II has given us good service.

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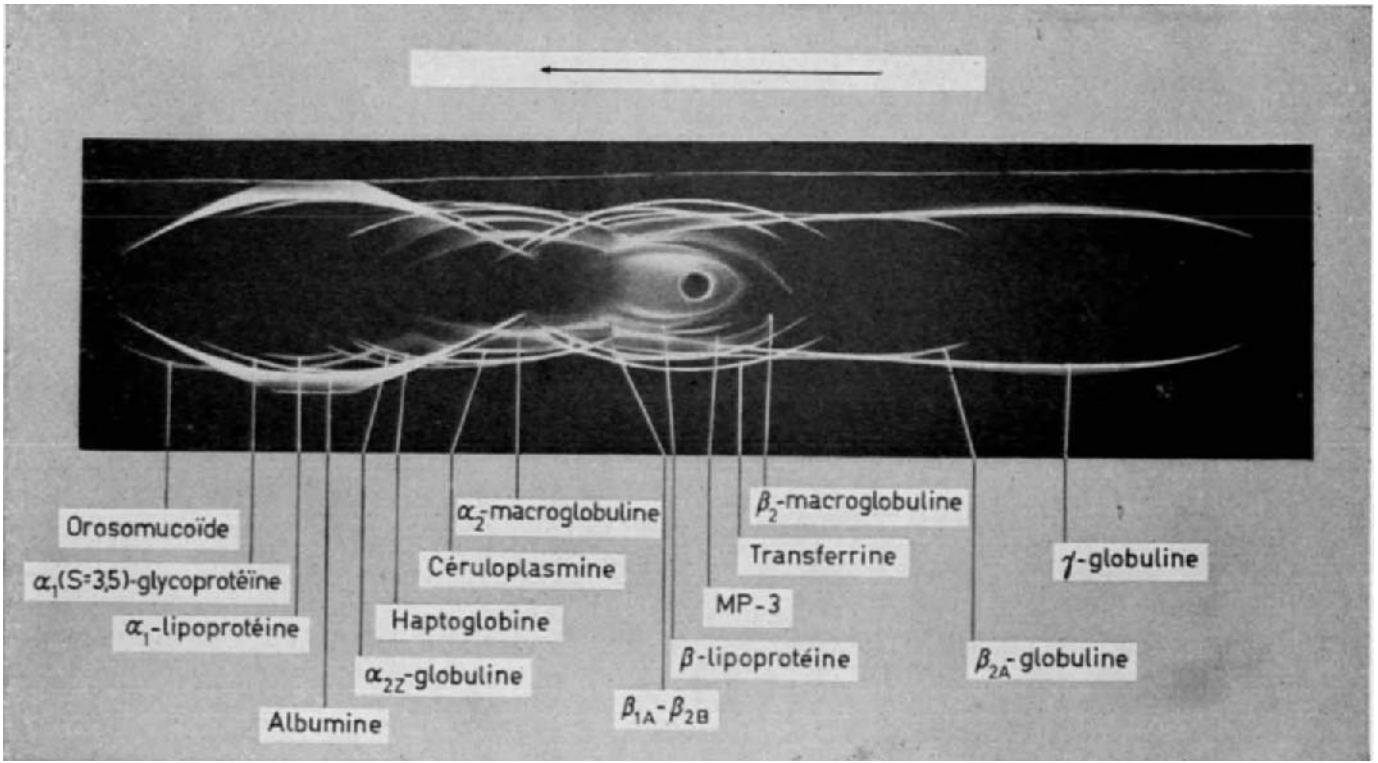

FIG. 9. The proteins of a normal human serum in agar gel as revealed by ImEl with a polyvalent antiserum (horse 491) from the Pasteur Institute. From J. Heremans (H4).

PART III. Results

10. Physiological Applications

10.1. Normal Constituents of Human Blood Plasma

Of all biological fluids human blood plasma is the most fully studied. Using several antisera containing a well-diversified number of antibodies of sufficiently high titer, it has become possible to identify 18–25 protein components, some of them only recently. As this advance in our knowledge was made possible by ImEl it is clearly desirable that details should be discussed here. The first thing is to survey what can be done using the micromethod with antiserum of the "Serpasteur" in agar gel (H4)

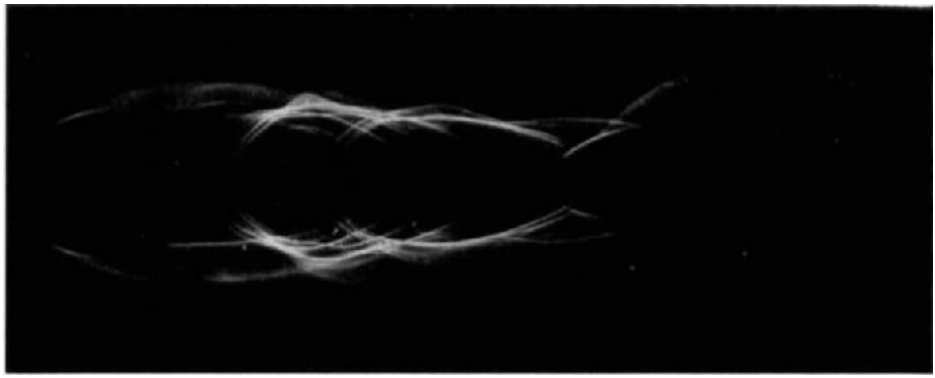

Fig. 10. The proteins of a normal human serum as revealed by ImEl in cellulose acetate; developed with a horse antiserum from Serpasteur, Paris. From Kohn (K4).

and in cellulose acetate (K4). Such a comparison shows that these two techniques are fairly equal from an analytical point of view (Figs. 9 and 10).

The fast-moving fractions, preceding serum albumin (G5, S7), contain normally only 0.12–0.31 % (A1) of the total serum proteins. This concentration is evidently too low to give an independent boundary in free electrophoresis or on paper. Grabar and his colleagues distinguish them by the letter ϱ (rho); they contain 1.1 % hexoses, 0.15 % acetylhexosamine (S9), and relatively much tryptophan; the sedimentation constant is 4.2 S and the molecular weight is 61,000. With the high sensitivity of ImEl, several lines are obtained preceding the albumin, but not regularly as Burtin (B4) has pointed out (Fig. 11).

Sometimes, a lipoid-containing line ϱ_2 is obtained; the acid seromucoid (B4) is apparently identical with the orosomucoid (H4). The latter sub-

stance has a sedimentation constant of 2.9 S, a molecular weight of 40,000–44,000 (S9), and contains many acidic groups, which explains the isoelectric point of 2.7. Albumin shows a sharp front on the anodic and cathodic side in agar electrophoresis. Whereas the α_1-globulin is satisfactorily separated from the albumin, an acid low molecular α_1-glycoprotein with a carbohydrate content of 38 % and a molecular weight of 40,000 has an identical mobility. Adjacent is an α_1-lipoprotein (U1), which belongs to the group of high-density lipoproteins in the ultracentrifuge; it needed ImEl to reveal the heterogeneous character of the α_1-group of proteins.

The α_2-group is even more complex, and it is very possible that new constituents will be discovered in the near future. Depending upon the

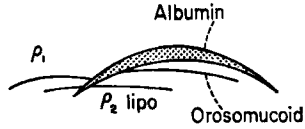

FIG. 11. The lines of the ϱ-group. From Burtin (B4).

serum specimen and its age, two fractions show up, but sometimes only the faster one has sharp fronts. In rare cases, even three lines of precipitate are formed. The slower α_2-globulins contain haptoglobin (N1) (see Fig. 12), a protein which is able to bind hemoglobin (B2). It seems that haptoglobin influences the migration velocity of α_2-globulin in a varying degree and causes the reported variations. Wieme (W1) has developed a method for locating haptoglobin by adding hemoglobin. The distance between the complex thus formed and the nonreactive α_2-globulins is observed; he stresses the point that enough hemoglobin must be added to saturate all the haptoglobin present (approximately 8 mg hemoglobin to 1 ml serum). The complex which is formed has a mobility equal to that of β_1-globulin. Haptoglobin is a glycoprotein containing 11.3 % hexoses and 5.7 % hexosamine; in monomer form, with a molecular weight of 85,000, it binds one molecule of hemoglobin, while the dimeric form binds two (H8, H9). Using starch gel electrophoresis, Smithies* (N1) discovered that constitutional differences exist in man (H9).

In between the lines of *haptoglobin* and α_2-macroglobulin, the light line caused by *ceruloplasmin* is just visible (U2). It is the principal carrier of copper, 8 atoms per molecule; besides, it contains 9.5 % of hexoses and has a molecular weight of 150,000 (S9). Its polyphenoloxidase activity can be used for localization and, coupled with ImEl, for identification.

* For references by Smithies, see (S2, S3) on page 220.

Parallel to this line, there is the distinct line of α_2-macroglobulin (Fig. 12); the convex curvature of the precipitin line is connected with the high molecular weight of the antigen (approximately 900,000) (K5) and its slow diffusion. Filitti-Wurmser *et al.* (F2) have demonstrated in a double cell of the ultracentrifuge the two heavy protein fractions in normal serum, i.e., α_{2M} and β_{2M}. The α_2-macroglobulin normally contains 3.6 % hexoses and 2.9 % *N*-acetylhexosamine and represents 2 % of all the serum proteins (S9). Finally, there is the α_2-lipoprotein (Fig. 12) with the high lipid content of 88.4 % (S9). The molecular weight is 1,300,000, and the diameter of the spherical molecule is 200 Å. It corresponds to the β_1-lipoprotein in paper electrophoresis (P3). The mobility in agar gel depends on the concentration (U1) and the number of acidic groups (W6).

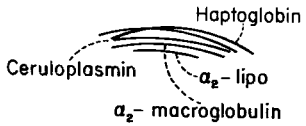

FIG. 12. The lines of the α_2-globulin group. From Burtin (B4).

Among the β-globulins, two fractions, β_1 and β_2, are well separated in agar gel. A slow component of the β_1-globulin close to the antibody channel contains the *siderophilin* (S8) (transferrin, Fig. 13). This protein has a molecular weight of 88,000 and binds two atoms of iron per molecule (S9, G4). Besides this line, there are three further lines of β_1-globulins (W6). In the following section, covering the β_2 and γ-globulins, Heremans (H4) succeeded in differentiating immunologically γ-, β_{2A}-, β_{2M}-, β_{2B}-, and β_{2X}-globulins; in certain sera there is further a γ_X-globulin (S10) which is immunologically (G2) similar to C-reactive protein (CRP) (Fig. 13). This CRP, which is increased in the early stages of inflammation and often in rheumatic diseases, belongs to the group of rapidly moving γ-globulins (H4). In zone electrophoresis CRP has been frequently isolated among β_1-globulins as the mobility depends on the support medium. The location of this group of globulins is schematically given in Fig. 13 (H4). The scale at the top of Fig. 13 represents the mobility multiplied by $cm^2\ volt^{-1}\ sec^{-1} \times 10^{-5}$.

Heremans (H4) considers the γ-, β_{2A}-, and β_{2M}-globulins as a functional entity and calls them *antibody globulins.* The antigenic relationship was proved with the Ouchterlony gel test. Kunkel *et al.* (K6) have located in the β_{2M}-globulins the high molecular weight antibodies of the 19 S class, such as the macroglobulin of Waldenström, rheumatoid factors, cold agglutinins, lupus factors, isoagglutinins, leucoagglutinins,

thyroid autoantibodies, and Wassermann antibodies. This compilation may prove the clinical interest in further investigation of the group of "antibody globulins." With a sufficiently polyvalent antiserum, the lines of γ-, β_{2A}-, and β_{2M}-globulins are regularly revealed, whereas β_{2B} is revealed only if the serum specimen is quite fresh; the lines of β_{2X} and γ_X are more difficult to obtain and are only faint. In Fig. 13, the extraordinary length of the precipitate line of γ-globulin is well rendered; technically, it is the result of a whole group of proteins with identical antigenicity but with different migration velocities (S3); in fact, the line

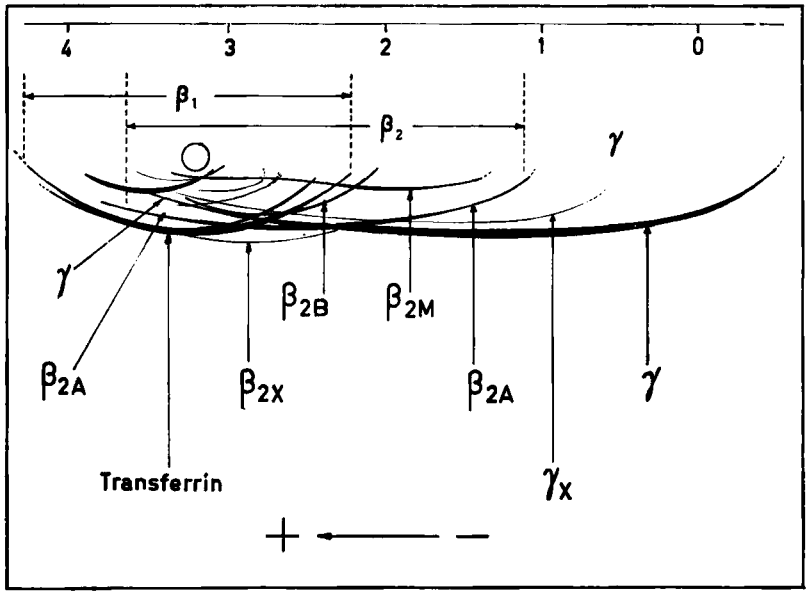

FIG. 13. The lines of the proteins of the β_2- and γ-region. From J. Heremans (H4).

often reaches into the region of the α_2-globulins. The γ-globulins have a molecular weight of 160,000 and constitute the principal component of the 7 S peak in ultracentrifugation diagrams.

A similar extension of the line of precipitate has been described by Burtin (B4); in the rare cases where albumin splits up into two peaks of slightly different mobility, the antigen is identical but the line of precipitate shows a double curvature. Wunderly (W8) showed it in the case of Nennstiel and Becht (1957); paper electrophoresis separated both albumin components well, whereas simple agar electrophoresis did not reveal them. It is interesting to note that in egg white two components of albumin are always resolved.

Further, there are the following protein fractions which are not regularly revealed by ImEl (W6): the ϱ-lipoprotein, postalbumin; a fraction between α_1- and α_2-globulins, a third α_2-globulin, a β_0-globulin, and a separate γ_1-globulin.

Fibrinogen, with a molecular weight of approximately 400,000 and a length of about 700 Å, hardly moves to either side in agar electrophoresis (S1, S12). In consequence, a short line can be obtained near the starting point with an antiserum containing antifibrinogen antibodies. Salmon (S1) has established three antigenic groupings in the human fibrinogen. Globin can be identified by ImEl with a specific antiserum (R2).

10.2. Normal Protein Constituents of Other Human Biological Fluids

As Grabar and Burtin (G4) have pointed out, our present knowledge of the protein composition of human biological fluids other than serum indicates two categories. There is one in which the number of identified fractions as well as their mutual proportions are identical with those of the normal serum; ascites, lymph, synovial fluids, and the aqueous humor fall into this group. Other liquids, like the cerebrospinal fluid, the perilymph of the ear, saliva, seminal fluid, and most of all urines, contain fewer protein components and in a different proportion than serum. Certain components appear only in one liquid and are thus characteristic. In pathological cases the second group shows generally a greater variation of proportion than the first group.

If *ascites* or *lymph* is to be studied by ImEl, it must be cleared by centrifugation; the quantity used for electrophoresis must at least be double that of normal serum. The albumin to globulin ratio is consistently slightly higher than in plasma. In pathological cases it is necessary to determine the protein content (S4, H2).

The proteins in *synovial fluid* have been thoroughly investigated by Schmid (S5, S6). In order to reduce the high viscosity, he incubates the specimen of synovial fluid with hyaluronidase; thereupon, they are lyophilized and dialyzed against borate buffer of pH 8.6 and $\tau/2 = 0.07$. The subsequent electrophoresis in agar gel lasts for 17 hours with 180 volts and 40 ma applied. Generally, 12 lines of precipitate were obtained by ImEl. As with *cerebrospinal fluid*, the ϱ-group is relatively increased in synovial fluids obtained 5–10 minutes after death or from traumatic cases. Free electrophoresis had already proved that the albumin/globulin ratio is changed in favor of albumin; as a consequence, globulin lines appear considerably weaker (S6). A similar change of proportions takes place in the aqueous humor (M2); in free electrophoresis the ϱ-group

amounted to 16.4 % of the total proteins in the rabbit and to 9.3 % in cattle. The lens proteins were separated by Wieme and Rabaey (W2) into three fractions by ordinary agar electrophoresis, cattle giving eight lines of precipitate in ImEl. The fast-moving Fraction I (α-crystalline), seems to be responsible for the organ specificity of lens proteins (H1), while the other fractions have species specificity (W3).

Before ImEl can be applied to *spinal fluid* (CSF), the latter must be concentrated by pressure dialysis or dialysis against concentrated dextran solution. Paper electrophoresis revealed only quantitative differences from serum proteins (K2). Much as in *pericardial fluid,* there is more albumin and less globulin than in serum. The investigation with ImEl showed the following interesting differences; the ϱ-group and orosomucoid gives a strong precipitation line; the α_2-globulin group is bare of haptoglobin, ceruloplasmin, and α_2-macroglobulin, but shows instead 2–3 lines not yet identified (B4); there is no line in the β_2-globulin region; the γ-globulin line is much shorter; lipoproteins seem to be absent from the CSF (G3). In conclusion, only proteins with a molecular weight of 160,000 or less show up in the CSF. Chevance *et al.* (C1) have given a comparison of results on CSF and the perilymph of the ear with ImEl which is detailed in Table 4. If possible differences in the foregoing con-

TABLE 4

PROTEIN FRACTIONS AS REVEALED BY IMEL IN THE CEREBROSPINAL FLUID AND THE PERILYMPH OF THE EAR

Cerebrospinal fluid (CSF)		Perilymph of the ear (human)	
1	Fraction		—
1	Seromucoid, acidic		—
1	Albumin	1	Albumin
1	α_1-Globulin (faint)	3	α_1-Globulins
2–3	α_2-Globulins	4	α_2-Globulins
2	β_1-Globulins	3	β_1-Globulins
1	β_2-Globulin (faint)	1	β_2-Globulin
1	γ-Globulin	1	γ-Globulin

centration process and in the multivalent antiserum are considered, the composition seems to be fairly similar.

Gabl and Pastner (G1) have analyzed the proteins in mixed *saliva* of 10 normal persons with paper and agar electrophoresis and ImEl. The saliva samples were first centrifuged and filtered; later their protein concentration was increased to 1–3 % by ultrafiltration under pressure. Whereas in paper and agar electrophoresis five fractions were separated, ImEl showed up to 9. These were in locations identical with those given

by serum, but in different proportions. The lines of the albumin and γ-globulin region were constant, but the α-globulins were variable.

The proteins of *seminal fluid* have been separated with paper electrophoresis by various investigators, 6–8 fractions being obtained, though the fronts were often distorted. There is much less albumin and γ-globulin than in serum. Variation in the proportions is considerable from individual to individual, whether normal or patients with abnormally viscous seminal fluids or with poorly motile or nonmotile sperm (M2). Lately, Hermann (H6) has characterized seminal fluid with ImEl and established the existence of several protein fractions identical with those in serum by using adsorbed antisera (H5). A ρ-fraction of high mobility was, however, not identical with the serum analog. No lipoproteins were identified, but several glycoproteins were. It was, furthermore, not possible to establish a relation between protein components and fertility. The seminal fluid of animals (rabbits, cattle) has a distinctly different composition (P2).

TABLE 5

UROPROTEINS (HUMAN) IDENTIFIED AS BEING IDENTICAL WITH PLASMA PROTEINS

Plasma proteins	Identical uroproteins (+) as found by (H3) Heremans *et al.*	(V1) Vaux *et al.*	(B1) Berggärd
Prealbumin, ρ		—	+
Seromucoid, acidic	+	+	+
Albumin	+	+	+
α_1-Globulin	+	1–2 lines	2 lines
Haptoblogin	—	—	+
Ceruloplasmin	—	—	
Transferrin		+	+
α_2-Macroglobulin	—	—	trace
α_2-Globulin		1–2 lines	+
β_{1A}-Globulin		—	+
β_{1B}-Globulin		—	variable
β_{2A}-Globulin	+ faint	+	+
β-Lipoprotein	—	—	—
Fibrinogen			variable
γ-Globulin	+ faint	+	+

Finally, there are several ImEl investigations on the normal *urinary colloids*. These form three subsections: proteins which are identical with serum constituents, a nonidentical uromucoid, and uropolysaccharides. It is necessary to concentrate the urine samples 1500–3000 times by simultaneous ultrafiltration and dialysis (G7). Later investigators used

specific antisera against purified plasma proteins and antitotal human serum adsorbed with purified proteins in ImEl of normal urinary proteins. In many instances, the Ouchterlony technique was used simultaneously. The uroproteins listed in Table 5 were thus identified as being identical with plasma proteins.

Hermann (H7), working also with the antiserum of Serpasteur (Paris), found 7–8 lines of serum-identical proteins in normal urine. Comparing these results it is gratifying to note that the three investigators (Heremans, Hermann, Vaux) using the same antiserum obtain similar results; however, Berggärd, working with a different antiserum, obtained rather more lines. Hermann (H7), who studied the formation of urinary calculi, has proved that the serum-identical proteins form part of their matrix. The normal 24-hour urine contains approximately 50 mg of a salt-insoluble mucoprotein. Heremans *et al.* (H3) have isolated it by sodium chloride precipitation and have prepared an antiserum. Absorption with human serum did not influence the antiserum activity. This antiserum gave two distinct lines with normal urine, one being located between albumin and α_1-globulin, the other between α_1- and α_2-globulin. Three acid mucopolysaccharides, migrating faster than albumin, were selectively stained with Alcian blue by Heremans *et al.* (H3). Their presence has since been confirmed by Fishel (F3).

10.3. Hemoglobin and Ferritin

The initial application of electrophoresis in order to detect differences in the amino acid sequence of human hemoglobin was started by Pauling *et al.* (P1) with free electrophoresis. Later on, Ingram (I1) was able to show with paper electrophoresis combined with paper chromatography that in hemoglobin S (sickle cell) one glutamic acid residue is replaced by a valine residue; the loss of one carboxyl group causes a small but distinct change in electrophoretic mobility. Since then, a systematic study has revealed over 20 hemoglobin varieties in man. Several have identical mobility at pH 8.6 and it is necessary to adapt pH, ionality, or buffer system in order to get a sharp fractionation (W4, W5). Wieme (W6) attained, for hemoglobins A and F, relative mobilities of 0.46 and 0.42 in a 0.90 % gel prepared with Difco Special Agar Noble and barbital buffer at pH 8.4 and $\tau/2 = 0.05$; the separation was even better in 0.8 % Behring Rein Agar in citrate buffer at pH 6.5 and $\tau/2 = 0.15$ (R1). Monnier and Fischer (M1) have separated in 2 % agar gel and barbital buffer at pH 8.6 and $\tau/2 = 0.06$, fetal- and adult-type Hb; with 210 volts applied, a temperature of 4°C, and a duration of 3–6 hours, the migration takes place to the cathodic side; samples of 0.002 ml with approximately 10 %

Hb are required. Some of the results with Hb from man and different animals are given in Fig. 14.

Hb has not only been found in the α_2-region but to some extent also in the β-region. Tombs (T1) has studied the binding of Hb A and β-globulin with ImEl; a benzidine stain was used and sodium nitroprusside served as stabilizing agent.

Hemagglutinins have been investigated with ImEl by Faure *et al.* (F1). They were first separated by simple agar electrophoresis, followed by elution of the different fractions. The latter were examined by specific

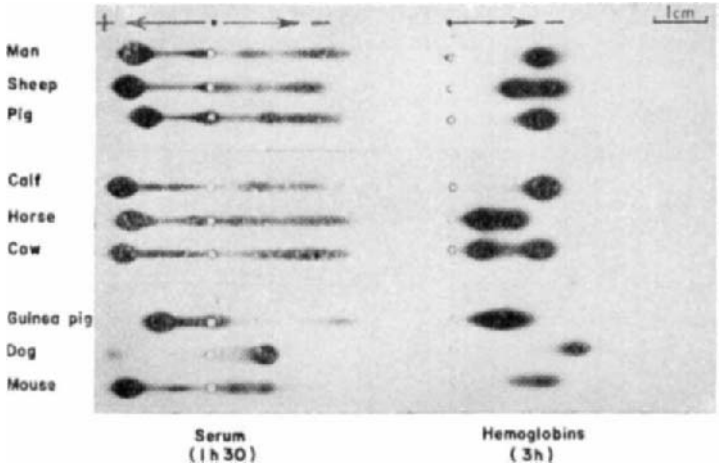

FIG. 14. Comparison of agar electrophoresis of human serum proteins and hemoglobins with those of several animals. From Monnier and Fischer (M1).

immunological reactions. The migration velocity of agglutinins was proved to be identical with that of β-globulins adjacent to the fastest γ-globulins. Identification was most successful with antisera which were absorbed with γ-globulin.

In order to gain an antiserum for *ferritin* studies, Wöhler and Schonlau (W7) injected rabbits with a 0.05 % isotonic solution of ferritin (20 % iron) at pH 7.2. Injections were made every fourth day, and 0.5, 1.0, 2.0, and (twice) 2.0 mg were given subcutaneously. The titer which was thus obtained was 1:500. If this antiserum was used in ImEl against separate sera with a content of at least 100 μg % ferritin, lines of precipitation could be stained with Berlin blue reaction. Ferritin shows a line which comes near β_1-globulin. A positive Ouchterlony test was obtained with most patients suffering from hepatitis epidemica or from liver cirrhosis.

10.4. Proteins of Cells and Tissues

Seligmann (S13) has studied extracts of well-washed human *leucocytes* with ImEl, and has proved the absence of the main serum components. Lymphocytes, whether normal or leukemic, circulating in the peripheral blood contain no components sharing the immunological specificity of serum γ-globulins. With ImEl, the ring test, and the Ouchterlony method, Seligmann (S14) was able to demonstrate in the serum of 10 patients suffering from *lupus erythematosus diffusus* the presence of at least one substance capable of provoking a precipitation reaction with normal or leukemic leucocytes. Thus, ImEl is an important method for the investigation of cellular components. But it is also possible to investigate *tissue fragments* as obtained by homogenization and subsequent extrac-

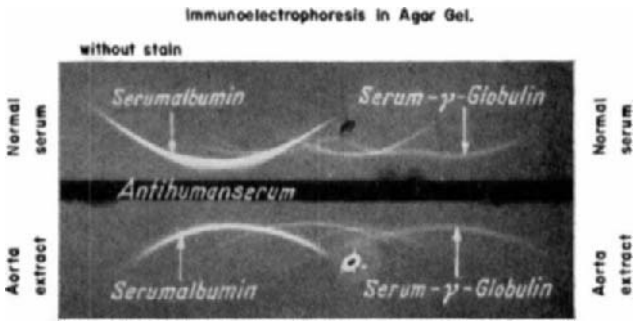

Fig. 15. Results of ImEl of a normal human serum and of an extract of the intima tissue of *aortae thoracales*. From Ott *et al.* (O1).

tion in the buffer used for ImEl. In this way, Ott *et al.* (O1) studied the proteins and lipoproteins in the intima of the *aortae thoracales*. The extract was centrifuged in the cold and then dialyzed under pressure until the protein content had risen to 2–4 %. A rabbit antihuman serum gave the lines of albumin and γ-globulin in the aorta extract; staining with Sudan black B revealed a lipoprotein in the α_2-globulin site, but several proteins of the extract were not precipitated (Fig. 15).

In order to make clinical biopsy material accessible to electrophoresis, Wieme (W6) developed the direct electrophoresis of *tissue proteins*. A comparison with the agar electrophoresis of a muscle extract showed good agreement, and it was concluded that the migrating proteins were contained in the cytoplasm. Kessel (K3) has demonstrated that agar electrophoresis is of advantage for the separation of organ proteins, as neither porosity nor adhesion hinders the migration; as is well known, the solubility of such proteins is limited. Here, then, a new field has

opened for ImEl which should yield interesting results if coupled with morphological analysis.

10.5. Fetal Serum and Milk

A study of the results of ImEl on serum of the newborn was of special significance to find out which lines were lacking, for such findings ad-

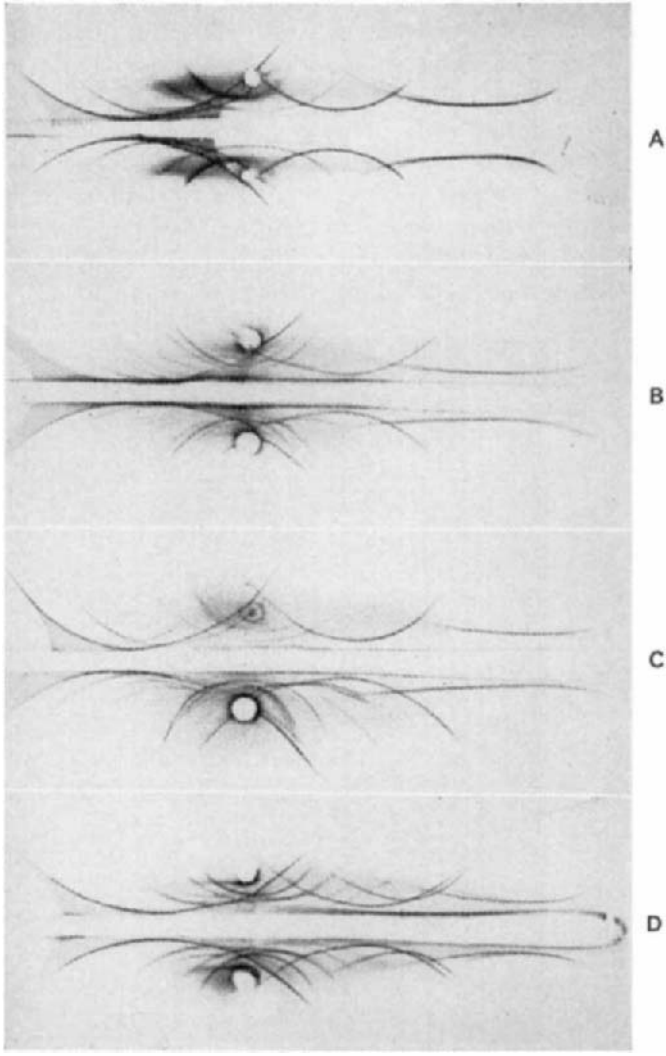

Fig. 16. The development of the lines of ImEl as a function of age. Serum proteins from the newborn (human) after 4 days (*A*), 4 weeks (*B*), 8 weeks (*C*), and 5½ months (*D*). From Hitzig (H10).

vance our knowledge of the permeability of the placenta. In *fetal blood* of the eighth week there are only 5 fractions: ϱ, albumin, α_1, α_2, and β_1 (S2). After the twelfth week the pattern becomes more complete as the γ-globulin appears, but *neonatal serum* at birth is still qualitatively incomplete. According to Hitzig (H10), there are lacking at least three α_2-globulins, two β_1-, and two β_2-globulins. All these protein fractions tend to appear during the 1–9 months after birth, so that the protein spectrum of an infant of one year is hardly different, qualitatively, from that of an adult. The exact composition of the γ-globulin group still awaits a detailed immunological investigation. The development of the protein spectrum during early life is well illustrated in Fig. 16.

When the proteins of *human milk* were studied against horse antihuman serum, four distinct precipitation lines were obtained by Gugler *et al.* (G8). Among them, serum albumin, α_1-globulin, and β_{2A}-globulin could be identified, but the immune globulins were of different antigenic structure from serum γ-globulins. Colostrum contains an additional

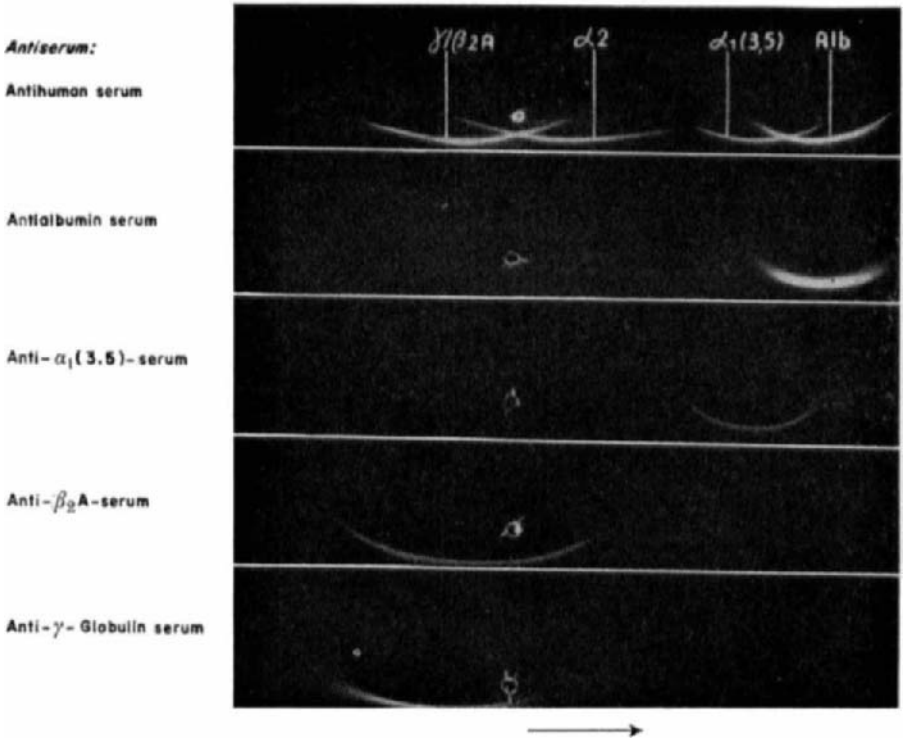

FIG. 17. ImEl of milk (human) with different specific antisera. From Schwick *et al.* (S11).

protein, with a determinant corresponding to the β_{2M}-macroglobulin of blood serum. Schwick *et al.* (S11) have followed quantitatively the albumin and γ-globulin content of human milk during 1–26 days of lactation (Fig. 17). With free and with paper electrophoresis, up to eight components have been separated in human milk; new antisera must be developed before ImEl can reveal a complete spectrum of the milk proteins. Besides individual variations, cow's milk should show significant breed or strain differences.

10.6. Serum Proteins of Animals

Making use of several antisera containing different antibodies, Marie Kaminsky and J. Durieux (K1) have investigated various sera from animals. They studied the constituents of serum of the *hen*, the *rooster*, and

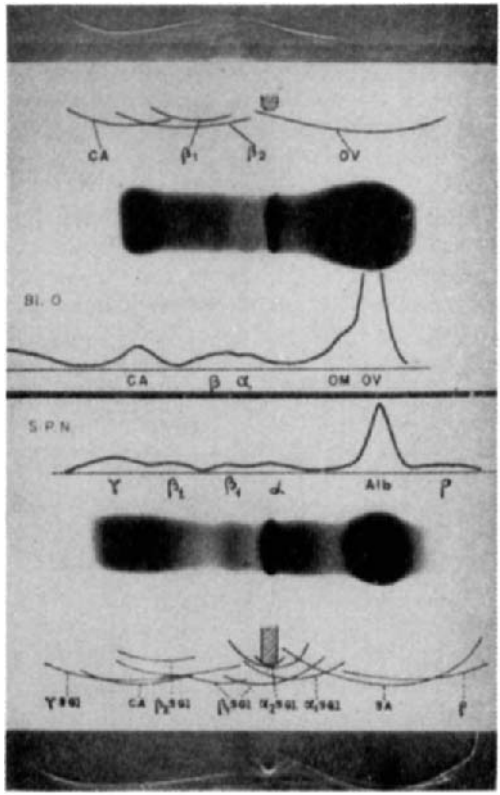

Fig. 18. A comparison of the protein composition of the serum of the hen (SPN) and of egg white (Bl O) with Tiselius electrophoresis, agar electrophoresis and staining of the proteins, ImEl as obtained with a homologous antiserum. From Kaminsky and Durieux (K1).

the newly hatched *chick* and compared them with those of chick embryos of 8, 11, 14, and 17 days and further with the constituents of hen egg white by immunochemical precipitation by double diffusion in agar, ImEl, and zone electrophoresis in agar gel (Fig. 18). Ovalbumin and ovomucoid of egg white are present only in *embryo serum*, but all the sera contain conalbumin, immunologically the same as in egg white.

Grabar *et al.* (G6) studied the serum of mice irradiated by lethal doses of X-rays and protected by rat bone marrow. Dray and Young (D1) pointed out differences in the antigenic components of sera of individual rabbits as proved by induced isoprecipitins.

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10.7. Pathological Human Plasma

Changes in plasma protein composition, arising from illness are not specific for any single disease. But if such changes are assessed in relation to other clinical data, they supplement the information which lies at the base of diagnosis and prognosis. Only for a limited group of diseases are extreme forms of more or less well-circumscribed changes in the blood

protein composition characteristic. They occur predominantly in systems or organs which have long been accepted as the site of significant functions of protein metabolism. Only organs which guide and accomplish the interplay of resorption and utilization, storing and degradation of the food and body proteins have a direct bearing. As paper electrophoresis gives a quantitative account not only of the serum protein fractions but also of lipoproteins, glycoproteins, and mucoproteins, it engenders a better clinical interpretation of the findings. The subsequent results for internal medicine are compiled in the book of Wuhrmann and Wunderly (W2).

ImEl has made possible another step forward. Giving qualitative results of a high sensitivity, it serves particularly well in cases of defect dysproteinemia (agammaglobulinemia, analbuminemia) or in cases of immunological deficiency, be it in the γ-globulin group or in the β_2-globulins. Thus, it complements efficiently the analytical data of the clinical laboratory in certain disease states, without being employed as a routine method. A complete ImEl reveals several antigens not previously known, whether by their chemical nature or their biological function. For the time being, ImEl is in advance of protein chemistry. Also, it should be stated that some sections of internal medicine have as yet not been investigated with ImEl. First studies with ImEl of the serum of patients with various diseases were made by Grabar and Burtin in 1955 (G3), Grabar *et al.* (G5), Burtin in 1956 (B3), followed by Martin and Scheidegger in 1957 (M3). Reviews were given by Wunderly in 1958 (W3) and by Wunderly and di Guglielmo in 1959 (W4).

10.7.1. *Defect Dysproteinemias*

A physiological or transient form of agammaglobulinemia occurs in infants. Gitlin *et al.* (G2) studied the congenital form in 8 patients and the acquired form in 3 patients with ImEl. Rabbit and horse antisera against human γ-globulin were used; it was proved that at least 2 plasma proteins (mobility of β-globulins) besides γ-globulin are absent or deficient in agammaglobulinemia. Transferrin and ceruloplasmin are somewhat increased. The high sensitivity of ImEl reveals the fact that the deficiency of γ-globulin is often not complete. The following year, Giedion and Scheidegger (G1) described the case of a boy of 4 years; his serum contained a normal amount of γ-globulin, but was lacking β_{2A}- and β_{2M}-globulin, as well as the isoagglutinins (Fig. 19). All these fractions are usually lacking in agammaglobulinemia.

Resulting immunological paresis was proved by the absence of a rise of antibody titer after attempts at immunization with tetanus and per-

tussis inoculations. Plasma cellular response of bone marrow and lymph nodes after antigenic stimulation appeared also insufficient. De Muralt *et al.* (D1) developed the antiglobulin inhibition test; it serves well for the quantitative determination of γ-globulin in cases of antibody deficiency syndrome and also for determining the lifespan of passively acquired γ-globulin in patients with such defects. It needed ImEl to show that in most cases of the antibody deficiency syndrome the lines for β_{2A} and β_{2M} are lacking, independent of the γ-globulin level. Further

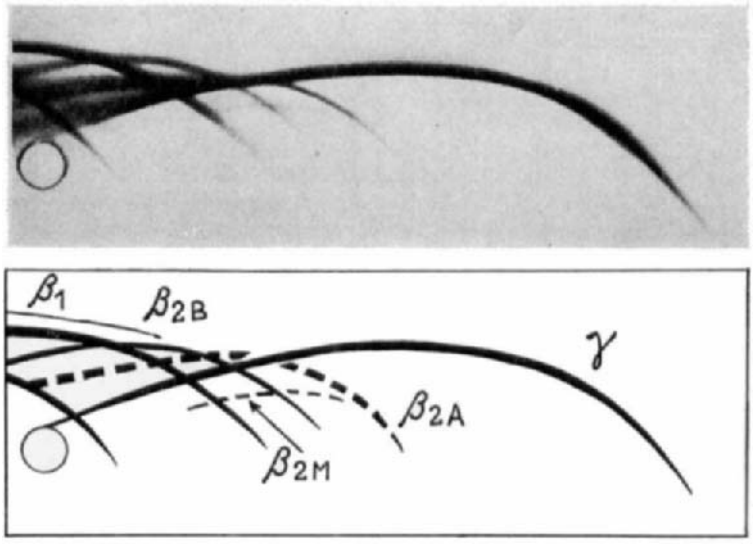

FIG. 19. Cathodic section of a microscale ImEl. Above: normal serum (human); below: a patient's serum where the lines of β_{2A}- and β_{2M}-globulin are lacking. After Giedion and Scheidegger (G1).

studies of cases of agammaglobulinemia have been reported by Marie and Etienne (M2), Grabar *et al.* (G6), and Gitlin (G2).

10.7.2. *Macroglobulinemia*

It has been suggested that 10 % of the serum proteins should have a sedimentation value greater than $s_{20} = 16$ S before a diagnosis of essential macroglobulinemia is considered (M4). Normally, the total concentration of heavy components of the serum proteins rarely rises above 3–4 % of the total protein; their electrophoretic mobility places them among the group of α_2-globulins and the β_2/γ_1-globulin section. Ultracentrifuge studies showed them to be heterogeneous (S7), having sedimentation constants of $s_{20} = 10.6$–28.8 S. In another case, the macro-

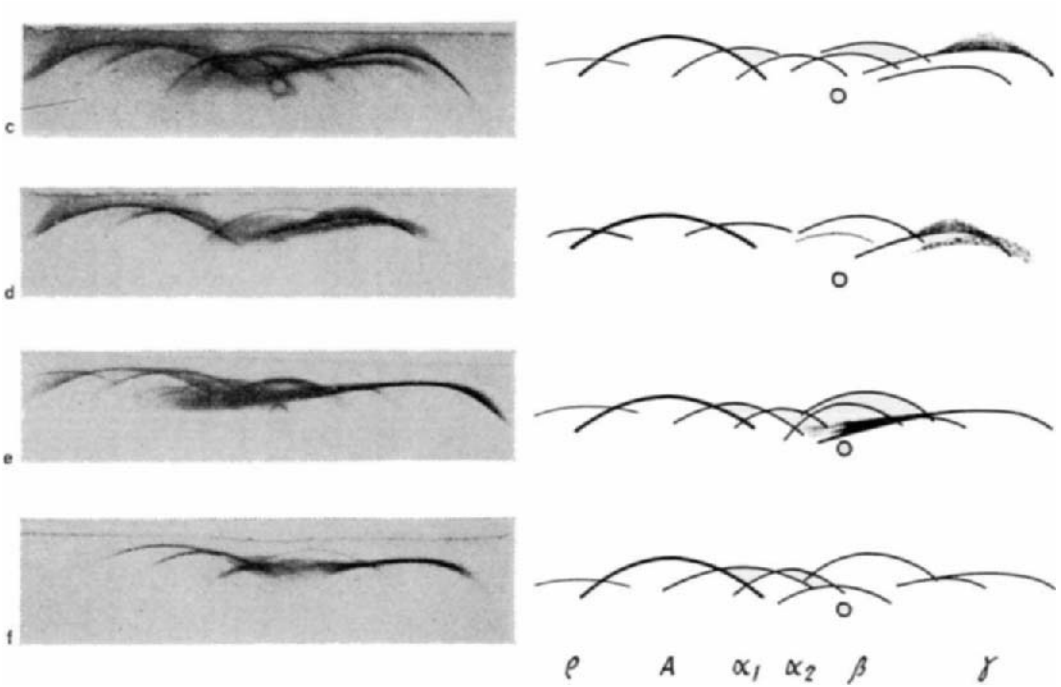

FIG. 20. Examples of ImEl (microscale) with sera of patients with outspoken dysproteinemia: (*c*) myeloma of the γ-type, (*d*) macroglobulinemia, (*e*) hyperglobulinemia, (*f*) normal serum pattern as comparison. More lines become visible upon optical enlargement. From Martin and Scheidegger (M3).

globulin migrated as one component in agar electrophoresis, although it showed 4 components in the ultracentrifuge with $s_{20} = 7$, 18, 21, and 26 S (K5). The high degree of electrical homogeneity is unexpected in view of its molecular complexity; thereupon, Kratochvil and Deutsch (K5) discussed the possibility that the higher molecular weight portions are polymers of smaller molecules. Fluctuations of weight were also found in the macroglobulin of a patient with Waldenström's syndrome observed during 4 years of his illness (U1). Most cases of macroglobulinemia show an increase of the β_2-globulins; ImEl, being more precise, located it with the β_{2M}-globulins. Burtin *et al.* (B5) as well as Hartmann *et al.* (H3) believe that the increased fraction is immunologically identical with the physiological β_{2M}-globulin. With the Ouchterlony gel diffusion technique, Korngold and van Leeuven (K3, K4) were able to prove that highly purified pathological macroglobulins are antigenically related to normal γ_1-macroglobulin with $s_{20} = 49$ S as well as the γ-globulins with $s_{20} = 7$ S (Fig. 20).

Korngold and van Leeuven (K2) as well as Hässig *et al.* (H4) have stressed the point that the pathological macroglobulins differ among each other (individual specificity) and that they are antigenically deficient when compared with the normal macroglobulin. These results may illustrate what ImEl can establish if combined with further immunological techniques and ultracentrifugation (P2). Di Guglielmo *et al.* (D3) and also Mallarmé *et al.* (M1) have built these results into their clinical reviews on the macroglobulinemia of Waldenström.

10.7.3. *Myeloma and Bence Jones Protein*

Combining the results of moving boundary and paper electrophoresis, ultracentrifugation, and Ouchterlony gel plate technique, Korngold and Lipari (K1) characterized the antigenic structure of 24 cases of multiple myeloma; mobilities ranged from 0.7 to 3.4 $cm^2\ volt^{-1}\ sec^{-1} \times 10^{-5}$ and s_{20}-values from 6.1 to 12.0, with an average of 7.1. In contrast to such a considerable variation in physicochemical values, all the myeloma globulins cross-reacted with an antiserum against normal gamma-globulin, and there was clearly a possibility that the myeloma globulins are altered γ-globulins.

The same investigators studied the antigenic relationship between purified Bence Jones uroproteins, multiple myeloma globulins, and normal γ-globulin. All 18 Bence Jones proteins cross-reacted with anti-γ-globulin sera; they showed mobilities between 1.4 and 4.7 $cm^2\ volt^{-1}\ sec^{-1} \times 10^{-5}$ and s_{20}-values from 2.14 to 4.60. Bence Jones proteins were found to be antigenically more deficient than either myeloma globulins

or normal γ-globulins; Bence Jones proteins contain some of the antigenic determinants present in the homologous myeloma proteins but absent from normal γ-globulin.

In immunoelectropherograms Grabar *et al.* (G4) showed that of cases of multiple myeloma there is still one line of γ-globulins stretching, often, into the α_2-globulin range; but in the region of γ-globulin mobility the curvature is more pronounced locally and the line is much thicker than in normal serum. If the antihuman serum from the horse is first absorbed with pure γ-globulin, a secondary line is often found in the vicinity of the γ-globulin in myeloma sera (see Fig. 21).

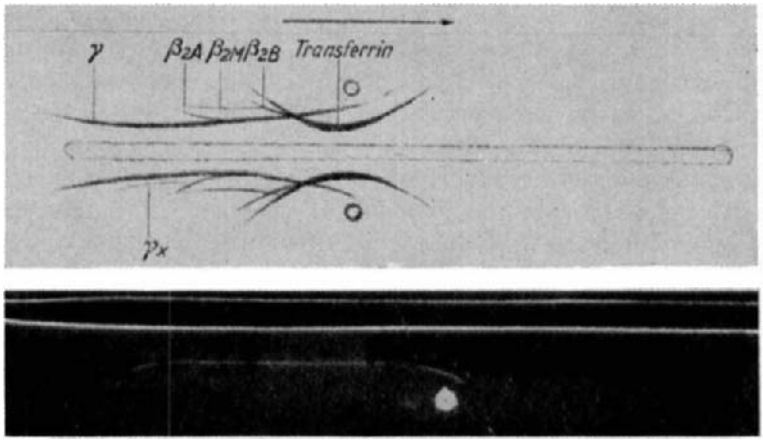

FIG. 21. Lines of the γ-globulin region after ImEl. Above, the newly found γ_x-line is only faint; below, the line of isolated γ_x-globulin begins right from the starting line. From Schultze *et al.* (S6).

This faint line of γ-protein (S6) is also found in cases of inflammation and in cancer; in comparison with normal γ-globulin, the γ_X-globulin contains more carbohydrates and has in consequence an increased solubility. γ-Globulin myelomas, which are the most frequent, show a very similar pattern of lines in ImEl (S4); if investigated with an adsorbed antiserum containing no γ-globulin antibody, it will be noted that the β_{2A}-line is often lacking or only very faint. In cases of myeloma, where the principally increased fraction shows an intermediate mobility between β- and γ-globulin, the ImEl with adsorbed antiserum often reveals a very strong line of β_{2A}-globulin. This applies also to the rare cases where simple electrophoresis shows greatly increased gradients of both β- and γ-globulin. As in myeloma sera with a pattern of β-, or in rare cases the α_2-globulin type, the antigenic structure can only be examined

with several absorbed antisera besides the generally used polyvalent antiserum. A classification of the divers types of myeloma by their antigenic structure was started by Heremans (H6), but more systematic investigations of this complex problem will be necessary. A preliminary survey seems to show that pathological alterations of the protein pattern are more marked in cases with an increase of β- or α_2-globulins than with

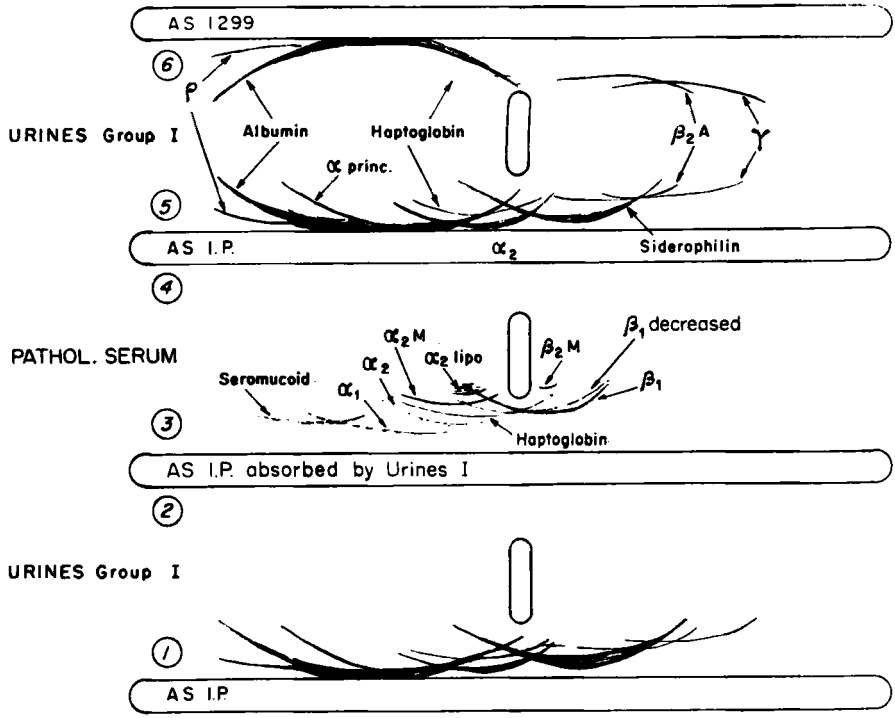

FIG. 22. Schematic drawing after ImEl of the lines of uroproteins of group 1 in comparison with the lines of the serum of the patient (middle section). From Patte *et al.* (P1).

γ-globulin. The ϱ-group is always lacking. With an antiserum against Bence Jones protein, ImEl often reveals traces of this uroprotein, which were not shown by simple electrophoresis or chemical fractionation (B4).

Analyzing 112 cases of *pathological proteinuria* with ImEl, Patte *et al.* (P1) stress the point that the use of an antinormal human serum, previously absorbed by the proteins of the urine, greatly facilitates the qualitative study of the proteinurias; it reduces the difficulty due to the quantitative variation in the excretion of each protein fraction from one subject to the other. They detected in normal urine, the ϱ-group, al-

bumin, acid seromucoid, some α_1 and α_2, siderophilin, β_{2A}, and the γ-globulins. Absent were some of the α_1 and α_2, two β-globulins (mol. wt. 150,000), macroglobulins α_2 and β_2, and the α- and β-lipoproteins (mol. wt. 200,000 and 1,300,000). The pathological proteinurias have been divided into group I, containing proteins with a composition qualitatively identical to that of normal urine, and group II, in which all the fractions are present (including the two β_1-globulins) except macroglobulins and lipoproteins. Variations of mobility were noted in many cases. Figure 22 gives the effective arrangement of parallel ImEl for the study of proteinurias, normal and pathological (P1).

Use is made of the polyvalent antiserum (Serpasteur) ASIP, another antiserum from the horse with the number AS 1299, and the former antiserum previously absorbed with the uroproteins of group I. Investigations with ImEl on the proteins of normal urines have been done by Grant (G7) and on myeloma proteins by Serre *et al.* (S10).

10.7.4. *Diverse Diseases*

Nephrosis is generally associated with a marked hypoalbuminemia, whereas the concentrations of the globulin fractions are variable. Cleve *et al.* (C2), who studied 12 sera from nephrotic patients with ImEl, were not able to establish a characteristic pattern. An increase of α_2-macroglobulin seems to be important in the cases of α_2-hyperglobulinemia, which are frequent. Both α_2- and β-lipoprotein are much increased, whereas transferrin is reduced and ceruloplasmin remains stationary. Thus, differences from the composition of a normal serum are of a quantitative order only; the same applies to the uroproteins (Fig. 23).

Rowe (R1) was able to prove that purified albumin prepared from nephrotic serum and urine had the same molecular weight as that from normal serum; he concludes that the increased excretion of albumin by nephrotic patients is not the result of a reduction of its molecular dimensions, but rather that the abnormality resides in the kidney. Ultrafiltration experiments showed (Fig. 23) that a selective molecular filtration may be reproduced by a suitable membrane *in vitro*. Rowe concludes that it is not necessary to invoke tubular activity to account for the composition of the uroproteins.

A constant hypoalbuminemia was found by Seligmann and Hanau (S8) in 30 cases of *lupus erythematosus*; α_2-globulins are frequently increased, whereas the content of γ-globulins is variable and goes parallel with the content of β_2-globulins. ImEl showed a constant decrease of β_1-globulins, especially the β_{1A}-globulin.

The different forms of leucoses and sarcomatoses have been inves-

tigated by Seligmann *et al.* (S9) with ImEl. They found marked variations in the magnitude of the β_2- and γ-globulin fractions from patient to patient; this nonuniformity seems to be a characteristic feature. Also, it was not possible to establish a clear correlation between the type of dysglobulinemia and the type of cells which are the basis of proliferation.

The report of Franklin *et al.* (F2) is interesting in relation to other instances of hyperglobulinemia, as in hepatitis and amyloidosis; they

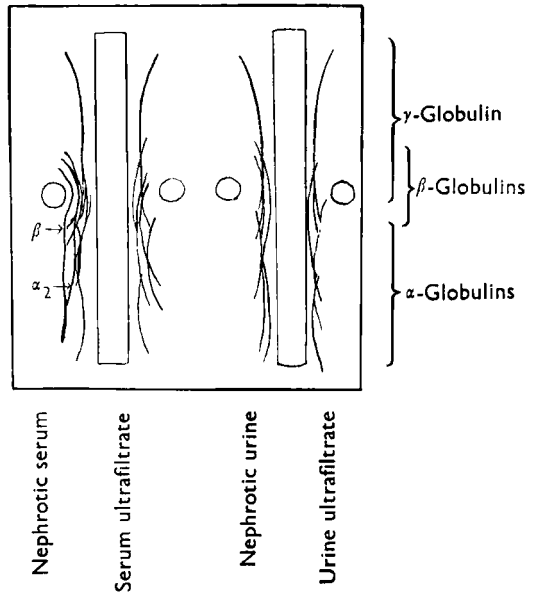

FIG. 23. A line diagram of an immunoelectrophoretic analysis of nephrotic serum and urine from case IV and the proteins appearing in their ultrafiltrates through a selective nitrocellulose membrane. Arrows indicate an α_2-globulin, present in high concentration in the serum and in low concentration in the ultrafiltrate and the urine, and the β-lipoprotein, apparently absent from the serum ultrafiltrate and the urine. The urine ultrafiltrate showed a relatively increased α_1-globulin concentration. From Rowe (R1).

showed the immunological differences between the 19 S and 7 S components of normal γ-globulin. In almost all liver diseases, cirrhosis included, the γ-globulin band is considerably broadened in electrophoresis, thus indicating a heterogenous group of globulins. ImEl proved that this phenomenon is not caused by a simple elevation of an intermediate fraction, such as H or T, or of γ_1-globulin, but by an interference of β_2-globulins (S1). Two subfractions of the β_2-group have antigenic groups identical with the γ-globulins. Whereas the content of β_{2A}- and β_{2M}-

globulins is generally increased in infectious hepatitis and cirrhosis, β_{1A}-globulin decreased in the former disease. However, these patterns are not characteristic etiologically, as other illnesses, such as infectious mononucleosis, chronic polyarthritis, and certain forms of sclerosis, have similar compositions of the globulin subfractions (B1, H2). In cases of hepatic disorders, flocculation tests are often used in a supplementary way; thymol turbidity or flocculation (Maclagan), cephalin/cholesterol flocculation (Hanger) and cadmium sulfate flocculation (Wunderly/Wuhrmann) have given good service as screening tests in the routine laboratory. ImEl will be a better tool than paper electrophoresis for elucidating the mechanism of these tests. Other nonspecific serum reactions, as used in cases of *rheumatism,* have been successfully investigated with ImEl. Scheiffarth *et al.* (S2) fractionated serum of patients with rheumatism in agar gel. Thereupon, they allowed streptolysin to diffuse into the gel and observed precipitation lines in the region of the α_2- and β_1-globulins. As these often increase in rheumatic diseases, the authors are doubtful whether the precipitates derive from a streptolysin-antistreptolysin reaction or an unspecific precipitation; Oudin and Ouchterlony tests were used for screening purposes in 126 cases. The identification of the C-reactive protein (CRP) by ImEl is not yet well established; most investigators believe it to be in the group of β-globulins, but the site changes in zone electrophoresis according to the carrier medium used.

Cleve (C3) found alterations of the α_1-, α_2-, and β_2-globulins with ImEl in active *rheumatic joint diseases,* but significant differences between acute and subacute rheumatic fever and rheumatic arthritis could not be established; proteins in sera and in synovial fluid showed serological identity. The author concludes that ImEl is not useful for clinical routine diagnosis of rheumatic disorders.

In certain cases of hyperglobulinemia, a protein precipitates from the serum if the latter is kept for some time at a temperature markedly below 37°C. Such *cryoglobulins* (E1) have been found mostly in cases of myeloma and macroglobulinemia. An excessive increase of one fraction often leads to a spontaneous precipitation in agar gel (C4). Most cryoglobulins observed in cases of myeloma are normal γ-globulin, but sometimes they contain β_2-macroglobulin or a mixture of β_{2M}- and γ-globulin (D2). Sladky *et al.* (S11) have described a case of essential cryoglobulinemia where the cryoglobulin had the mobility of a slow γ-globulin.

Summing up the results achieved with ImEl in clinical chemistry, it appears that the method is best used in cases where a protein com-

ponent is lacking or almost lacking (L4). In cases of severe dysproteinemia it is of great advantage to use ImEl in combination with the Ouchterlony gel plate, making use of several absorbed antisera. Thus, it is possible to establish the antigenic structure. It will only be possible to make full use of these immunological techniques in cases of special interest, but they do lead to a new characterization of protein anomalies which the sensitivity of the physicochemical methods of classic protein chemistry was insufficient to detect.

11. Biochemical Applications

11.1. Control of Protein Fractionation

The various stages of purification of a protein by salt or ethanol precipitation can be very well controlled with ImEl. It is necessary, of course, that the antiserum contain antibody against the protein which is being observed; in order to get optimal proportions, it is necessary to work with suitable dilutions. Nitschmann *et al.* (N1) removed the heat-labile globulins by complete desalting of the blood plasma with a mixture of cation and anion exchange resins. The goal was to obtain a plasma product which could be guaranteed free of hepatitis virus (Fig. 24).

The remaining solution, which has been termed PPL, withstands heating at 60°C for 10 hours and is still qualified as a substitute for plasma as far as oncotic and nutritive effects are concerned.

Stahmann *et al.* (S12) studied the antigenic action of synthetic polypeptides with ImEl. The latter were composed of leucine, phenylalanine, glutamic acid, and lysine; these polypeptides were coupled to a homologous protein, the serum albumin of the rabbit. From the pattern of the precipitate lines, it followed that antigenicity decreased in the following order: leucine > phenylalanine > glutamic acid > lysine.

Schultze and Schwick (S5) developed a quantitative immunological method. With suitable antisera of known titer, the following fractions were estimated in normal and pathological sera: ϱ-group, albumin, α_2-macroglobulin, ceruloplasmin, β_1-siderophilin, γ-globulin, fibrinogen, α_2-lipoprotein, and β-lipoprotein.

Figure 25 shows the result of ImEl of a protein mixture of seven isolated and highly purified serum protein fractions. Heide *et al.* (H5) controlled the purity of a γ-globulin ($s_{20} = 7$) with a low carbohydrate content. The group of γ-globulins was separated beforehand by zone electrophoresis in polyvinyl chloride; the Longsworth buffer had a pH of 8.6; $\tau/2 = 0.1$, and the field strength was 4.4 volts/cm.

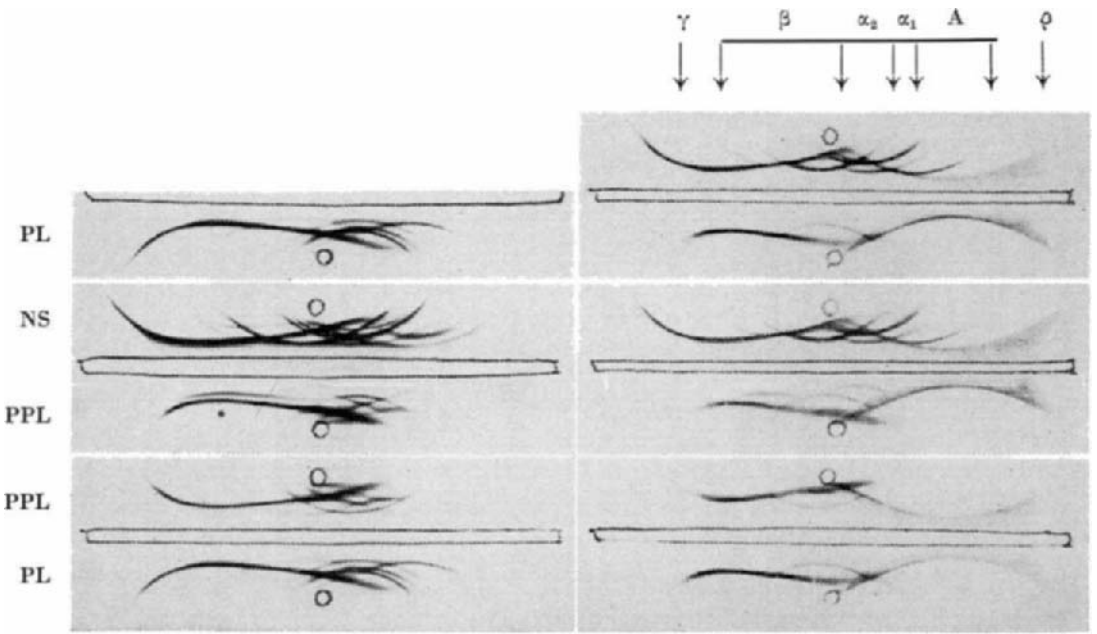

FIG. 24. Control by ImEl of the desalting of blood plasma. Such patterns (PL and PPL) are compared on the left with the lines of a normal serum. On the right side are the same sera but in a dilution of 1:4. From Nitschmann *et al.* (N1).

Vaux St. Cyr (V1) studied perchloric acid extracts of normal and of pathological sera. All of them contained seromucoid acid, and the latter sera contained in addition γ-globulin, β_{2A}-globulin, and albumin, but in variable amounts.

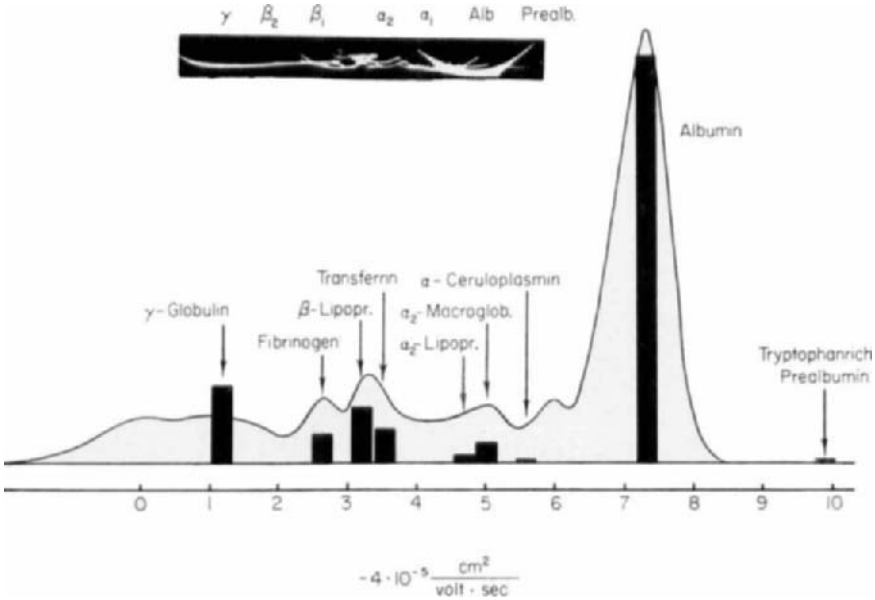

FIG. 25. ImEl of a model composition of a serum consisting of a mixture of 9 isolated and purified protein fractions (above). The quantitative amount of these fractions can be seen below. From Schultze and Schwick (S5).

11.2 CONTROL OF ENZYME ACTION (ENZYMOELECTROPHORESIS)

Lapresle (L1) made an early use of ImEl for identification of the degradation products of serum albumin by an extract of rabbit spleen. Upon the enzymatic hydrolysis at pH 3, several degradation products with different electrophoretic mobilities were obtained. Rabbit serum immunized with normal human serum albumin contains three different antibodies, corresponding to the three specific groupings of the albumin. In a later study, Lapresle and Durieux (L3) studied the antigenicity of degraded albumin with ImEl. Such products remain antigenic for the rabbit even if there is no native albumin left. The rabbit immune serum so obtained contains two different types of antibody: one, corresponding to the specific group of the native albumin; the other, to new specific groups unmasked by the enzymatic degradation. The "spontaneous" and enzymatic cleavage (proteolytic digestion) of human

γ-globulin was followed by Augustin and Hayward (A1) with ImEl. Hanson *et al.* (H1) studied the products of hydrolysis of human γ-globulin by crystalline trypsin and chymotrypsin; the duration of hydrolysis was 24 hours at 25°C and pH 8.2. ImEl has also been used for detecting antigenic impurities in insulin perparations (L2). Miller and Owen (M5) proved the existence with ImEl of insulin-binding antibodies. "Ionagar" from Oxo Ltd., London, was used as supporting medium and the insulin was labeled with I^{134}. The specific activity was 3.2 mC/mg. The self-decomposition of compounds labeled with radioactive isotopes, leading to the disintegration of unstable atomic nuclei, has been described by Bayly and Weigel (B2). Further studies lead to a better knowledge of the properdin-zymosan and properdin-inulin complex (S3).

In several papers, Wieme (W1) has given an account of enzymo-electrophoresis and its applications; the action of sorbitol dehydrogenase (SDH) and that of malate dehydrogenase (MDH) were studied, but foremost that of lactate dehydrogenase (LDH). The latter test is pointed out as a sensitive and selective investigation of actual parenchymal activity. Further applications of agar electrophoresis and ImEl have been made in virology (F1) and in experimental pathology (C1). It is clearly probable that the scope of applications where ImEl is used as a particularly valuable analytical tool will steadily expand.

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BIOCHEMICAL ASPECTS OF PARATHYROID FUNCTION AND OF HYPERPARATHYROIDISM

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1. Introduction

The parathyroid hormone regulates ionic calcium homeostasis in extracellular fluids by a direct action on the skeleton. It also promotes the renal excretion of phosphorus and produces other physiological effects which are not obviously related to its calcemic action.

Disorders of parathyroid function are relatively uncommon, which may explain why the isolation of the hormone, its estimation in body fluids, and elucidation of its mode of action have lagged behind corresponding

work on the secretions of the other endocrine glands. Recently, however, more attention has been paid to disorders of calcium metabolism in general and to the parathyroid glands in particular, and it now appears that solution of some of the major problems is imminent.

The present article reviews current knowledge of the properties and physiological effects of parathyroid hormone and compares the latter with the biochemical disturbances of clinical hyperparathyroidism. In the course of this review, the concentrations of calcium and phosphate in extracellular fluid are considered and related to the solubilities of the calcium phosphate salts and to the solubility of bone mineral itself.

The normal range of plasma or serum calcium concentration varies slightly with the method of estimation. The ranges are 9.0–11.4 mg/100 ml by the classic oxalate precipitation method (K1); 9.6–11.2 by flame photometry (M4); and 9.0–10.5 by chelation with EDTA in the presence on an indicator (author's observations). For the purpose of this article, it will be assumed that the normal calcium concentration in plasma is 10.0 mg/100 ml. The normal concentration in a plasma ultrafiltrate is about 6.5 mg/100 ml, of which about 6.0 mg is ionic (F1). The normal ionic calcium concentration in plasma and extracellular fluid is therefore about 1.5×10^{-3} mole/liter.

The serum concentration of inorganic phosphate is about 2.5–4.5 mg/100 ml in normal adults but varies slightly with age and sex (G7). It is frequently assumed that all the serum inorganic phosphate is ultrafiltrable (H10), but a number of reports have indicated that this is not the case (G3, M7), and work now in progress at various centers suggests that it is in fact about 90 % ultrafiltrable. There are three ionic species of inorganic phosphate. At pH 7.4, about 20 % exists as monovalent $H_2PO_4^-$; about 80 % as divalent HPO_4^{--} and about 7 ppm as trivalent PO_4^{3-}.

2. The Parathyroid Hormone

2.1. Physical Properties

For a long time very little progress was made with the purification and isolation of the parathyroid hormone; the acid extraction procedure of Collip (C11) provided the standard commercial extract for over 30 years.

The introduction of phenol extraction by Aurbach (A4) has enabled Rasmussen (R3) to produce a much more active principle with a potency of about 2500 units (USP)/mg dry weight when assayed by the method of Munson (M11). This material possesses both phosphaturic and calcium-mobilizing activities. Its minimum molecular weight, calculated from its amino acid composition, is about 8500. Rasmussen interprets his latest

data as indicating that the hormone exists in the gland as a protein having at least this molecular weight and that the greatest yields of this protein are obtained with phenol extraction. Fractions extracted with acid are fragments which are less active and extremely labile.

The purification of the parathyroid hormone represents a major advance which will undoubtedly have repercussions in every aspect of calcium metabolism.

2.2. Physiological Effects on Bone

2.2.1. *Local Effects*

Parathyroid implantation (B2) and tissue culture experiments (G1, G2) have shown that the secretion of the parathyroid glands exerts a direct action on bone which results in resorption and destruction. Injection of the hormone produces definite cellular changes within a few hours in the osteoblasts and osteocytes (H3). There is also an increase in the number of osteoclasts. It is impossible to say, however, whether bone mineral or organic matrix is primarily affected, as they appear to be mobilized simultaneously (C6, M5).

2.2.2. *Calcemic Action*

Experimental work has demonstrated the vital role of skeletal mineral in the homeostatic control of blood calcium (H2, C12, T2). The absolute calcium concentration at which homeostasis is preserved is governed, however, by the parathyroid glands. Experimental or accidental parathyroidectomy results in a fall of ionic calcium concentration from about 6.0 to about 3.5 mg/100 ml. It remains relatively steady at its new equilibrium value and returns to this value if it is artificially raised by intravenous infusion of calcium or lowered by infusion of EDTA or sodium citrate (C12, C13, S3). There is also a transient fall in plasma phosphate which is soon reversed by the fall in phosphate excretion (M12). Continuous infusion of parathyroid extract, 1.0 unit/kg body weight/hour, raises the plasma calcium of a parathyroidectomized dog to a near normal level within about 15 hours, and 0.1 unit/kg/hour holds it at this level (C13). The parathyroid hormone therefore appears to regulate the absolute calcium concentration at which equilibrium is established between tissue fluid and bone mineral.

It is some hours before injected parathyroid extract raises plasma calcium, and the parathyroid mechanism is therefore almost certainly too slow to govern small short-term changes in extracellular calcium. These are probably corrected before they are measurable by the migration of calcium into or out of bone mineral, but the parathyroids provide a slowly

adjustable mechanism to regulate this maintenance of serum calcium by the skeleton. (On the other hand, Sanderson *et al.* (S3) express a contrary view and believe that rapid control of plasma calcium is effected by the parathyroids.) This variable parathyroid action is superimposed on the fixed calcemic action of vitamin D, which appears to be similar to that of parathyroid hormone (C2, C16, N13, N14), without possessing its flexibility. It may be significant that fish which do not possess parathyroid glands have very large hepatic stores of vitamin D (B11, T6).

It is generally assumed that it is the concentration of ionic calcium in the extracellular fluid which the parathyroid glands control by this action on the skeleton and that the variations in total plasma calcium are strictly proportional. However, Freeman and Breen (F11) have shown that parathyroidectomy is followed by a rise in the proportion of protein-bound calcium and that this can be reduced by injection of the hormone. This accords with the observations of Lloyd and Rose in hyperparathyroidism (L6; see Section 4.2) and with the clinical observation that hypoparathyroid patients may develop tetany at surprisingly high levels of total plasma calcium. The effects of the hormone on acid-base balance may be concerned in this phenomenon since acidemia reduces the proportion of protein-bound calcium.

2.3. Physiological Effects on the Kidney

2.3.1. *Phosphate Excretion*

Parathyroidectomy is followed by a fall in urine phosphate excretion and a consequential rise in plasma inorganic phosphate concentration until a new equilibrium is established (T1). In clinical hypoparathyroidism, plasma inorganic phosphate may be as high as 10 mg/100 ml, but the amount of phosphorus excreted in the urine by such patients really depends upon dietary intake. In these cases, therefore, phosphate clearance is usually low and is always low when considered in relation to the plasma phosphate concentration (K4, N6, N11).

Intravenous injection of parathyroid extract is generally followed by an immediate phosphaturia (H6, B7, G4). Figure 1 shows that intravenous injection into 20 normal subjects of 2.5 ml of commercial parathyroid extract caused a mean rise of about 100 % in phosphate excretion (N12). The apparent rise in the proportion of divalent phosphate ion excreted is due to the simultaneous rise in urine pH.

The effect of acute injection of parathyroid extract on phosphate excretion is reported to be largely or wholly due to a rise in glomerular filtration rate (F3), but repeated injections produce a definite reduction in tubular resorption of phosphate (F4, H6). Experiments involving selec-

tive destruction of kidney tissue in dogs have suggested that parathyroid hormone promotes tubular secretion of phosphate rather than inhibiting tubular resorption (N3, N4). Stopflow studies, on the other hand, appear to refute this hypothesis (S2).

Variations in plasma phosphate concentration produced by parathyroid activity are often greater than can be accounted for by the changes in urinary excretion of phosphate. This had led to the suggestion that parathyroid hormone may facilitate the migration of phosphate into cells (F3). The evidence on this point is not conclusive, but increased labeling

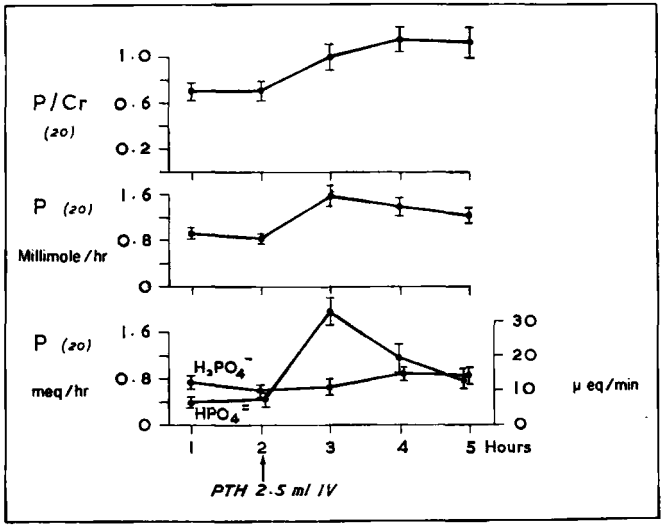

FIG. 1. Effect of 2.5 ml of commercial parathyroid extract (250 USP units) on urine phosphate excretion and the ratio of phosphorus to creatinine (P/Cr) in 20 subjects (mean values ± SE). Reproduced by permission of *Clinical Science*.

of phosphoproteins with P^{32} under the influence of parathyroid hormone has been reported (D6).

2.3.2. *Calcium Excretion*

The fall in serum calcium which follows parathyroidectomy is associated with a rise in calcium excretion (T1). Taken in conjunction with the hypocalcemic hypercalcuria of clinical hypoparathyroidism (L5), this suggests that the apparent calcuric effect of parathyroid extract is simply a reflection of its calcemic action. In fact, administration of the hormone appears if anything to reduce urinary calcium. Evidence to this effect has been produced by Bernstein *et al.* (B6), who have demonstrated a steep rise in calcium/inulin clearance ratio in dogs after parathyroidectomy

(Fig. 2). Similar results have been obtained in frogs (C14). Corresponding observations have been made in man by Kleeman *et al.* (K3), who have infused calcium with and without parathyroid extract into 4 normal subjects. There was a substantial reduction in the proportion of the infused calcium which appeared in the urine when it was given with parathyroid extract (Fig. 3). The rise in urine calcium usually seen after

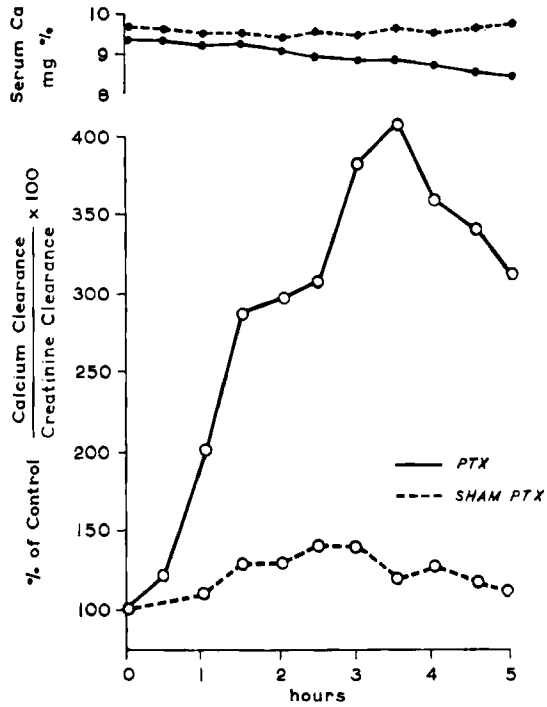

FIG. 2. Effect of parathyroidectomy on serum calcium and calcium/creatinine clearance ratio in a dog compared with the effect of sham operation [Bernstein *et al.* (B6)].

injection of parathyroid extract can therefore be regarded as secondary to the hypercalcemia produced by the hormone.

2.3.3. *Electrolyte Excretion*

Intravenous injection of parathyroid extract is followed by an immediate increase in the excretion of sodium, potassium, chloride, and bicarbonate (G4, N12). In 9 normal subjects there was a tenfold increase in urine bicarbonate and a corresponding rise in urine pH after injection of 2.5 ml of the commercial extract. Figure 4 shows that there was a fall

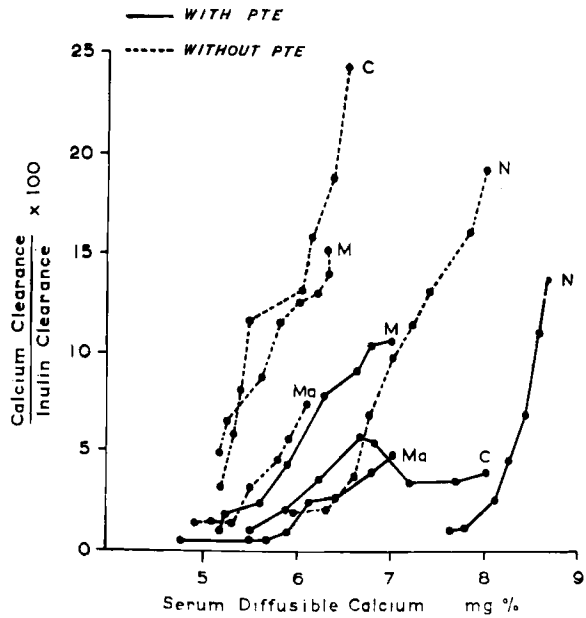

FIG. 3. Calcium/inulin clearance ratios during calcium infusions given with and without parathyroid extract in 4 normal subjects [Kleeman *et al.* (K3)].

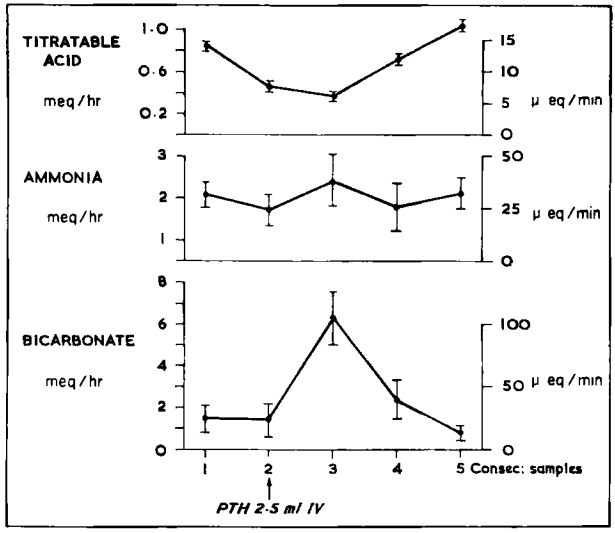

FIG. 4. Effect of 2.5 ml (250 USP units) of commercial parathyroid extract on titrable acid, ammonia, and bicarbonate excretion in 9 subjects (mean ± SE).

in titratable acid excretion without any change in ammonia output and therefore a net fall in hydrogen ion excretion in the urine. *In vitro* experiments have failed to demonstrate carbonic anhydrase inhibition by parathyroid extract (N12).

2.3.4. *Water Excretion*

Renal concentrating ability is reduced by parathyroid extract. This was first demonstrated in dogs (E6) and has been confirmed in man by Lambie and Robson (L2). Table 1 shows the effect of daily intramuscular injections of parathyroid extract on the tubular maximum water reabsorptive capacity. The two subjects were given 10 units of Pitressin prior to 20 hours of water deprivation, after which water reabsorptive capacity was measured during mannitol diuresis. There was a substantial reduction in water conservation after 6000 units had been given in 6 days. It is possible that this effect is the result of the hypercalcemia produced by the extract (B5, C4).

2.4. Physiological Effects on the Digestive Tract

2.4.1. *Gastric Secretion*

Injection of large doses of parathyroid extract (1000 units in 24 hours) increased gastric secretory volume and total pepsin in normal subjects (D8). This effect was not observed in rats (B8). It is believed that the rise in gastric secretion is due to hypercalcemia (L4) although parathyroidectomy in man does not consistently lower it (D7).

2.4.2. *Calcium Absorption*

Experiments with everted sacs of rat intestine have shown a fall in the transport of calcium-45 across the intestinal wall after thyroparathyroidectomy (D9) and after parathyroidectomy (R2), and it has been suggested that the hormone may promote calcium absorption. It is said that calcium is particularly well absorbed in hyperparathyroidism, but this may be a nonspecific response of the absorptive mechanism to hypercalcuria (J1).

2.5. Assay Procedures

Parathyroid hormone can be assayed by its phosphaturic effect in mice (D2), its calcemic effect in parathyroidectomized rats (D1, M11), its effect on serum calcium in the dog (U1), and its effect on the excretion of isotopic calcium or phosphate in the urine after the labeling of rats with Ca^{45} or P^{32} (C8, C9). None of these methods is particularly sensitive, and for this reason little work has been done on the estimation of parathyroid hormone in biological fluids. An attempt has been made to meas-

TABLE 1

EFFECT OF PARATHYROID EXTRACT ON RENAL POWER TO CONSERVE WATER[a]

Experimental conditions	Osmolal ratio U/P	$T^{C}_{H_2O}$ (ml/min)[b]	Plasma calcium (mg/100 ml)	24-Hour urinary calcium excretion (mg)	Creatinine clearance (ml/min)
Subject: A.F., 43 years					
Control	3.9, 4.0	6.7	9.1	218	99
Parathormone, 1000 units imi[c]/day, 3½ days	3.0, 2.4	5.3	12.5		92
Parathormone, 1000 units imi[c]/day, 6½ days	1.5, 1.4	2.9	11.7	498	98
Five days after stopping parathormone	2.2	4.5	10.0	235	120
Six months after stopping parathormone	3.2, 3.3	6.2	8.9	107	104
Subject: B.M., 55 years					
Control	3.1, 3.3	4.7	10.7	225	93
Parathormone 1000 units imi[c]/day, 6½ days	1.9, 2.0	3.2	11.9	490	117

[a] Data of Lambie and Robson (L2).

[b] Average of 6–9 clearance periods.

[c] By *imi* is meant intramuscular injection.

ure its excretion in urine. This suggested that the excretion is less than 100 units/day in normal subjects and considerably more in hyperparathyroidism (D3). Much work needs to be done in this field, and hormonal assay should certainly form the basis of selection of cases for parathyroidectomy in the future.

3. The Mechanism of Parathyroid Hormone Action

3.1. Alternative Hypotheses

There are two principal hypotheses to explain the action of parathyroid hormone on bone. The first is that of McLean and Urist (M6), who postulate a "feedback" mechanism. They suggest that the labile and accessible mineral of the skeleton can only sustain, by a physicochemical action, the hypoparathyroid plasma calcium concentration of about 6–7 mg/100 ml and that the high concentrations associated with normal parathyroid function are maintained by active resorption of mineral from a stable bone mineral pool into a labile pool. They therefore imply the existence of different mechanisms for supporting the hypoparathyroid and the normal concentrations of plasma calcium. The known association of osteoclasis with parathyroid activity tends to support this hypothesis, although the role of the osteoclast in bone resorption is controversial. Gaillard (G1, G2) has demonstrated stimulation of osteoclasis in embryonic bone incubated with parathyroid tissue, and Talmage finds the response of the osteoclast to parathyroid action so reliable that he has made it the basis of his assessment of parathyroid function (T3). However, the restoration of a depressed tissue fluid calcium concentration by bone is so rapid that it seems unlikely to be mediated by osteoclasts; it is much more suggestive of a state of physicochemical equilibrium. Moreover, although the tissue fluid ionic calcium concentration is low after parathyroidectomy, it is nonetheless maintained at this low level by the skeleton (C12, C13). It is clear, therefore, that mineral can be mobilized from the skeleton even in the absence of the parathyroids. The difference between the hypoparathyroid and the normal states is consequently more suggestive of a difference in degree than of a difference in kind.

The second hypothesis to explain parathyroid action is that a physicochemical steady state exists between bone mineral and the calcium and phosphate ions of the bathing fluids at all levels of parathyroid activity. On this view, the parathyroids determine by their effect on bone the calcium concentration at which bone and tissue fluid come into equilibrium (M3, N1, N8). Equilibrium studies with bone *in vitro* have shed some light on the plausibility of this hypothesis.

3.2. Bone Solubility *in Vitro*

Equilibrium studies with both dead and surviving bone have shown that bone mineral possesses a definable solubility. Powdered calf (N17) and human (M3) bone equilibrated for 24 hours in buffer solutions yield a reproducible "solubility product" in terms of calcium and phosphate concentrations whether the initial concentrations in the buffer are high or low. This product can be expressed in the terms $[Ca]^3[P]^2$*

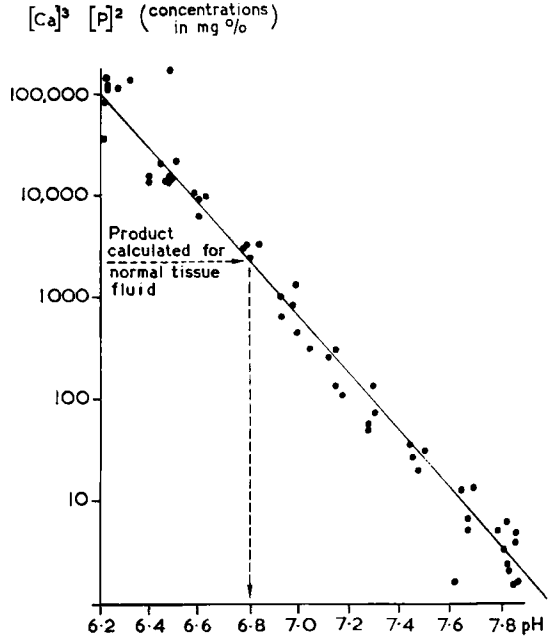

Fig. 5. Relationship between pH and $[Ca]^3[P]^2$ product in buffer solutions after 24-hour equilibration with powdered bone. At pH 6.8 the equilibrium product corresponds to that in normal tissue fluid. Reproduced by permission of the *Journal of Biological Chemistry* from MacGregor and Nordin (1960)(M3).

and at pH 7.4 is very much lower than the corresponding product in mammalian extracellular fluid. At pH 6.8, however, the *in vitro* product is similar to that in tissue fluid, as shown in Fig. 5. The implication is that bone mineral would maintain the tissue fluid concentrations of calcium and phosphate by a purely physicochemical equilibrium if the pH at the interface were 6.8. It is considered not impossible that this

* [Ca] is the concentration of free calcium in mg/100 ml; [P] is the concentration of inorganic phosphate in mg P/100 ml.

may be the pH within bone, particularly since intracellular pH is also about 6.8 (C1).

Surviving human bone chips reproduce at pH 7.4 higher concentrations of calcium and phosphate than dead bone (B4). The mean 24-hour product $[Ca]^3[P]^2$ is about 1500, which is only slightly lower than that in normal tissue fluid. In similar experiments with rat bone, prior treatment of the rats with parathyroid extract yielded a higher calcium concentration and parathyroidectomy a lower calcium concentration in the buffer at equilibrium (R1).

These experiments show that the "solubility" of bone mineral can be defined in terms of the concentrations of calcium and phosphate; that its "solubility" is governed by the constant value of the ion product $[Ca^{++}]^3[PO_4^{3-}]^2$; that the product $[Ca]^3[P]^2$ approximates the calculated tissue fluid product at pH 6.8; and that surviving bone can sustain a higher product than dead bone at pH 7.4. The results are summarized in Table 2 and compared with the values in typical mammalian extracellular fluid.

TABLE 2

CALCIUM AND PHOSPHATE CONCENTRATIONS AND THEIR ARITHMETICAL PRODUCTS IN SOLUTION AT EQUILIBRIUM WITH DEAD AND SURVIVING BONE AND IN TYPICAL HYPOPARATHYROID, EUPARATHYROID, AND HYPERPARATHYROID EXTRACELLULAR FLUID AND PLASMA[a]

System	Ionic concentrations (mg/100 ml)		Products in buffer solution and ECF		Equivalent products in plasma	
	[Ca]	[P]	[Ca] [P]	$[Ca]^3[P]^2$	[Ca] [P]	$[Ca]^3[P]^2$[b]
Dead bone, pH 7.4	4.4	0.6	2.5	26.4		
Dead bone, pH 6.8	16.0	0.7	13.6	2050		
Surviving bone, pH 7.4	5.5	3.0	16.5	1500		
Hypoparathyroid ECF	3.1	6.0	18.6	1070	31	5100
Euparathyroid	6.0	3.1	18.6	2130	31	9700
Hyperparathyroid	8.4	2.2	18.6	2900	31	11,500
"Calcium phosphate," pH 7.4				5000–10,000		25,000–50,000

[a] The suggested ceiling for the precipitation of "calcium phosphate" is also shown (Table 9).

[b] Including protein-bound calcium.

It will be seen from Table 2 that bone is more "soluble" in the hyperparathyroid than in the euparathyroid state if it is assumed that the relevant product to be considered is $[Ca]^3[P]^2$ rather than [Ca][P]. Some of the evidence for this assumption will be discussed in Section 4 below; at this stage, it is only necessary to observe that in all the bone

powder work the ion product $[Ca^{++}]^3[PO_4^{3-}]^2$ is more constant than $[Ca^{++}][HPO_4^{--}]$. Whatever salt bone mineral may be, it certainly is *not* secondary calcium phosphate. Its Ca/P ratio approximates that of tertiary calcium phosphate, $Ca_3(PO_4)_2$, but whether it is in fact this salt or—more probably—a form of hydroxyapatite is outside the scope of this discussion and in either case equally compatible with the assumptions made above.

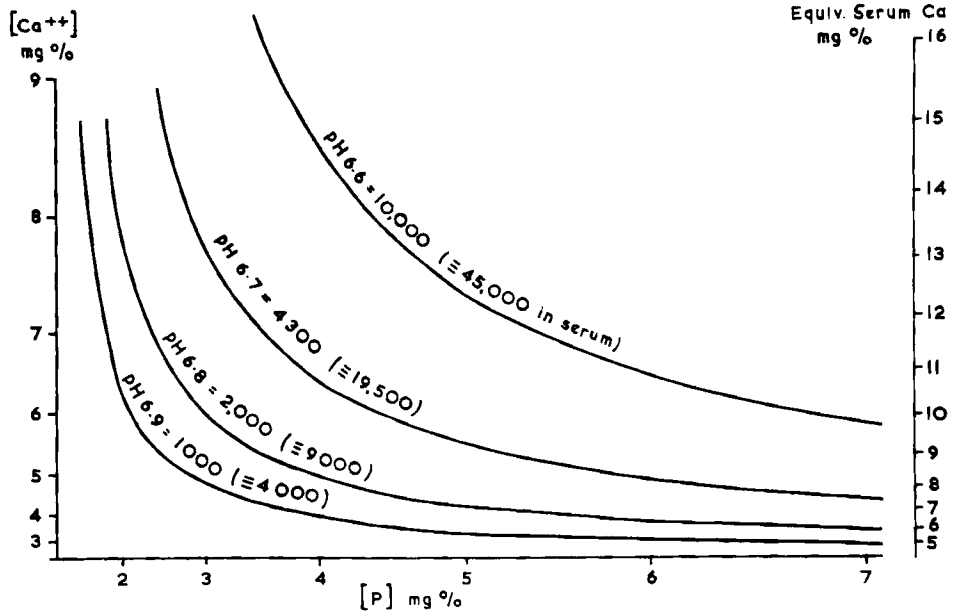

FIG. 6. Relationship between calcium and phosphate concentrations in buffer solutions of different pH's in equilibrium with dead bone. The curves are based on the $[Ca]^3$ $[P]^2$ values shown in Fig. 5 and indicate that at any given phosphorus level a small change in bone pH causes a large change in calcium concentration. The ordinate on the right takes into account the protein-binding of calcium in serum and indicates the approximate equivalent calcium concentration if the *in vitro* observations were applicable *in vivo*.

The bone solubility data are therefore compatible with the concept that the equilibrium between bone and tissue fluid mineral can be explained in terms of pH gradient. It is a natural extension of this hypothesis to suggest that the effect of parathyroid hormone on plasma calcium could be mediated by small alterations in pH at the surface of bone crystal. This concept is supported by the tendency of radioyttrium to seek resorbing bone surfaces (J2). Neuman (N2) has shown that this can be more easily explained if the pH at the resorbing surface is below

7.0. Figures 5 and 6 show that only very small changes in pH would be required to produce the range of calcium concentrations seen at different levels of parathyroid activity. How the hormone would regulate pH is not known; it has been suggested that citric acid (N1) or lactic acid (N17) might be the organic acid responsible, but no satisfactory evidence that parathyroid hormone causes accumulation of either of these acids has yet been forthcoming. A citric acid cycle certainly exists in bone (D7), which also contains a considerable amount of lactate (C5), but the evidence that parathyroid hormone increases the concentration of either in bone is unconvincing (N1). Parathyroid extract raises plasma citrate in hypoparathyroid animals but proportionately less than plasma calcium; and this effect on citrate is less marked than when vitamin D is administered (E2, E3). Parathyroidectomy causes a fall in plasma and a rise in urine citrate (K4), but these effects were abolished by prior adrenalectomy. Their significance in relation to bone resorption is obscure.

The role of the osteoclasts is also uncertain. It is not known whether they are concerned with mineral removal, matrix removal, or both processes, which appear to proceed *pari passu* (C6, M5).

3.3 The Physiological [Calcium] [Phosphate] Product

Bone solubility studies suggest that the equilibrium between tissue fluid calcium and phosphate and bone mineral, the crystals of which possess a very large surface area (E5), is a physicochemical one. This is compatible with the classic experiments of Hastings and Huggins (H2), amplified by the elegant work of Copp (C12, C13) and Talmage (T2), who have shown that the removal of ionic calcium from extracellular fluid is followed by its rapid replacement from bone mineral stores whatever the level of parathyroid activity. The intimate relationship between blood and bone calcium has also been confirmed by the use of isotopes which demonstrate rapid ionic exchange between tissue fluid and bone mineral (L1).

The relationship of the inorganic phosphate concentration to bone mineral is more complex. The normal range of plasma phosphate concentration is much larger than that of calcium, presumably because the phosphate concentration (unlike that of calcium) is influenced by dietary intake of phosphorus and by urinary excretion and because the parathyroids intervene to correct major changes in calcium concentration. The skeleton thus controls plasma calcium at a constant level but permits relatively large variations in plasma phosphate; there is therefore no constant [calcium] [phosphate] product in plasma or tissue fluid

which would represent a fixed "solubility product" of bone mineral, and no simple reciprocal relationship between the plasma levels of calcium and phosphorus in normal subjects. The range of normal plasma products of $[Ca]^3[P]^2$ extends from about 4500 to 27,500, as shown in Table 3.

It is a natural corollary of the arguments in the previous section to suggest that this variation represents the normal variation in bone solubility under the influence of physiological variations in parathyroid activity. Figure 6 shows that the normal range of the calcium and phosphate concentrations would represent a range of pH at the crystal surface of only about 0.1 pH unit.

TABLE 3

LOWER AND UPPER LIMITS OF [CALCIUM] [PHOSPHATE] IN NORMAL ADULT PLASMA

Limits	[Ca] mg%	[P] mg%	Products	
			[Ca] [P]	$[Ca]^3 [P]^2$
Lower limit	9.0	2.5	22.5	4550
Upper limit	11.0	4.5	49.5	27,500

The apparently reciprocal relationship between plasma calcium and phosphate which is seen in clinical practice is largely accounted for by the opposite effects of parathyroid activity upon the calcium and phosphate concentrations. Thus, hyperparathyroidism is associated with high calcium and low phosphate concentrations and hypoparathyroidism with the reverse; this is not a biochemical but a physiological reciprocity. A true biochemical or physicochemical reciprocity exists above the solubility product of tricalcium phosphate and presumably explains the irreversible depression of serum calcium by phosphate in renal failure (see Section 4.2).

No similar reciprocity can exist below the bone-mineralizing product where the tissue fluid must be undersaturated with respect to bone salt and inorganic calcium phosphate. Within the normal range of products, reciprocity with respect to bone as the solid phase is probably masked, as already explained, by varying bone solubility. However, if parathyroid activity regulates bone solubility, then states of fixed parathyroid activity, i.e., parathyroid tumor and hypoparathyroidism, might be associated with fixed bone solubility and therefore with a reciprocal relationship between calcium and phosphate concentrations.

In support of this suggestion, it is known that high phosphate feeding may produce tetany and that low phosphate feeding or Benemid administration may relieve it in hypoparathyroidism (P2). Conversely, Albright (A2) has demonstrated that in primary hyperparathyroidism

plasma calcium can be lowered by high phosphate feeding. Similarly, the data of Chambers *et al.* (C7) suggest a rise in serum calcium when patients with hyperparathyroidism are given a low-phosphorus diet. Thus there is a little evidence to suggest that states of fixed parathyroid function are associated with fixed bone "solubility," but the question clearly requires further study.

3.4. The Control of the Parathyroid Glands

The secretion of the parathyroid glands is probably controlled by the concentration of ionic calcium concentration in the tissue fluid. Artificial elevation of plasma calcium suppresses the parathyroids (H12, K5, N5), and parathyroid size varies inversely with the level of plasma calcium (H1, S6). Patt and Luckhardt (P3) demonstrated an increase in parathyroid hormone secretion in response to the perfusion of parathyroid glands with fluids of low calcium concentration. Talmage's osteoclast count, which is believed to measure parathyroid activity, rises in response to rapid removal of calcium from the extracellular fluid of intact rats (T2). The precise ionic calcium levels which stimulate and suppress the parathyroids are not known, but calcium infusions suggest that parathyroid suppression occurs when total plasma calcium rises above about 11 mg/100 ml. Stimulation probably occurs when the concentration falls below about 9 mg/100 ml.

The parathyroids are also stimulated by phosphate administration and by elevation of the plasma phosphate. Carnes *et al.* (C3) demonstrated a relationship between phosphorus intake and parathyroid volume in rats. Drake *et al.* (D10) produced parathyroid hyperplasia by parenteral phosphate administration in rabbits. Crawford *et al.* (C15) have shown that there is a fall in tubular reabsorption of phosphorus on high-phosphate diets, which they attribute to parathyroid stimulation. Their observations have been confirmed by Goldman and Bassett (G6). In Fig. 7, these two sets of data have been converted into the Phosphate Excretion Index (PEI) (N11) (see Section 4). The normal range of PEI is -0.09 to $+0.09$. High values are usually seen in hyper- and low values in hypoparathyroidism. Judged by this criterion, high phosphate feeding does in fact stimulate the parathyroids, since after the first 24 hours it causes an abnormally high PEI. This high PEI can be suppressed with calcium infusion, as can the high PEI of the secondary hyperparathyroidism of osteomalacia (N5).

It seems inherently improbable that the parathyroid glands would be sensitive both to depression of calcium and to elevation of phosphorus concentration and more likely that phosphorus stimulates the para-

thyroids by depressing ionic calcium. This has been shown to be the case, both by Ham *et al.* (H1) and by the careful work of Talmage in nephrectomized rats (T3). Talmage has shown that the rise in osteoclast count produced by nephrectomy can be prevented by elevation of serum calcium.

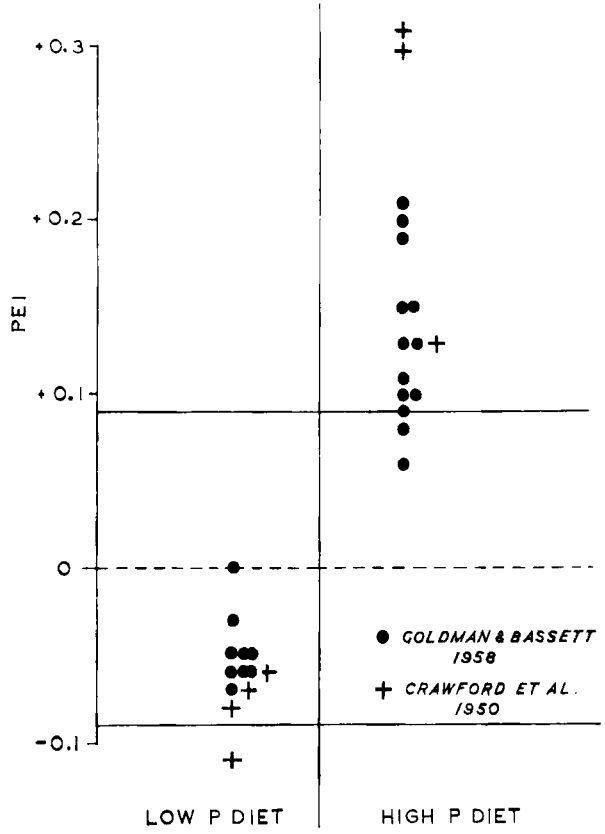

FIG. 7. Phosphate Excretion Index in subjects after more than 24 hours on low and high phosphorus diets.

It seems paradoxical that whereas high phosphate feeding stimulates the parathyroid glands, low calcium feeding does not do so in animals (C16, N14), nor do low-calcium diets raise the PEI in man (personal observation of the author). The probable explanation is summarized in Table 4, which should be studied in conjunction with Fig. 6. Primary depression of ionic calcium (as on a low-calcium diet) would cause undersaturation of tissue fluid and immediate shedding of bone mineral with restoration of calcium level. There is no reason to assume that this

requires *additional* parathyroid activity. Secondary depression of calcium concentration by elevation of phosphorus concentration, on the other hand, represents supersaturation of tissue fluid; shedding of bone mineral to restore the calcium level therefore requires an increase in "solubility," i.e., an increase in parathyroid activity. Similar considerations apply to the reverse changes. The implication is that tissue fluid phosphorus is the normal regulator of parathyroid activity, albeit via the concentration of ionic calcium.

TABLE 4

EFFECTS OF CHANGES IN TISSUE FLUID CALCIUM AND PHOSPHORUS CONCENTRATION ON BONE MINERAL AND ON PARATHYROID ACTIVITY

Primary change	Effect on tissue fluid in relation to bone salt	Effect on skeleton	Net result	Effect on parathyroid function
Fall in Ca conc.	Undersaturation	Shedding of bone mineral	Ca level restored, P level raised	No change
Rise in Ca conc.	Supersaturation	Formation of bone mineral	Ca level restored, P level depressed	No change
Fall in P conc.	Undersaturation	Shedding of bone mineral	P level restored, Ca level raised	Suppression
Rise in P conc.	Supersaturation	Formation of bone mineral	P level restored, Ca level depressed	Stimulation

It has been suggested that the parathyroid glands are under the control of the pituitary (E4, T10). This is not generally accepted, partly because hypocalcemia is a very rare complication of panhypopituitarism. This does not, however, exclude the possibility that the parathyroids, though largely autonomous, may also be influenced by the secretion of the pituitary, just as adrenal secretion of aldosterone appears to be partially influenced by the pituitary or hypothalamus (B3). Injection of human growth hormone causes a considerable increase in urine calcium (I1). Fraser and Harrison (F8) have suggested that this is due to stimulation of the parathyroids. In particular, they have shown that growth hormone does not increase calcium excretion in parathyroidectomized rats.

4. Primary Hyperparathyroidism

4.1. Introduction

Primary hyperparathyroidism is essentially a biochemical disturbance in the normal equilibrium between bone mineral and tissue fluid. It may remain undetected for many years before the development of complications [frequently, renal calculi (F2)] lead to its discovery, by which time it may be too late for effective treatment. It threatens health and even life, and early diagnosis and treatment are therefore of prime importance.

The responsibility for this diagnosis rests almost entirely on the clinical chemist, on whose findings and recommendation surgical exploration must be carried out. Until the hormone can be assayed easily and precisely in body fluids, the diagnosis will depend on the secondary biochemical disturbances which will now be described.

4.2. Hypercalcemia and Hypercalcuria

The most important single biochemical estimation in primary hyperparathyroidism is the concentration of calcium in the plasma. Since the elevation above the normal value may be very small indeed, it is essential that every laboratory should know with the greatest possible precision the upper limit of the normal range of plasma calcium by its own particular method. In appropriate cases plasma calcium should be estimated not once but three or four times, and any abnormal values should be regarded with the greatest suspicion (M2).

Various attempts have been made to improve on the estimation of plasma calcium by determining the ultrafiltrable or ionic calcium fractions (F1, H10, T9). Since it is almost certainly the tissue fluid ionic calcium which is controlled by the parathyroid hormone, and since about 90 % of the ultrafiltrable calcium is ionic, the estimation of ultrafiltrable calcium might be expected to be rather more valuable than the estimation of total plasma calcium of which some 30–40 % is protein-bound. This is probably correct, despite the numerous reports that total calcium is almost invariably directly proportional to ultrafiltrable calcium (H10). In borderline cases, or if there is a disturbance of plasma proteins or of acid-base balance, the measurement of ultrafiltrable calcium is desirable and the method should therefore be available in every laboratory. Recent studies indicating that parathyroid hormone reduces the protein-binding of calcium confirms this view (F10, L6). The procedure used may be mechanical ultrafiltration (H10, T9) or a very elegant venous occlusion method (G3). The exact upper limit of ultrafiltrable calcium concentration cannot be stated with precision because it will vary with the tech-

nique used for calcium estimation, but it is about 7 mg/100 ml. The Donnan effect and the concentrating effect of ultrafiltration are approximately equal and opposite and can be ignored.

It is doubtful whether the measurement of ionic calcium in the ultrafiltrate is called for at the present time other than for research purposes. The method devised by Rose (R9) is available, but although it represents a considerable contribution to calcium research, it is slow and laborious and the results obtained with it have confirmed that it offers little or no advantage over the much easier measurement of ultrafiltrable calcium.

The hypercalcemia of hyperparathyroidism may lead to hypercalcuria,

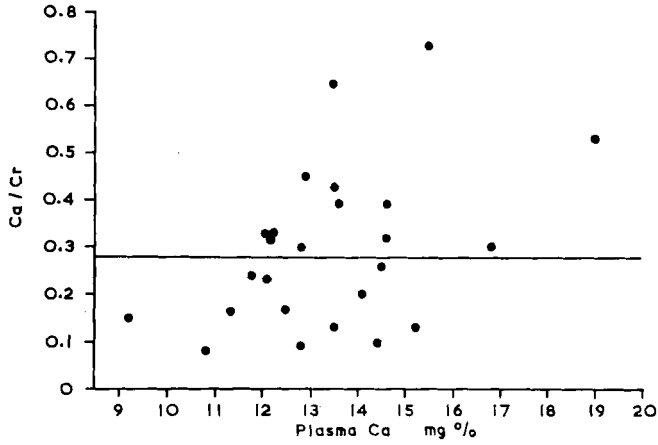

FIG. 8. Relationship between plasma calcium and calcium/creatinine ratio (N9) in cases of primary hyperparathyroidism (some of the data supplied by Dr. I. Anderson).

the urinary concentration of calcium being more or less proportional to the plasma calcium (Fig. 8). Frequently, however, urine calcium is normal, as might be expected from the action of the hormone on the kidney which tends to reduce calcium clearance (Figs. 2 and 3). This being the case, the determination of urine calcium in primary hyperparathyroidism, though of interest, is of limited diagnostic value, and its determination after a fixed period on a low-calcium diet is very seldom necessary. If calcium excretion is very high, a low-calcium diet will not restore it to normal, whereas if it is within the normal range nothing more will be learned by studying it on a low calcium intake. It is of interest, though of no known diagnostic value, that hypercalcuria, when present in primary hyperparathyroidism, can be suppressed by the daily administration of about 100 meq of alkali. Calcium excretion returns to normal within

a few days, although no convincing change has been seen in plasma calcium (personal observation). This is compatible with the concept that parathyroid hormone operates through an acid-base effect (see Section 3).

The tendency to renal calculus formation in hyperparathyroidism is not simply the result of hypercalcuria, since stone formation is rare in other conditions, such as hyperthyroidism, in which urine calcium may be much higher. It is more probably connected with the tendency to an alkaline urine (F5, N12), which, when combined with a rather high urine calcium concentration, predisposes to stone formation. The cases with the highest urine calcium are not necessarily those that form stones (N16).

4.3. Hypophosphatemia and Hyperphosphaturia

Hypophosphatemia is a less constant feature of primary hyperparathyroidism than hypercalcemia, despite the well-documented effect of parathyroid hormone upon phosphate excretion. Figure 9 shows the plasma calcium and phosphorus values in 346 cases of primary hyperparathyroidism diagnosed at the Mayo Clinic. It will be seen that the diagnosis was rarely made in the presence of a normal plasma calcium, although this occasionally has been done (M8), but was frequently made when the phosphorus concentration was normal. The data also suggest a slight inverse correlation between the plasma calcium and phosphorus concentrations; in cases with normal renal function the phosphorus was usually low when the calcium concentration exceeded 12 mg/100 ml.

A wide range of plasma phosphorus concentration has been observed by other workers in primary hyperparathyroidism (C7) and explained in terms of diet and renal excretion. Unlike the calcium concentration, which is normally very constant regardless of dietary intake and urinary excretion, the concentration of inorganic phosphate in plasma is the resultant of the rate of phosphorus absorption from the gut and protein catabolism, on the one hand, and of renal excretion, on the other. Although the parathyroid hormone promotes phosphorus excretion, this is only one of the factors governing plasma phosphate concentration. Plasma phosphate in cases of hyperparathyroidism on a relatively high phosphorus intake may therefore not be distinguishable from that in normal subjects on a lower intake.

Several phosphate excretion tests have been devised to assist in the assessment of parathyroid activity from the study of the renal excretion of phosphorus. All workers are agreed that plasma phosphate tends to be high and phosphate clearance low in hypoparathyroidism and that

the reverse is usually true in hyperparathyroidism (N11, R4, S4). False negatives and false positives may, however, be obtained when these determinations are used as a diagnostic test in primary hyperparathyroidism (M1, R5, T5).

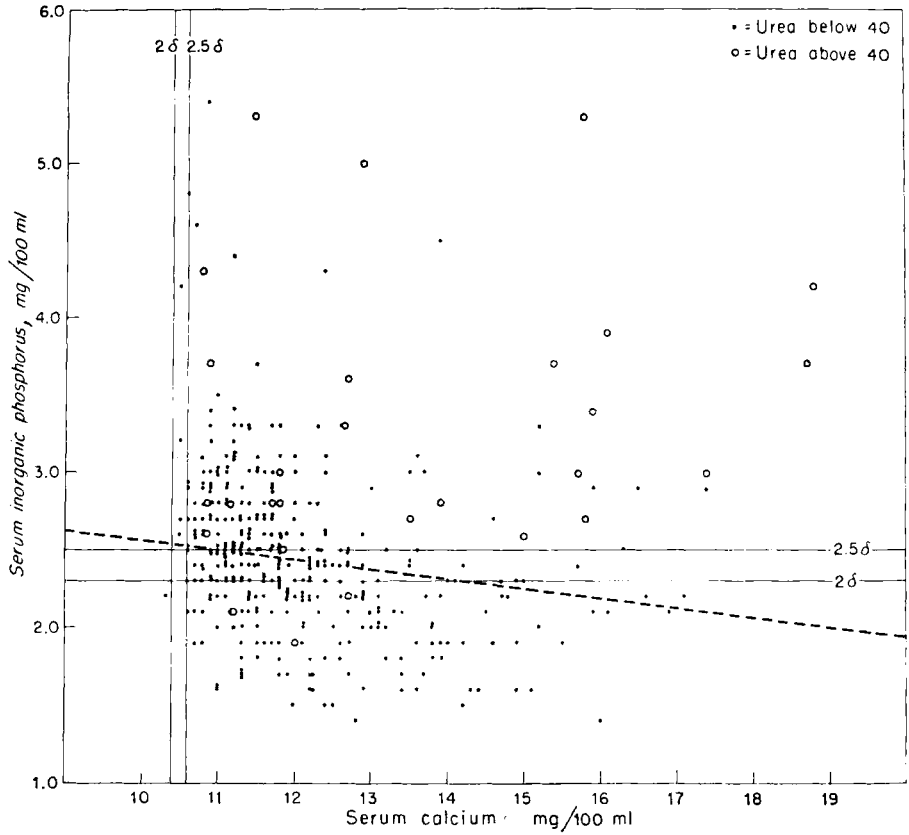

FIG. 9. Plasma calcium and plasma inorganic phosphate in 346 cases of primary hyperparathyroidism (data supplied by Dr. R. Keating).

Phosphate excretion tests can be classified as follows:

A. *The measurement of phosphate clearance* from a timed sample of urine and a simultaneous blood sample (K6). The normal range is reported as 6.3–15.5 ml/min. Higher and lower values were found in a series of cases of hyper- and hypoparathyroidism, respectively. The authors have made no attempt to correct their clearances to take into account the effect of plasma phosphate concentration upon phosphate clearance (see below).

B. *The measurement of the phosphate/creatinine clearance ratio* (C_p/C_{cr}) from the phosphate and creatinine concentrations in an untimed urine sample and simultaneous blood sample (N5). Tubular reabsorption of phosphate (TRP) can be derived from this figure ($TRP = 1 - C_p/C_{cr}$) and has been widely used as a measure of parathyroid activity (C7, R5, T5). Its normal range is reported as 78–90 %. The authors do not make any correction for plasma phosphate concentration.

C. *The measurement of the phosphate excretion index* (N11). Since the phosphate/creatinine clearance ratio is normally directly related in a known manner to plasma phosphate concentration (M10), the observed C_p/C_{cr} in any given case can be compared with the normal mean value at the same plasma concentration. The difference is called the Phosphate Excretion Index (PEI) and its normal range is —0.09 to +0.09. The values in hyperparathyroid cases usually lie above the upper normal limit and in hypoparathyroid cases below the lower limit (N11) (Fig. 10).

D. *Phosphate reabsorption per 100 ml of glomerular filtrate.* If the plasma phosphate concentration is multiplied by the phosphate/creatinine clearance ratio, an approximate figure is obtained for the amount of phosphate reabsorbed per 100 ml of glomerular filtrate (T7). The normal range of this value is approximately 2–4 mg/100 ml filtrate (N11).

E. *The theoretical phosphorus threshold* can be determined by phosphate infusion, but this is considerably more laborious than the other methods. The normal range is 2–4 mg/100 ml (H14).

F. *The maximum tubular reabsorptive capacity for phosphate* (Tm_P) can be determined by a more elaborate phosphate infusion procedure (A3, T8). The normal range is reported as 1.4–4.9 mg/min (R6). It has frequently been shown that the Tm_P is not reduced in primary hyperparathyroidism (T8, R6).

In considering the relative merits of these procedures, the last two can probably be excluded on the grounds that they do not appear to provide any additional information which would justify their greater complexity.

The determination of phosphate clearance is a very simple procedure which involves only phosphate estimations. However, it is almost certainly too crude to be useful. In a recent series (R5), it yielded very poor discrimination between hyperparathyroid and euparathyroid subjects (Fig. 11). It would probably be improved by the introduction of a correction for plasma level, but, even so, precision requires catheterization which is undesirable in a routine test.

C_p/C_{cr} and TRP are virtually interchangeable, except insofar as the derivation of the latter (C7) from the former (N5) by the formula

TRP $= 1 - C_p/C_{cr}$ depends on the assumption that creatinine clearance is a precise measure of glomerular filtration rate. This is an unjustified assumption (H8), and it is therefore probably wiser to use the term C_p/C_{cr}. Whichever term is used, discrimination between euparathyroid

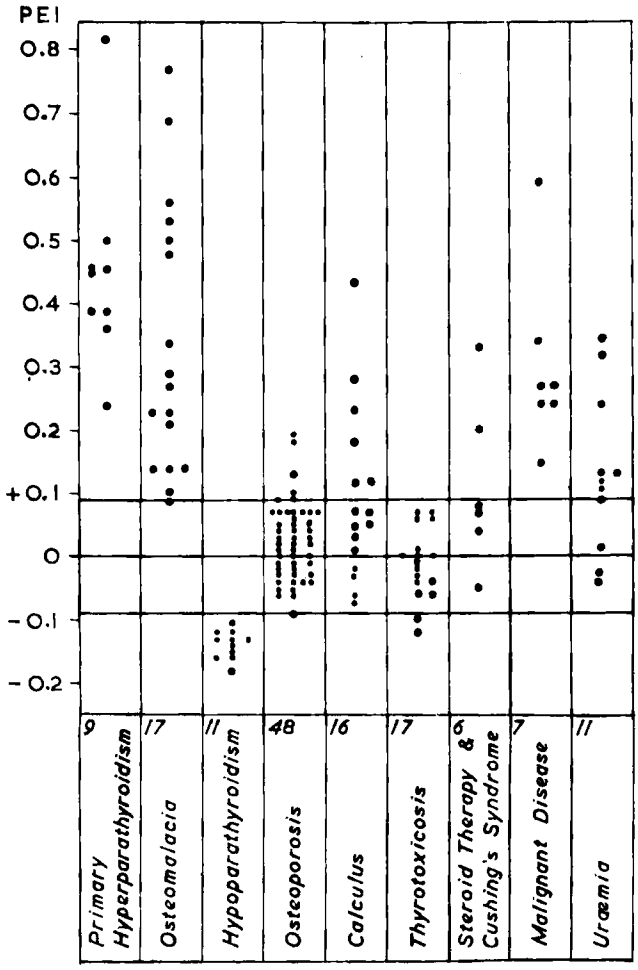

FIG. 10. Phosphate Excretion Indices in various diagnostic groups (N11). (Reproduced by permission of *Lancet*.)

and hyperparathyroid subjects on a free diet is relatively poor (Fig. 11). This is probably because the calculation includes no correction to allow for the normal dependence of renal phosphate clearance upon plasma phosphate concentration (M10). When such a correction is introduced

by calculating the PEI, some improvement in discrimination is obtained as shown in Figs. 10 and 11. Even with this calculation, however, complete discrimination is not always obtained and this may well be due to variations in the dietary intake of phosphorus.

To eliminate dietary variations, Chambers *et al.* (C7) have suggested that TRP should be measured after 6 days on a low-phosphorus diet and

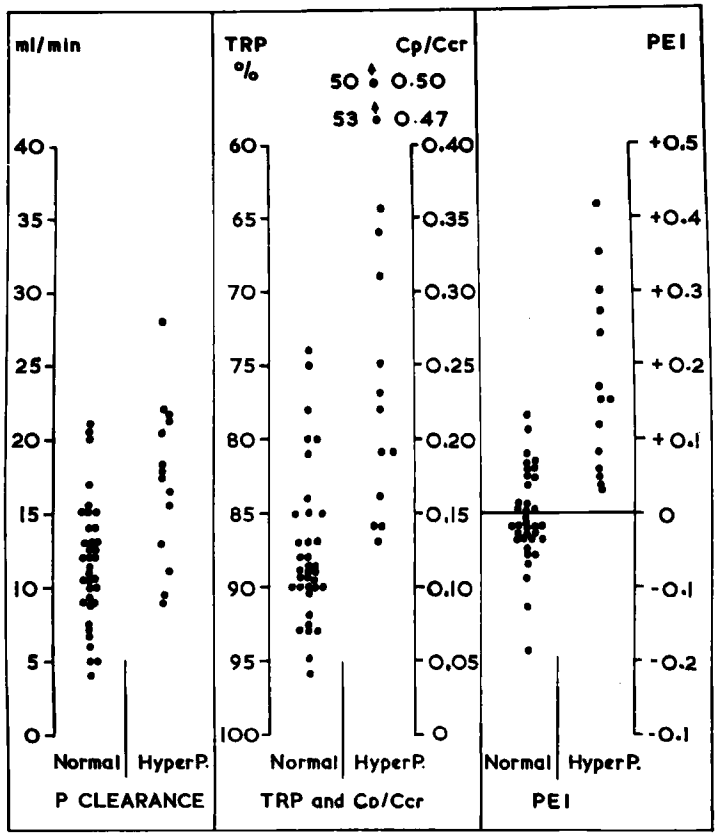

FIG. 11. Comparison of various indices of phosphate excretion applied to the data of normal and hyperparathyroid subjects of Reynolds *et al.* (R5).

aluminium hydroxide supplements. Objections to the TRP calculation have already been mentioned, but the dietary phosphorus restriction, which reduces parathyroid stimulation to a minimum in normal subjects (see Section 3.4), is probably a desirable refinement in the assessment of phosphate excretion. The PEI calculation is, however, almost certainly preferable to the TRP.

The recommended procedure for the assessment of phosphate excretion is therefore as follows:

The patient should be placed on a low-phosphorus diet with 20 ml of aluminium hydroxide suspension before and after meals for 3 days before the test. The test should be carried out fasting. A urine sample covering approximately a 2-hour period is collected as well as a blood sample obtained near the midpoint of the urine collection. Inorganic phosphate and creatinine are measured in plasma and urine, the plasma creatinine

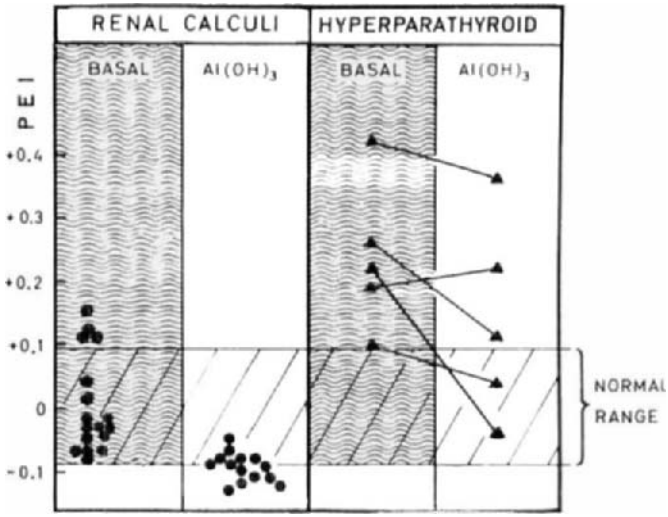

FIG. 12. Phosphate Excretion Index in cases of renal calculus and of hyperparathyroidism on basal diet and on aluminium hydroxide showing reduction in PEI and improved discrimination between the two groups [data supplied by R. Fraser (F9)].

by the method of Owen *et al.* (O1). The phosphate/creatinine clearance ratio is calculated from the formula:

$$C_p/C_{cr} = \frac{\text{urine P} \times \text{plasma Cr}}{\text{plasma P} \times \text{urine Cr}}$$

where P is phosphate and Cr is creatinine concentration in mg/100 ml.

The phosphate excretion index is calculated as follows:

$$\text{PEI} = C_p/C_{er} - (0.05 \times \text{plasma P}) + 0.07$$

This procedure and calculation have been applied by Fraser (F9) to normal subjects and cases of parathyroid tumor and renal stone with the improved discrimination shown in Fig. 12. Figure 13 shows the PEI and

serum calcium in 35 cases of primary hyperparathyroidism collected from three different sources. There is a highly significant correlation between these two determinations, which suggests that the PEI does reflect parathyroid activity.

Suppression of phosphorus excretion by infusion of calcium can also be used as a diagnostic test. It was first introduced for this purpose by

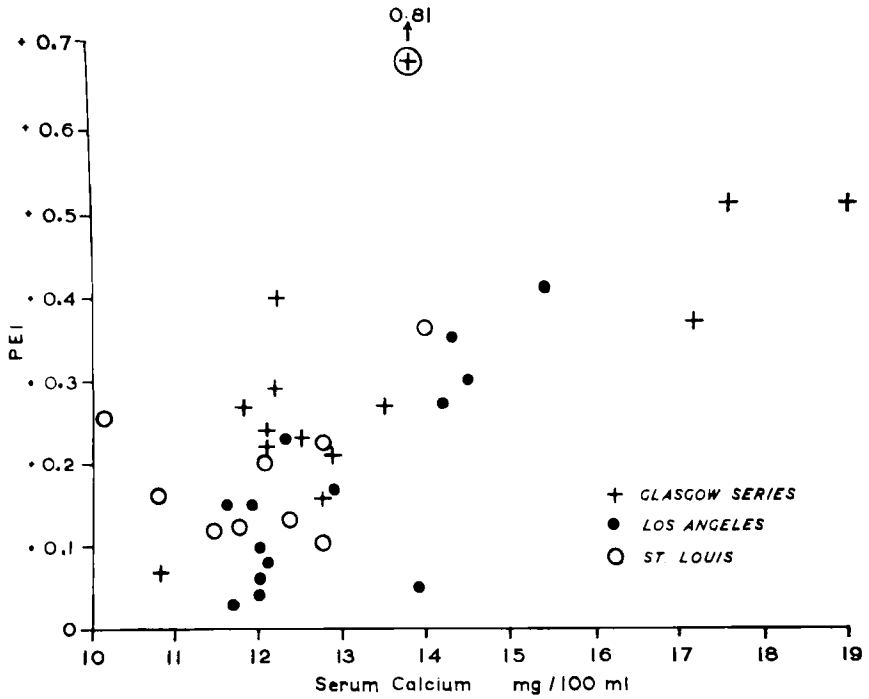

FIG. 13. Plasma calcium and Phosphate Excretion Index in 34 cases of primary hyperparathyroidism ($r = 0.65$; $p < 0.01$). (Circled case in renal failure and omitted from calculation.) [Including data of Reiss (R4) and Reynolds (R5).]

Howard *et al.* (H11, H12), who suggested that the rise in plasma phosphate produced by intravenous calcium was due to parathyroid suppression, and that this rise was smaller than normal in hyperparathyroidism. It has since been shown that the effect of intravenous calcium on plasma phosphate is not a measure of parathyroid activity (N5), and this may explain why calcium infusions have tended to fall into disfavor. The fall in urine phosphate which follows calcium infusion does, however, indicate parathyroid suppression (K5, N5). Table 5 shows the effect of a standard calcium infusion on the urinary phosphate/creatinine ratio

TABLE 5

EFFECT OF FOUR-HOUR INFUSION OF CALCIUM GLUCONATE[a] ON URINARY PHOSPHATE/CREATININE RATIO IN 14 NORMAL SUBJECTS (N5, N15)[b]

Time (hours)	Urine P/Cr	2 SE ±
—4–0	100	—
0–4	88.1	14.4
4–8	100.0	28.4
8–12	47.3	16.0
12–16	32.3	10.6
16–20	37.6	16.0
20–24	40.0	14.2

[a] 15 mg calcium/kg body weight at 0–4 hours.

[b] All values as per cent of starting value ± 2 standard errors (SE).

in 14 normal subjects, and Fig. 14 compares the mean normal values with those in three cases of parathyroid tumor. It will be seen that urine phosphate falls less in hyperparathyroidism than in normals but that the discrimination is most useful about 18 hours after the start of the in-

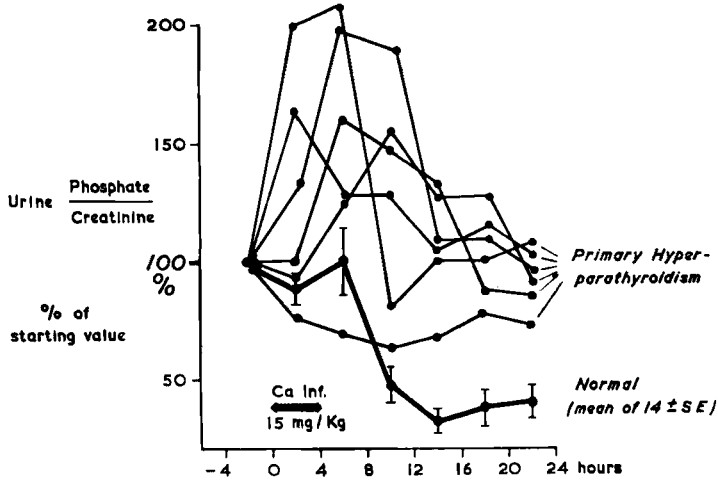

FIG. 14. Effect of calcium infusion upon urinary phosphate/creatinine ratio in six cases of primary hyperparathyroidism compared with same effect in 14 normal subjects.

fusion. This procedure may be helpful in suspected cases of hyperparathyroidism where hypercalcemia is slight or of doubtful significance, but its diagnostic value is very limited if the urine collections are made on a 24-hour basis (H12).

TABLE 6

RENAL POWER TO CONSERVE WATER BEFORE AND AFTER PARATHYROIDECTOMY

[Data of Robson (R8)]

	Before operation					After operation				
		Calcium				Calcium				
Patient	Creatinine clearance (ml/min)	Plasma (mg/100 ml)	Urine (mg/24-hrs)	Osmolal ratio U/P	TC_{H_2O} ml/min	Plasma (mg/100 ml)	Urine (mg/24-hrs)	Osmolal ratio U/P	TC_{H_2O} (ml/min)	Days post-operative
1	19	16.8	213	119	0	9.6	176	152	0	7
2	65	12.0	219	127	11	7.9	81	165	26	7
3	83	15.8	630	164	24	11.1	108	256	39	10
4	84	14.5	306	177	25	9.5	140	284	54	7

4.4. Polyuria

Polyuria is a recognized clinical feature of primary hyperparathyroidism (C10). It has been shown to be associated with a reduction in maximum tubular water reabsorptive capacity which cannot be explained by general impairment of renal function and which has been found to improve after parathyroidectomy, as shown in Table 6 (R8). Hellstrom (H4) has found that it is unusual for a case of primary hyperparathyroid-

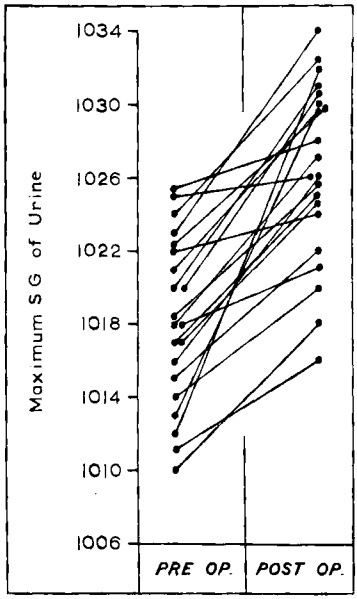

Fig. 15. Maximum specific gravity of urine in cases of parathyroid tumor before and after parathyroidectomy (calculated from data of Hellstrom, 1958). Reproduced by permission of *Postgraduate Medicine.*

ism to be able to concentrate urine to a higher specific gravity than 1022 and that improved concentrating power usually follows parathyroidectomy (Fig. 15).

4.5. Electrolyte and Other Disturbances

Patients with primary hyperparathyroidism tend to suffer from some degree of metabolic acidosis even when they are not uremic (T7). Fourman *et al.* have shown that failure of acidifying power may be a feature of primary hyperparathyroidism (F5); but if it is a specific effect of the hormone, it is surprising that this function does not always improve after operation. It is possible that it may be the result of structural damage

to the renal tubules rather than of parathyroid activity per se, or it may be the counterpart of the effect of the hormone on urine bicarbonate (N12).

Potassium depletion has been reported in hyperparathyroidism (B9). This would be expected if there was impaired hydrogen ion secretion into the renal tubule; such impairment may be associated with a compensatory increase in potassium secretion in exchange for sodium. Magnesium depletion has also been described, and parathyroidectomy is followed by magnesium retention (B1). It is thought that these effects may be the result of the action of the hormone on bone (F6).

Despite the postulated connection between parathyroid hormone activity and citric acid metabolism, plasma citrate concentration is normal in cases of hyperparathyroidism unless there is active bone disease present, in which case it may be raised (W1). Plasma alkaline phosphatase is also raised only in the presence of active osteitis fibrosa.

There have been very few measurements of bone formation rate in primary hyperparathyroidism. Bone turnover determined by the stable strontium test of Fraser *et al.* (F10) is reported to be high in cases of parathyroid tumor, but it is not known whether this is true of radioisotopic tracer tests.

There is a high incidence of peptic ulceration in primary hyperparathyroidism (H5, S1), but the cause of this is not established. Parathyroidectomy did not change gastric secretion in 4 out of 5 patients (D8), but did do so in one who had a duodenal ulcer. Four cases of hyperparathyroidism were identified in a series of 300 consecutive cases of peptic ulcer who were screened for this purpose (F7).

4.6. Differential Diagnosis

The diagnosis of primary hyperparathyroidism should not be made until the following causes of hypercalcemia have been excluded.

Sarcoidosis. It is reported that the hypercalcemia of sarcoidosis is invariably restored to normal by cortisone, 100 mg daily for 10 days, and that the hypercalcemia of primary hyperparathyroidism never responds to this therapy (B10, D5).

It is not known how far phosphate excretion studies can help to distinguish between the two conditions.

Malignancy. Hypercalcemia may occur in association with cancer without bone metastases (P4), and cases have been reported with a raised phosphate clearance (T7). This type of hypercalcemia does not usually respond to cortisone, but the hypercalcemia of myelomatosis may do so.

Vitamin D poisoning. Plasma phosphorus is high rather than low and plasma calcium returns to normal within a few weeks of withdrawing vitamin D.

Milk-alkali syndrome. The patients are always uremic. The hypercalcemia responds to withdrawal of milk and alkalis and the renal failure usually does the same (R7).

Hypercalcemia is occasionally seen in *hyperthyroidism* (K2) and in *adrenal deficiency* (L3). These conditions should present no diagnostic problem.

5. Secondary Hyperparathyroidism

5.1. Vitamin D Deficiency: the Low [Calcium] [Phosphate] Product

Vitamin D contributes to the calcemic action of the parathyroid hormone (C2, N14). Deficiency of vitamin D therefore results in a fall in ionic calcium concentration which stimulates the parathyroids. Parathyroid hypertrophy is seen in vitamin D-deficient animals (C3, H7, S7) and man (P1) and can be prevented by exposure to ultraviolet light (H7). A low plasma phosphorus concentration and high C_p/C_{cr} are characteristic of osteomalacia and rickets, and the PEI is of the same order as that in parathyroid tumor (N11) (Fig. 10). However, this high phosphate clearance, unlike that of parathyroid tumor, can be reduced to a normal or subnormal value by intravenous infusion of calcium (N5).

The effect of vitamin D deficiency upon plasma calcium is not primarily due to malabsorption of calcium from the gastrointestinal tract. In addition to its well-known effect on calcium absorption, vitamin D contributes directly to the maintenance of plasma calcium by the skeleton by a direct action on bone similar to that of parathyroid hormone (C2, C16). The vitamin D effect may be mediated by an action on the Krebs glycolytic cycle, resulting in inhibition of citrate oxidation (S6).

The combination of a reduced plasma calcium concentration (due to vitamin D deficiency) and a reduced plasma phosphate concentration (due to secondary hyperparathyroidism) produces a calcium $\times$ phosphate product below the threshold for calcification of new bone.

It has been known for 40 years (H13) that rickets develops in children when the plasma [Ca][P] product falls below about 30, and is either absent or healing when the plasma product exceeds 40. In adults the critical value of the product is about 20, below which level osteomalacia results. There are, however, exceptions to these general rules and, in particular, osteomalacia has been described in a case of postoperative hypoparathyroidism in which the plasma calcium was low but the plasma phosphate correspondingly high. The [Ca][P] product in this case was

apparently normal (A2). This observation is hard to explain in terms of a precipitation of secondary calcium phosphate; but if the material involved were related to tricalcium phosphate, then the reduction in plasma calcium concentration would not be offset by an equal rise in plasma phosphate concentration and a fall in the product would follow

TABLE 7A

CALCULATION OF RACHITIC PRODUCT $[Ca]^3\,[P]^2$ IN INFANT PLASMA [FROM DATA OF HOWLAND AND KRAMER (H13)]

Subject	[Ca] (mg/100 ml)	[P] (mg/100 ml)	$[Ca]^3\,[P]^2 \times 10^{-3}$
Untreated			
P.B.	4.9	5.0	3.2
G.B.	6.7	3.6	3.6
A.B.	7.2	3.6	3.7
W.B.	5.6	5.1	4.5
E.W.	6.2	5.5	8.0
Calcium-treated			
P.B.	8.0	3.0	4.5
W.B.	9.7	3.0	8.0
E.W.	8.5	3.5	8.0
G.B.	10.1	3.4	11
A.B.	9.5	3.8	12
Vitamin D-treated			
S.S.	8.7	4.7	15
E.V.	9.8	5.3	26
W.C.	9.8	6.1	33
B.F.	9.7	6.2	33

TABLE 7B

CALCULATION OF OSTEOMALACIC PLASMA PRODUCT $[Ca]^3\,[P]^2$ IN ADULTS [FROM DATA OF NORDIN AND FRASER (N7)]

Patient	[Ca] (mg/100 ml)	[P] (mg/100 ml)	$[Ca]^3\,[P]^2 \times 10^{-3}$
1	9.4	1.8	2.7
2	10.2	2.4	6.0
3	10.0	2.6	6.8
4	7.7	2.6	3.1
5	8.4	2.2	2.8
6	9.3	2.6	2.9
7	8.9	1.9	2.5
8	7.1	2.3	4.0
9	10.0	1.6	2.5
10	7.5	2.2	2.0
11	9.2	2.9	6.7
12	7.9	3.4	5.8

(Table 2). Recalculation of the original data of Howland and Kramer (H13) indicates that the lower limit of the normal $[Ca]^3[P]^2$ product in children is about 12,000 (Table 7A; Fig. 16). The corresponding lower limit in normal adult plasma is about 5000–6000 (Tables 3 and 7B; Fig. 16).

Although the actions of vitamin D and parathyroid hormone are very similar (C2, C16, N13), the latter is relatively more phosphaturic (A1).

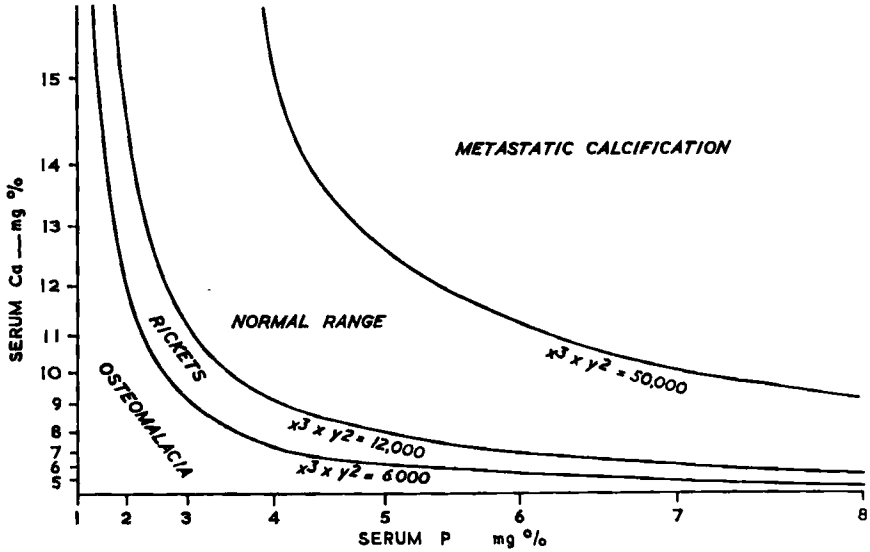

FIG. 16. Relationship between plasma calcium and phosphorus concentrations in osteomalacia, rickets, normal subjects, and renal failure. Reproduced by permission of *Clinical Endrocrinology*.

This may explain why secondary hyperparathyroidism causes rickets, whereas vitamin D cures it.

5.2. GLOMERULAR RENAL FAILURE: THE HIGH [CALCIUM][PHOSPHATE] PRODUCT

It is generally believed that the solubility product of some calcium phosphate salt must represent a ceiling above which the ion product of calcium and phosphate in the tissue fluid cannot rise without producing precipitation (H9). It is not certain whether the relevant solubility product to be considered is that of calcium acid phosphate, $CaHPO_4$, or that of tricalcium phosphate, $Ca_3(PO_4)_2$. Most workers have favored the former, but recent evidence rather suggests that the significant ion species is the trivalent form of phosphate. However, no really acceptable solu-

bility products exist for the more basic forms of calcium phosphate.

Talmage (T4) has determined the concentrations of calcium and phosphate in rat plasma after high-speed centrifugation. He has studied normal plasma and plasma to which calcium, phosphate and calcium orthophosphate had been added as well as plasma obtained after nephrectomy. There was a substantial variation in the final [Ca][P] products (82–120); high products were obtained when the plasma phosphate concentration was high, as after nephrectomy. However, when his data are recalculated as $[Ca]^3[P]^2$ as shown in Table 8, there is much less varia-

TABLE 8

[Ca] [P] AND $[Ca]^3[P]^2$ PRODUCTS IN CONTROL AND SUPERSATURATED RAT PLASMA AFTER HIGH-SPEED CENTRIFUGATION[a, b]

	Control	Added Ca	Added P	Added Ca and P	Nephrectomized
[Ca] mg. per 100 ml	11.3	15.0	9.7	11.4	8.0
[P] mg. per 100 ml	6.7	5.5	10.3	9.0	14.9
[Ca] × [P]	76	82	100	103	120
$pK_{i.p.}$ $[Ca^{++}] \times [HPO_4'']$	5.5	5.5	5.4	5.4	5.3
$[Ca]^3 \times [P]^2 \times 10^{-4}$	6.5	11	9.7	12	11
$pK_{i.p.}$ $[Ca]^3 \times [PO_4''']^2$	23.9	23.7	23.7	23.7	23.7

[a] Calculated from data of Talmage *et al.* (T4).

[b] pK-Values assume 60% of calcium and all phosphate is ionic.

tion, and the ion product $[Ca^{++}]^3[PO^{3-}_4]^2$ is the same in all four experimental groups of supersaturated plasma ($pK_{ip} = 23.7$). In contrast, the calculated ion product of secondary calcium phosphate in these experiments varies by a factor of almost 2 ($pK_{ip} = 5.3$–5.5) and is virtually the same as the initial value in rat plasma.

The precipitation of calcium phosphate in inorganic solutions by the addition of alkali (N10) also suggests that a tertiary rather than a secondary salt of calcium phosphate is the first to form at pH values greater than about 6.5. There is a relationship between the calcium and phosphate concentrations in ionic solution and the pH at which a precipitate first appears on the slow addition of alkali. The nature of this relationship is such that it can be explained on the basis of the dissociation of trivalent phosphate ion, but not of that of divalent phosphate ion. These data suggest that the negative logarithm of the solubility product of tricalcium phosphate lies between 23 and 24 (N10).

Finally, it has already been pointed out that bone itself does not appear to contain secondary calcium phosphate, which exists at pH levels lower than the physiological (< 6.2). The behavior of bone *in vitro* indicates that a more basic form akin to tricalcium phosphate or an

apatite is in direct equilibrium with the ions of calcium and phosphate in tissue fluid.

It therefore appears that the ceiling for the [calcium] [phosphate] product in extracellular fluid should correspond to the solubility product of tertiary rather than secondary calcium phosphate, i.e., that the ceiling can be expressed in the terms $[Ca]^3[P]^2$ rather than [Ca][P]. The difficulty remains of placing this ceiling in the absence of a reliable solubility product for tertiary calcium phosphate. A pK_{sp} of 23.1 (L7) would signify that plasma phosphate could rise to 20 mg/100 ml before causing metastatic calcification by precipitation of calcium phosphate (Table 9). This is not compatible with the facts (N8). The pK value of 23.7 derived from the work of Talmage (Table 8) would imply that precipitation would occur at a plasma phosphate concentration of 10.0 mg/100 ml, assuming that rat and human plasma phosphate are equally ultrafiltrable. The largest body of data on the reciprocal relationship between plasma calcium and phosphate in humans at the "ceiling" are those of Meroney and Herndon derived from cases of anuria (M9). Their work suggests that plasma calcium starts to fall when plasma phosphate exceeds about 5.0 mg/100 ml. This is, however, within the normal range of plasma phosphate in human infants and in many animal species and therefore seems too low. In the absence of the necessary data, it is thus suggested that the plasma phosphate "ceiling" is about 7.0 mg/100 ml at a plasma calcium of 10.0 mg/100 ml, making a $[Ca]^3[P]^2$ product of about 50,000 (Fig. 16). In adults, a plasma phosphate concentration as high as that is seen only in renal failure, in which metastatic calcification is a common feature. Generalized metastatic calcification is not seen in primary hyperparathyroidism without renal failure, even when plasma calcium is very high, presumably because the low plasma phosphate keeps the product below the critical value.

When the concentration of phosphate in plasma rises to the level where precipitation of calcium phosphate occurs, ionic calcium is depressed and parathyroid stimulation follows. This stimulation is continuous and progressive because the parathyroids are unable to restore the calcium concentration to normal, and enormous enlargement of the parathyroids follows (N8). Continuing parathyroid overactivity makes the bones more "soluble" than calcium phosphate at pH 7.4 with the result that there is a continuous transfer of mineral from the skeleton (which develops osteitis fibrosa) to blood vessel walls and soft tissues in general (which become calcified). Parathyroidectomy is the only logical treatment and this has been done by Stanbury *et al.* (S5).

The increased parathyroid activity in renal failure is reflected in a rise

TABLE 9

CALCULATION OF PLASMA PHOSPHATE CONCENTRATION FROM THE ION PRODUCT $[Ca^{++}]^3\,[PO_4^{3-}]^2$, ASSUMING A PLASMA CALCIUM CONCENTRATION OF 10 mg/100 ml

Calculation	Plasma Ca[b] (mg/100 ml)	Plasma ionized Ca (moles/liter)	$[Ca^{++}]^3$ (moles/liter)	pK_{ip} $[Ca^{++}]^3\,[PO_4^{3-}]^2$	$[PO_4^{3-}]^2$ (moles/liter)	$[PO_4^{3-}]$ (moles/liter)	Total [P] (moles/liter)	Total [P] (mg/100 ml)
A[a]	10.0	1.5×10^{-3}	3.4×10^{-9}	2.0×10^{-24}[c]	6×10^{-16}	2.4×10^{-8}	3.3×10^{-3}	10.0
B[a]	10.0	1.5×10^{-3}	3.4×10^{-9}	8.0×10^{-24}[d]	2.4×10^{-15}	4.9×10^{-8}	6.8×10^{-3}	21.0
C[a]	10.0	1.5×10^{-3}	3.4×10^{-9}	4.8×10^{-25}	1.4×10^{-16}	1.2×10^{-8}	1.6×10^{-3}	5.0[e]

[a] In A and B, the plasma [P] at the "ceiling" is calculated from the ion product; in C, the ion product is calculated from the plasma [P].

[b] Assuming a plasma Ca concentration of 10 mg/100 ml, the concentrations in the other columns follow.

[c] Talmage (T4).

[d] Logan and Taylor (L7).

[e] Meroney and Herndon (M9).

in PEI and in the development of osteitis fibrosa (N8). These signs of parathyroid overactivity may appear before the plasma phosphate has risen to 7.0 or even 5.0 mg/100 ml. The mechanism suggested in Section 3.4 for the stimulation of the parathyroids by the reciprocal behavior of calcium and phosphate in relation to bone mineral as the solid phase probably explains why parathyroid stimulation may occur before the critical $[Ca]^3[P]^2$ ceiling is reached and so before metastatic calcification begins.

5.3 Secondary Hyperparathyroidism with Hypercalcemia

Very occasionally, patients are seen in whom hypercalcemia is apparently due to secondary hyperparathyroidism. This is in a sense a contradiction in terms, since the essence of secondary hyperparathyroidism is the continuous stimulation of the parathyroid glands by a reduced ionic calcium concentration. However, at least two such cases with steatorrhea have now been described (D4, E1), and in each case a parathyroid adenoma was discovered. The implication is that parathyroid stimulation can in certain circumstances go on to adenoma formation; this also occurs occasionally in prolonged renal failure (G5).

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ULTRAMICRO METHODS

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1. Introduction

In the past 10 years the need for clinical chemical methods which require minimal quantities of body fluids has become increasingly urgent.

The three main stimuli for the development of these ultramicro methods are:

(a) difficulties encountered in obtaining enough material for chemical analysis in pediatrics;

(b) the increasing necessity for determining several constituents in one sample;

(c) the desirability of replacing venipuncture by fingerprick.

The micromethods in use in clinical chemistry require as a rule samples of the order of 0.1 ml. Ultramicro techniques should use samples of the order of 0.01 ml (10 µl). This is not always possible, e.g., the determination of serum iron- or protein-bound iodine requires much more material.

It is obvious that the development of ultramicro methods depends almost entirely on the development of apparatus which can handle minute quantities of samples, solutions, etc. In most cases the methods already in use in microanalysis can be adapted to an ultramicro scale.

The main purpose of this article is therefore to give a review of the most important apparatus necessary for the performance of ultramicro chemical analysis.

2. General Procedures

2.1. Collection of Material and Centrifuging

Although this detail of the work is only partly dependent on specialized apparatus and is described in various handbooks, it seems wise to give an outline of the possibilities of modern techniques, emphasizing some new ideas. Collecting "material" usually means collecting blood. For microtechniques two drops is enough for each analysis, so cumbersome venipunctures can be replaced by earlobe, heel-, or fingertip prick; the differences in composition between venous and capillary blood are very small for the majority of constituents (K1).

The side of the finger or the heel is commonly used. Massaging, a warm bath, or waving of arm or leg is allowed, but it is advisable not to exert too much pressure when the blood is not flowing freely (V3). The blood is taken up in a tube with a small bore by capillary force or by gentle suction. Glass tubes may be lined with heparin. After filling they are sealed by heat, with sealing wax, or with plastic caps. Collecting may also be done directly in small polyethylene centrifuge tubes, providing the inner diameter is not too small: after first snapping down a small drop so that the wall is wetted, more blood will flow in freely when the tube touches the drop on the finger.

Centrifuging of glass tubes may be done in any ordinary laboratory centrifuge which is capable of giving 5000 $\times$ g. Polyethylene tubes may be centrifuged in the same apparatus after folding the tube once. Modern plastic microcentrifuge tubes mostly contain about 400 µl blood, which means 200 µl serum, enough for 5 to 10 determinations. They are spun in specially developed centrifuges which accommodate about 20 to 25 tubes. Some have small holders for 5 tubes which are easily slid in and out of their places. Speeds are from 10,000 to 15,000 rpm. which

gives enough centrifugal force to separate erythrocytes or a protein precipitate in only a few minutes.

2.2. Pipetting

A great variety of ultramicropipets are commercially available at the moment. Glass pipets are generally washout pipets. Among the older

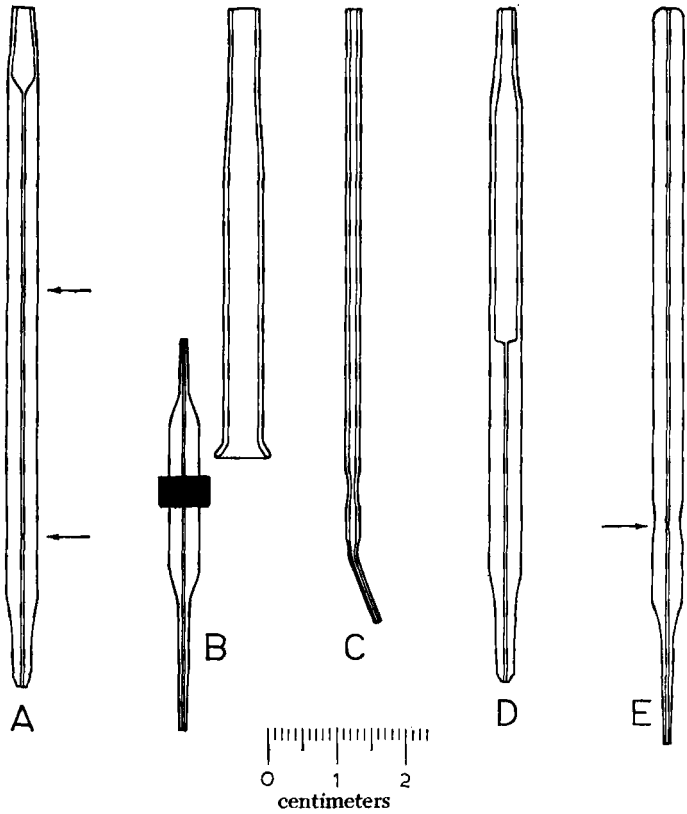

Fig. 1. Ultramicro pipets, self-zeroing. A, constriction pipet to deliver from mark to mark (N2); B, overflow type showing separate holder and rubber ring to connect holder to pipet; C, constriction pipet (Lang-Levy); D, capillary rise pipet (ABA-type); E, constriction pipet washout (N2).

types, the Sahli pipet is the best known. Filling these pipets exactly to the calibration line is time consuming and introduces the chance of making errors. This trouble is partly overcome in the self-zeroing pipets or pipets with an automatic stop, overflow pipets and constriction pipets (Fig. 1) (N2, K3).

Sahli pipets combined with a diluting chamber are sometimes convenient when the dilution can be made with one reagent and no other measured transfers are necessary. A modification of this dilution pipet, with detachable chamber, allows the precipitation of protein followed by centrifugation in one and the same apparatus (H1). Seligson (S7) introduced a sample pipet which is connected to a stopcock (Fig. 2). Washing out is done with a predetermined amount of liquid after turning the stopcock.

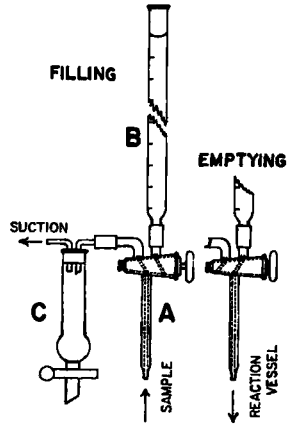

FIG. 2. Diagram illustrating the significant features of the automatic pipet: A, tip of the pipet and 3-way stopcock; B, calibrated buret; C, waste-receiver (S7).

Though these devices make sampling somewhat more easy, working with "washout" pipets is as a rule very time consuming and not suited for the routine laboratory. Moreover, dilution is often not wanted (for instance, when the light absorption of the resulting solution is to be measured).

It is therefore preferable for routine work to use pipets which are calibrated "to deliver" (C3). When these pipets are pre-rinsed with the solution to be measured and when the same pipet is used for the standard solution as well as for the unknown, it is not necessary to know exactly the delivered volume. This mode of working appreciably increases the accuracy and the number of determinations per hour. However, the necessity of rinsing makes it uneconomical with material and introduces an (sometimes neglected) error of different retention for different liquids. Siliconizing eliminates these drawbacks, but keeping pipets well siliconized is time consuming (D1).

For dispensing reagents the same type of pipets may be used, but for serial work a semiautomatic pipet according to Linderstrøm-Lang and

Holter (L5) is better, the reproducibility being 0.1% for a 30 µl pipet (Fig. 3).

The majority of difficulties encountered in the use of glass pipets of any form is eliminated by the use of the polyethylene pipet introduced

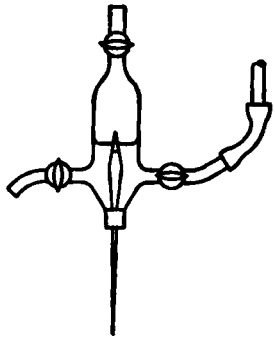

FIG. 3. Reagent pipet of Linderstrøm-Lang and Holter (K3).

by Sanz (S2) which is in fact a modification of the Linderstrøm-Lang and Holter pipet. This pipet consists of a piece of polyethylene tubing, drawn out at both ends to give fine, flexible tips, and fastened in a glass bulb by means of a small silicon rubber stopper. This bulb is

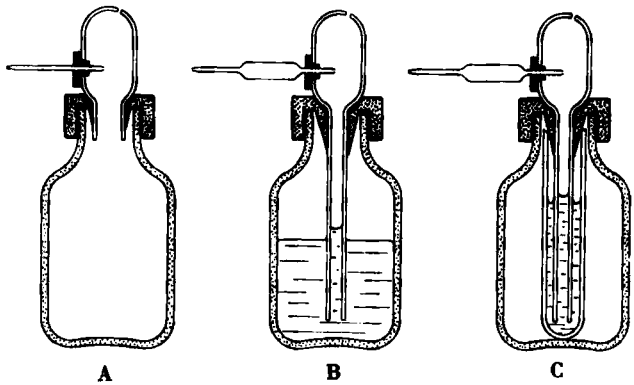

FIG. 4. Ultramicro pipets according to Sanz (S3). A, sample pipet; B, reagent pipet; C, reagent pipet for corrosive liquids.

mounted on a small polyethylene bottle which contains a reagent or, when used as a sampling pipet, collects overflowing material (Fig. 4). Manipulating these pipets is simple and easily learned, even by unskilled personnel. With some care the reproducibility is very good, the standard deviation for a 20 µl pipet in routine use being less than 0.3%.

Temperatures in the range 15°–35°C have no influence on the delivery, due to the very small differences in expansion coefficients between polyethylene and water (Sanz, private communication). These pipets may be used uncalibrated because the unknown sample as well as the standard solution is measured with the same pipet. Owing to the hydrophobic character of polyethylene the holdback of these pipets is negligible, provided they are emptied slowly.

Construction of these pipets can be done by every laboratory technician; the drawing of the polyethylene tubing requires only a little practice. Care should be taken that the tips have the right diameter and wall thickness, that the tubing is as clear as possible, and that mounting is done with a piece of clear silicon rubber. (Occasionally an air bubble will adhere on the inner surface and this should be clearly visible.) In the writers' experience handmade pipets are far superior to the mechanically made pipets which are commercially available. Calibration (after 2 weeks for shrinking) can be done by weighing with water on a good microbalance, while the adjustment to a special volume is easily performed by cutting small bits off the tip. For cleaning pipets, used for sampling serum, Alconox or pepsin-HCl solution give good results.

2.3. Titration

The titration of small volumes requires special burets. Modified stopcock burets have been described for use in the microliter range, but the accuracy is never greater than ± 1 µl (P4). Manipulation is very difficult and not suited for the routine laboratory.

Most types of ultramicro burets belong to one of two classes: (a) the capillary burets and (b) displacement burets. The best known of the capillary burets is the Rehberg buret (R1). Here the solution is expelled by a column of mercury which is controlled by a screw-operated plunger. Reading is done by measuring the displacement of the meniscus of the mercury in the calibrated capillary, 1 mm of the capillary equaling about 0.5 µl (Fig. 5). This type of buret is very reliable, although it has some disadvantages: it is fragile, bulky, has to be filled frequently, and the titrating solution is in continuous contact with mercury.

Several burets have been developed where the flow from the tip is controlled by surface tension: Schwarz (S6), Benedetti-Pichler (B2), Kirk (K3), and Pécar (P1). The main disadvantage is that delivery speed is very low due to a (necessarily) very narrow tip. Conway (C6) applies a small hydrostatic pressure to expel the solution from the cap-

illary. Flow is started and stopped by a stopcock (Fig. 6). This buret too is rather bulky; however, it has been successfully applied by many workers.

A modified Rehberg-buret has been developed by Kirk (K3) in which a number of disadvantages of the original buret have been elim-

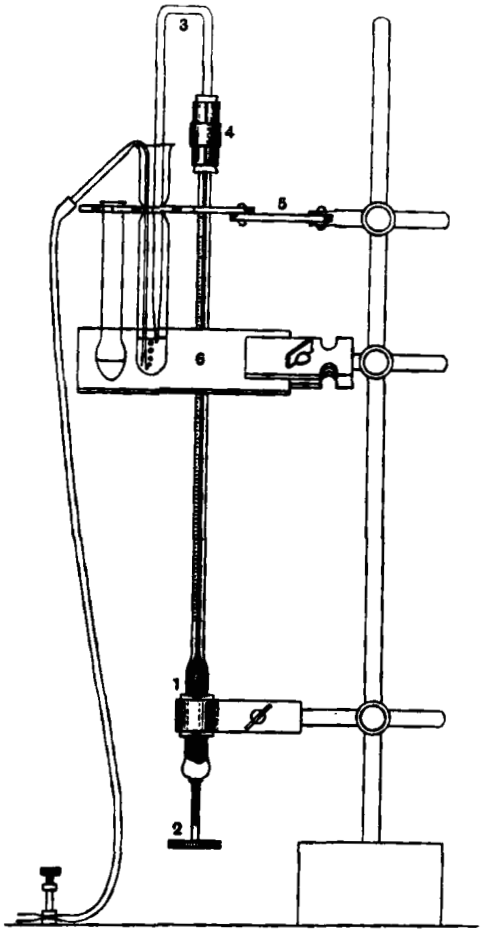

FIG. 5. Rehberg buret (R1).

inated. The form has been adapted to mounting on a stand which makes it more flexible so that it is possible to bring the tip of the buret to the vessel instead of bringing the vessel to the tip (Fig. 7). The bore of the capillary is 0.5 mm, so a 1-mm length corresponds to 0.2 μl. The titrating fluid is separated from the mercury by a column of

air. This avoids contamination of the solution, but on the other hand, it renders the buret sensitive to rapid changes in temperature and introduces errors due to different retention of titration fluid with different titration speeds.

In the displacement burets, the fluid is expelled by a plunger of known diameter, which is actuated by a micrometer screw from which

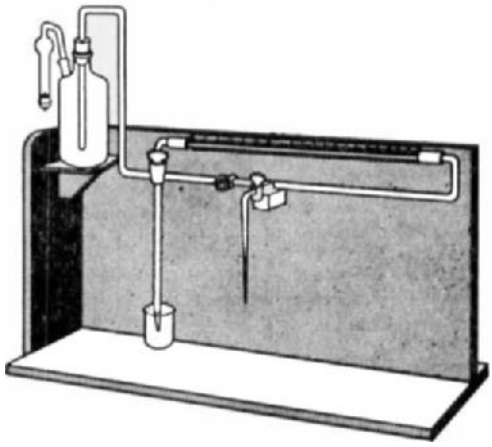

FIG. 6. Conway buret (K3).

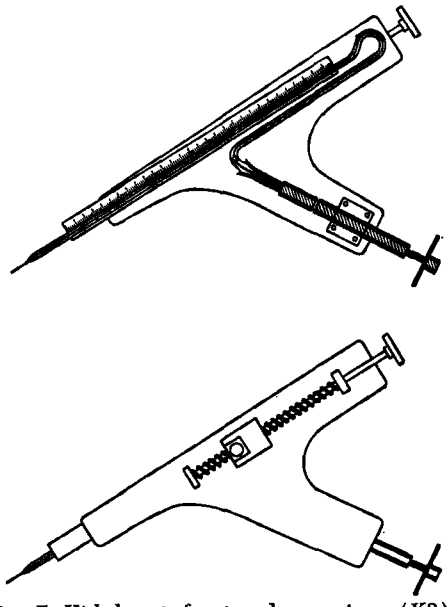

FIG. 7. Kirk buret, front and rear views (K3).

the reading is made (W2). The Scholander buret (S5), like the Rehberg buret, contains a large amount of mercury between plunger and titrating fluid (Fig. 8). Here too temperature changes may cause errors. Contact between mercury and titration fluid may be avoided by a layer of carbon tetrachloride (S8). Soft gaskets moving with the movement of the plunger can be a source of errors and the same holds for soft pistons (e.g., Teflon) in precision bore tubing, especially when the gasket, or the piston, has a large unsupported area.

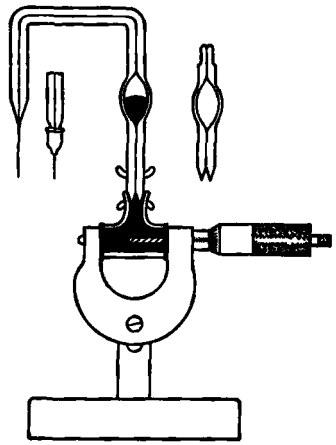

FIG. 8. Scholander buret (K3).

In syringe-type ultramicro burets, the plunger system has been replaced by an all-glass syringe. Reading is taken from the micrometer screw which moves the piston [e.g., the micrometer syringe developed by Trevan (T2) (Fig. 9)] or from a dial micrometer which is placed between screw and piston. The Trevan buret delivers 20 μl for each millimeter displacement with an accuracy of about 0.5%. The Natelson (N2) ultramicro buret uses a calibrated glass plunger. Parallel to the movement of the plunger a metal rod actuates a dial micrometer. One revolution of the large dial equals 100 μl. Plungers of highly chemically resistant material [synthetic ruby, Kel-F, stainless steel (G3, S2)] are used in several modern ultramicro burets now on the market. Up to now these types give the most accurate dispensing of liquids. The diameter of the plungers generally is only a few millimeters. Reading is done using special micrometers, dials, or mechanical counters, and most of these types can be zeroed, thus eliminating waste time and a source of errors (Fig. 10). Delivery of 10 μl is achieved with a standard deviation of 0.15%.

During the ultramicro titration the tip of the buret always dips under the surface of the liquid in the titration vessel. Hence, to minimize diffusion, the tip has to be very narrow. It is best made of hydrophobic material as only then will there be no liquid creeping up along the tip and thus escaping the titration (S3). It has to be straight: a tip which is curved will stretch under the pressure of the flowing liquid

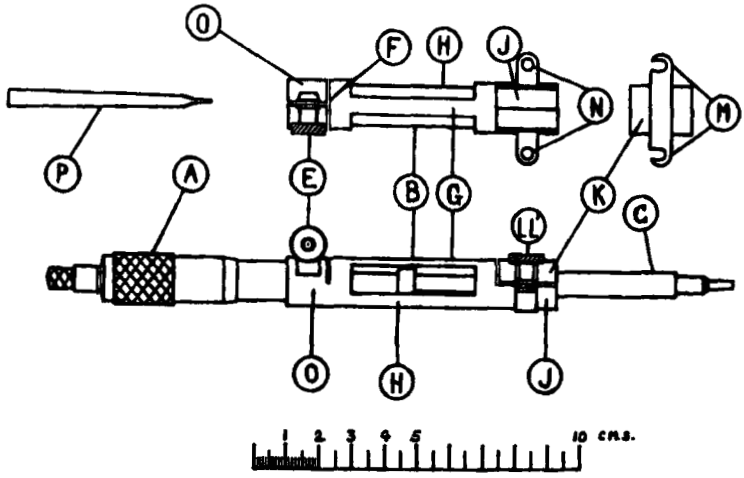

FIG. 9. Trevan buret/pipet (T2). A, micrometer head; B, syringe holder; C, glass "tuberculin" syringe; P, glass tube drawn out to a point, to be used instead of steel needle when desirable; O, end of syringe holder with slot F and screw E to fasten micrometer head. The slots between the bars G and H allow the head of the syringe piston to be drawn up and down; JK, slip to hold syringe; LL, milled heads to hold down K and J; M, lugs on K, fitting round screws N.

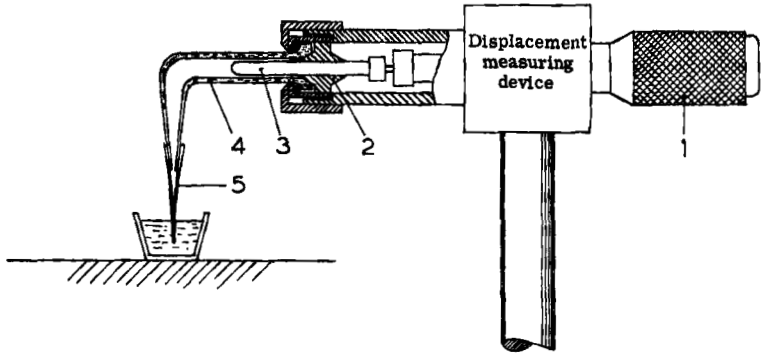

FIG. 10 Plunger-type ultramicro buret. 1, micrometer head; 2, packing gland (Teflon); 3, plunger (Kel-F, stainless steel, ruby); 4, glass reservoir; 5, capillary tip (polyethylene).

and will continue to deliver a small amount of liquid after the pressure is relieved.

Agitation during titration is very important and several principles have been applied. Bubbling devices for one or another gas have been frequently used and are especially convenient when a special atmosphere has to be maintained. Other apparatus for titrating under a gas blanket have been described by Berret (B3). Rotating stirrers are too bulky for titration in volumes smaller than 250 μl. Here magnetic stirring with small pieces of iron, enclosed in glass or polyethylene, is more convenient (O2). Also, stirring with vibrating rods (K3, S3) is excellent. Rotation of the vessel while the tip of the buret functions as a stirring rod gives good results only when the turbulence created around the tip of the buret has enough energy to stir the entire contents. This seems easy, but often very persistent flow patterns occur which enclose only a part of the volume. Titration vessels of different shapes are used, depending on the different circumstances. Small test tubes have long been in practice for titration, especially with Rehberg burets. However, when concentrations are low the recognition of the endpoint is very difficult.

White porcelain dishes are preferred by Kirk (K3). These have the advantage of allowing ample space for inserting different accessories; on the other hand, the ratio of surface to volume is very high, thus introducing the possibility of errors due to processes which are surface-area dependent (S3). With modern techniques, titrations are performed in small plastic cups. The price of these cups being very low, it may be even more economical to use them only once than to clean them. Titrating in these small volumes (100–300 μl) is generally done with solutions of a higher concentration than in older methods. This is made possible by the precision of the modern ultramicro buret and it is necessary on account of the visibility of the endpoint.

2.3.1. *Titration with Visual Endpoint Detection*

As mentioned before, the recognition of the endpoint is often difficult, due to the very small volumes that are used. Increasing the layer thickness means decreasing another dimension, which is always accompanied by some other inconvenience. Cups with a diameter of the same order as the height of the liquid in them seem to be well suited.

Matching faint indicator colors with a standard or a duplicate is possible only under adequate illumination which does not change from day to day or from hour to hour. So daylight has to play a minor part, though it is not necessary to exclude it completely. Illumination by

small 5-watt fluorescent "daylight" tubes has proved to be satisfactory in the authors' laboratory. The cups are placed on a white platform and examined against a normally white background. Depending on the color of the indicator we place contrasting filters on the lamp and/or change the color of the background. It is also possible to change the "daylight" tube by an UV tube and even mixed light is used with special indicators. Irritation of the eyes can be avoided by a sheet of UV-absorbing cellophane.

In order to have a good view *through* the cups, which is better than looking *on* them from above, a platform at eye level is preferable.

2.3.2. *Titration with Colorimetric Endpoint Detection*

One way of eliminating the difficulties described in the previous chapter is to use a photoelectric colorimeter for endpoint detection.

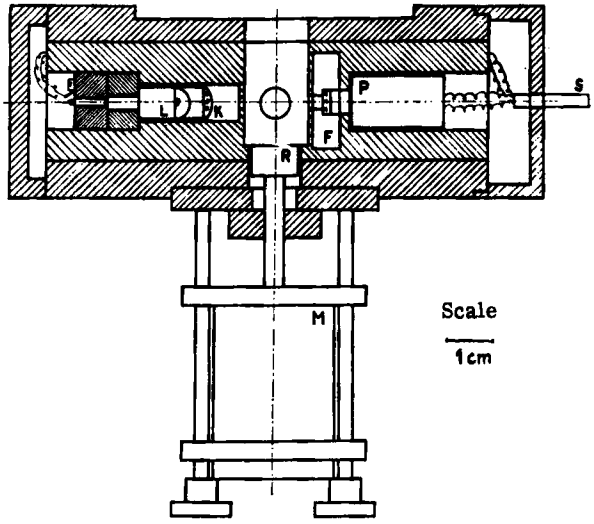

FIG. 11. Apparatus for colorimetric titrations according to Holasek (H6). L, light source; K, condenser; F, filter; P, phototube; R, stirring magnet; M, motor.

However, for the very small volumes which are used in real ultramicro analysis, this is far from easy. Some types of apparatus have been described (F1, M2), or are commercially available, but the required samples are large. A real ultramicro apparatus has been described by Holasek *et at.* (H6). Titration is performed with an ultramicro buret in a rectangular cell with a light path of 10 mm and a required volume of 500 μl. Stirring is done with a rotating magnet and a "flea" (Fig. 11).

2.3.3. *Titration with Potentiometric Endpoint Detection*

With the same intention as the colorimetric endpoint detection, a large number of potentiometric methods has been described.

Acid-base titrations can be performed in special hollow glass electrode cups which are available in different sizes. The increased sensitivity of modern pH meters allows the use of rather thick-walled electrodes which have a long life, even in routine laboratories. Sometimes it is desirable to exclude atmospheric carbon dioxide which necessitates special apparatus. See, for example, Sisco *et al.* (S10) and Berret (B3). Most potentiometric methods are developed for special determinations, e.g., the determination of Cl. The principle underlying these methods is nearly the same, differences are only found in the type of electrodes: Pt-$AgNO_3$-Ag (K2) or Ag-AgHg ["Clark" electrodes, see Seligson *et al.* (S8)]. Malmstadt and Winefordner (M3) developed a null-point detection method which should be very sensitive and rapid, giving relative errors of less than 0.5% using 20-μl samples. Automation of potentiometric titrations has been described by Robinson (R2). Electrodes can be brought in the cup from above. This allows good changing and cleaning possibilities. Cups with built-in electrodes are used by Bishop (B4), and burets with sealed-in electrodes have also been described. For determination of copper, Sadek and Reilley (S1) described a potentiometric method for very low concentration (0.006 μg in 100 μl).

Undoubtedly, these methods have their special merits and possibilities. However, it is wise to consider if the purchase of expensive apparatus is justified by increased accuracy and/or speed. In the case of the chloride titration, for instance, it is the writer's experience that the ultramicro titration according to Schales and Schales (S4) can be performed with nearly the same accuracy, reproducibility, standard deviation, and speed as is achieved potentiometrically.

2.4. Colorimetric and Spectrophotometric Procedures

The essential condition for the application of colorimetric and spectrophotometric procedures in ultramicro chemistry is the availability of suitable absorption cells. These cells should have the following properties:

(a) The volume necessary for a measurement should not exceed 100 μl.

(b) The lightpath should have a length of about 1 cm.

(c) The cell should be made from a material as resistant as possible toward the chemicals used.

(d) Filling and cleaning of the cell should be easy.

The points (a) and (b) in combination aim at an optical density not less than 0.1. Assuming an absolute error in determining the transmittancy of 1%, it is easily computed that at optical density 0.1 the relative error in optical density and consequently in the concentration determination is about 5%. This error rises steeply at lower optical densities, is minimal (2.7%) at optical density 0.4343, and rises slowly at higher optical densities (K3). In ultramicro chemical practice this means that only too low an optical density is to be avoided, as high

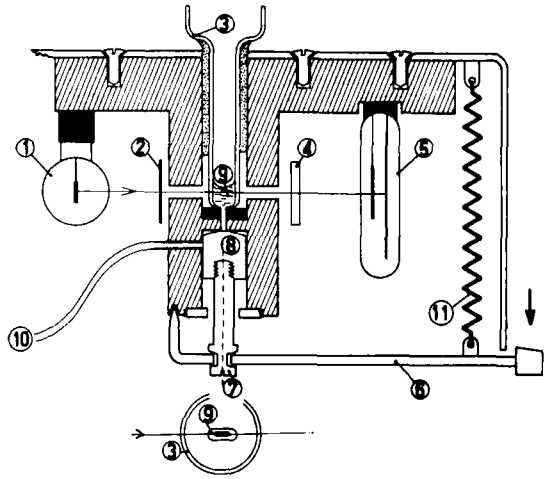

FIG. 12. Ultramicro colorimeter according to Sanz (S3). 1, light source; 2, diaphragm; 3, cuvette (lower figure in cross section); 4, filter; 5, phototube; 6, lever attached at 7 to plug 8 and kept in position by spring 11. On pressing down the lever 6, the solution 9 is removed by suction via tube 10.

densities will seldom occur. The absorption cell described by Lowry and Bessey (L7) fulfills the requirements (a)–(d) given above. This cell has parallel windows 1 cm apart and thickened side walls 2 mm apart. The result is a cell in the shape of a standard absorption cell in use with several commercial spectrophotometers and colorimeters, but with a considerably smaller capacity. By proper masking of the light beam it can be used with volumes as small as 50 μl. It can be made from transparent quartz [point (c)]. It is easy to fill and to clean [point (d)].

Sanz (S3) described an absorption cell to be used with a special

colorimeter (Fig. 12). This cell has the shape of a flattened glass tube and thus has no flat windows. The light path has a length of 1 cm. The cell has an outlet at the bottom. It can be used with volumes of 150 to 200 μl.

In the past year a few colorimeters for ultramicro determination have been put on the market. The absorption cells used with these colorimeters are more or less like the cells described above. Only one cell is used for all determinations. The cells are emptied by suction. This overcomes the removing of the cell from the colorimeter for emptying and refilling. It is self-evident that the use of one stationary cell enhances considerably the accuracy of the measurements. Care should be taken that the inner surface of the cells be perfectly wettable. This is necessary to avoid air bubbles which would interfere with the measurement of the optical density. Moreover, the formation of droplets sticking to the cell wall is prevented. This is essential for the removing of the liquid from the cell after a measurement. Sanz (S3) states that under good working conditions the volume remaining in the cell after removing a volume of 100 to 200 μl by suction during 5 seconds is about 1 μl, i.e., 0.5–1% of the initial volume. As a consequence, when measuring samples differing not too much in optical density, rinsing between the measurements is unnecessary.

Solomon and Caton (S12) described a recording colorimeter. Absorption cells according to Lowry and Bessey are used. Six of these cells (four samples of 30 μl, one standard, and one blank) are placed in a special carriage, which ensures that the position of each cuvette be reproducible within 0.1 mm. An accuracy of 1% at optical density in the 0.1 region and of 0.2% in the 0.4 region is claimed at a wavelength of 476 mμ. Six samples can be measured and recorded in less than 200 seconds. In our opinion this instrument is not to be recommended for routine work because of the use of six absorption cells instead of one stationary cell. The same remark applies to colorimeters or spectrophotometers which use capillary absorption cells closed by glass or quartz disks. However, capillary absorption cells are indispensable when only very small volumes are available.

A rather simple microspectrophotometer which permits accurate absorption measurements using a capillary cell with a content of only 10 μl was described by Wallach and Surgenor (W1). The cells are made from borosilicate glass tubing of 1-mm bore. They are precisely 10 mm long and are closed by quartz disks. These are held in position by capillary forces. A monochromatic light beam 0.4 mm in diameter is obtained from a glass prism monochromator. The cell is positioned

with the aid of a microscope, which is an essential part of the instrument. After precise alignment the transmitted light is deflected to a photomultiplier.

The use of infrared spectrophotometry in clinical chemical problems on an ultramicro scale has been described by Stewart *et al.* (S13, S14). Two drops of serum or other body fluids are spread out between the barium fluoride crystal windows of the sample cell. A light path of 0.06 mm is used. As the spectrometer used is a double-beam instrument, simultaneous scanning of sample and water results in an absorption spectrum of the solute.

2.5. Gasometry

Gasometric methods have long been practiced because of their simplicity and accuracy. For a long time however, these methods have been performed only on a macro or semimicro scale. (A remarkable fact: van Leeuwenhoek tried to measure blood gas on a true ultramicro scale in 1692: see Letter No. 72.) Manometric methods are preferred instead of volumetric methods because of their greater accuracy. A volumetric instrument for gasometry on ultramicro scale (samples of 20 µl) nevertheless was developed by Lazarow (L1, L2); and according to the author, the reproducibility for CO_2 determination in serum is better than 1 vol % (L3).

The ultramicro methods of Scholander and co-workers are very ingenious and very small samples of gas can be analyzed (K3). For the routine laboratory, however, the method is not suited as too much depends on the personal skill of the operator.

The manometric van Slyke apparatus has been modified by Natelson (N1) for the analysis of gases in serum samples of 30 µl (Fig. 13). The principle (measuring the pressure of the gas in a standard volume) is the same as the original van Slyke, but the operations are simplified. With this method the use of de-gassed reagents is necessary. Dispensing these reagents from syringes provided with a mercury seal was mentioned by Holaday and Verosky (H5).

Recently van Slyke too has modified his gasometer to handle samples of less than 100 µl (V1).

New possibilities for gas analysis are opened by the development of gas chromatography. The use in clinical laboratories generally depends on the commercial availability of well-suited apparatus. Recently an instrument was introduced which has been developed specially for the determination of respiratory and blood gases. It combines the use of a molecular sieve and real chromatography. Carbon dioxide and

oxygen can be determined in one sample of 100 μl in 6 minutes, carbon dioxide alone in 2 minutes.

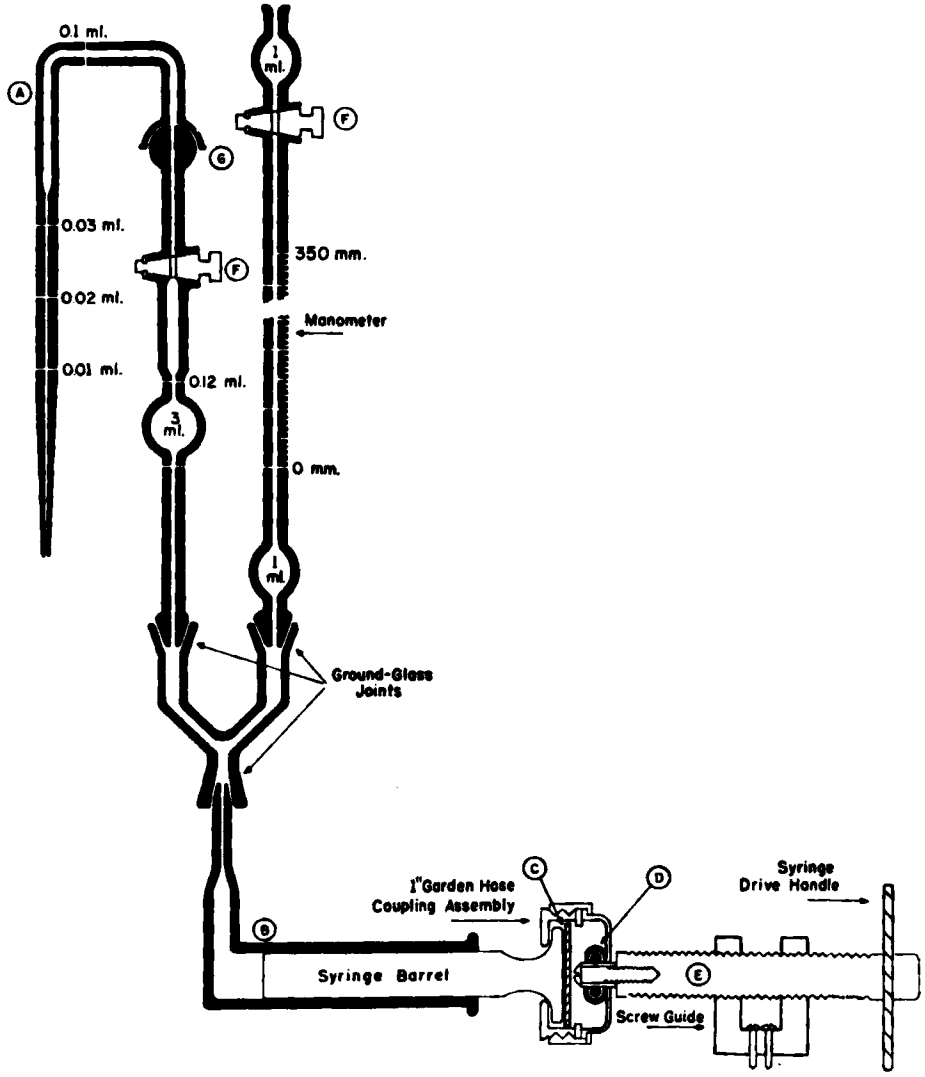

FIG. 13. Ultramicro manometric gasometer (N1). A, 0.5-mm bore Pyrex capillary tube joined to 1-mm bore Pyrex capillary tube; B, 10-ml syringe and side tube sealed on, as shown; C, metal blank (from electric junction box); D, ball bearing for rotating screw without turning coupling; E, machined screw ¾ inch diameter, 20 threads per inch, recessed and tapped at one end to receive machine screw; F, vacuum stopcock; G, ball and socket ground-glass joint.

2.6. pH AND pCO_2 DETERMINATIONS

Since the recognition of the importance of the study of the acid-base balance, the interest in these values has gradually increased. It was, however, soon recognized that conventional pH electrodes were not suited for the determination of blood or plasma pH due to the prolonged contact of the sample with air.

An electrode for measuring small samples in a sealed-in capillary was described by Pickford (P3) in 1937. The capacity was 60 µl and it could be filled from a Luer syringe.

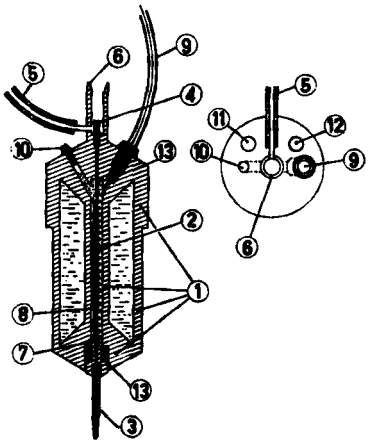

FIG. 14. Ultramicro glass electrode according to Sanz (S3). 1, electrode housing (polymethyl metacrylate); 2, glass capillary; 3, exchangeable polyethylene capillary tip; 4, overflow; 5, connection to vacuum pump; 6, female cone for connection with a syringe; 7, electrolyte solution; 8, chlorinated silver spiral; 9, shielded cable to compensator; 10, filling hole for electrolyte solution; 11 and 12, in- and outlet for water from thermostat; 13, elastic, high-resistance packing.

A method for determining the pH of much smaller samples was developed by Lloyd Claff and Swenson (L6). They considered the sealed-in capillary a disadvantage and consequently used loose capillaries (of 5-µl capacity). After filling these were brought in contact with electrode liquids in a special container.

A glass electrode for determining the intracellular pH has been described by Caldwell (C2). Total thickness of this electrode (complete with reference electrode) is 150 µ. It is constructed for special research work and not for clinical use, but it shows the possibilities of these instruments. A thermostated, sealed-in capillary electrode was developed by Sanz (S2). The volume of the capillary is about 10 µl.

With a good electronic compensator, determinations with an absolute accuracy of 0.01 pH are possible. Apparatus of this model or slightly modified [with horizontal capillary according to Astrup (A3)] are now commercially available.

In a more recent publication Sanz (S3) describes a new electrode housing which is made from polymethylmetacrylate (Fig. 14).

The electrode which is described by Wilkinson (W5) is in principle the same as others. However, a more robust capillary is used which is easily renewed, and special care is given to the connection with the reference electrode.

The determination of pCO_2 with electrodes is based upon the diffusion of dissolved CO_2 through a membrane into a solution surrounding a pH-sensitive electrode. The older electrodes as described by Stow *et al.* (S15), Gertz and Loeschke (G2), and Severinghaus and Bradley (S9) use the ordinary glass-bulb electrode. Consequently, the amount of sample is rather large.

Hertz and Siesjö (H3) constructed an electrode, based on the same principles, but the form of the glass electrode was modified in such a way that the enveloping Teflon membrane has a flat surface. Although the instrument was developed for continuous measurements, it will be possible to use it for small samples as well and by diminishing its dimensions the ultramicro range may be covered.

2.7. Zone Electrophoresis

2.7.1. *Paper Electrophoresis*

In 1950 Durrum (D2), Cremer and Tiselius (C8), and several other authors published papers on the electrophoretic separation of proteins on filter-paper strips soaked with a buffer solution. Since then the technique of paper electrophoresis has been introduced in most clinical chemical laboratories, where it is applied especially to the separation of serum proteins.

As only 10–50 μl of serum is required for this purpose, paper electrophoresis is a real ultramicro method.

The technique of paper electrophoresis need not be described here, as several excellent books and articles on this subject have appeared (see, e.g., M1, L4, W6, W7, P2).

A very sharp separation of serum proteins using 5–20 μl of serum has been achieved by Kohn (K4) using cellulose acetate instead of filter paper. Cellulose acetate has the advantage that the adsorption of proteins and the dyes used for staining them is less than on filter paper. However, it is rather brittle when dry and it is rather expensive.

These disadvantages are not important when strips of 12 × 2.5 cm are used. In this case, with only 1 to 3 μl of serum on a 1.5-cm streak, best results are obtained. It is even possible to use as little as 0.1 μl of serum (K5). Cellulose acetate strips may be scanned after clearing by immersion in a nonvolatile fluid of refractive index of about 1.47, when they become completely transparent (see, however, Section 2.7.2).

2.7.2. *Agar Gel Electrophoresis*

The use of agar gel as a supporting medium for electrophoresis of proteins, first described by Gordon *et al.* (G4), has become well known by the work of Grabar and Williams (G5).

Comprehensive books on agar gel electrophoresis have been written by Wieme (W3, W4). For detailed information on the subject the reader is referred to these books. Agar gel is mostly used as a 1% solution in a buffer solution. For ultramicro electrophoresis it is used in a layer of 1 mm thickness, which may be mounted on a microscope slide.

The material to be used for electrophoresis experiments is usually applied into a slit in the gel. The length of the slit determines the quantity of material to be applied (1–3 μl). It is even possible to apply very small tissue samples into the slit.

When only very small quantities of material are available it is possible to put a small drop, say 0.03 μl, *on* the agar gel.

During electrophoresis the agar is either placed in a closed atmosphere or, when using rather a high field strength, covered with, e.g., petroleum ether to prevent evaporation of water and to keep the temperature on an acceptable level.

Agar gel is excellently suited to the electrophoresis of proteins as there appears to be very little adsorption. However, β-lipoproteins are strongly adsorbed, presumably by the presence of sulphate groups in agar (W3, p. 200). Moreover, these groups cause a strong electroosmotic effect.

When the sulphate groups are removed using the procedures described by Araki (A2), the adsorption effects as well as the electroosmotic flow are considerably reduced. As a consequence, β-lipoproteins move at nearly the same rate as in paper-electrophoresis experiments. Moreover, sulphate-free agar is very suitable to electrophoretic analysis of mucopolysaccharides, as the background of electrophoresis diagrams stained by metachromatic techniques is practically colorless (R.E.P.A. Ballieux, personal communication). As stained electrophoresis diagrams

on agar gel are perfectly transparent, they can be scanned directly. However, the results obtained by scanning procedures are reliable only when:

(a) the slit of the scanning apparatus has a length not greater than the length of the band;

(b) the bands have all the same length; and

(c) the dye has a uniform distribution in the band (G6).

The last two requirements are difficult to fulfil, especially in experiments with a mixture of components differing much in concentration.

2.8. Miscellaneous

Gravimetric methods have seldom been used in routine clinical chemistry. This is mainly due to difficulties encountered in the preparation of the precipitate (purification) and in weighing. However, gravimetric methods are used in many other laboratories and, when searching for a special determination, it can be enlightening to take cognizance of this mode of working.

Microbalances used to be very tricky instruments and only very skilled workers could hope to obtain reproducible results. The basic principle of most types is the torsion of a fiber (steel or quartz), its deflection being read with special optics. Cefola (C5) described a "fish-pole" balance which has been used in the isolation of plutonium. (A great number of ultramicro techniques now generally known originate from research on radioactive chemicals.) The development of these balances and the use of new principles (electric balances) bring the possibility of accurate weighing to the level of the laboratory technician.

Micro and ultramicro balances are now commercially available and, although rather expensive, working facilities are much better than 10 years ago. Weighing the sample may be done directly on the pan of the balance. When the sample is a solution which has to be concentrated, a very convenient way is described by Thomas (T1). In this method use is made of very small cubes of polystyrene foam which are soaked in the sample and dried repeatedly. In gravimetric technics use is often made of filtration instead of centrifugation. This is done with small tubes with a glass-sintered disk of 1 to 3 mm diameter, or by pressing the suspension through polystyrene foam.

For incineration of products very thin platinum foil may be folded to form micro-"crucibles" as described by Cahn and Cadman (C1).

These authors also described the use of very small weighing tubes and micropycnometers, made from melting-point capillaries.

In spite of all the accessories, gravimetric methods are up to now not suited for routine work in the clinical laboratory.

A great number of publications deal with automation of one or the other instrument. It is beyond the scope of this article to give a complete review of these "improvements," because most of these are not of outstanding importance. Some of them may be mentioned, perhaps not for their intrinsic value, but more to give an example of the possibilities.

For the Natelson gasometer it is possible to purchase a "shaking attachment" which takes over the manual shaking. O'Mara and Faulkner (O1) describe another way to achieve the same purpose by magnetic mixing. They also diminish the time for one determination by coupling a motor with appropriate switches to the micrometer screw.

The use of a motor-driven micrometer buret was described by Cartier and Clément-Métral (C4) in their apparatus for automatic calcium titration. Irving and Pettit (I1) went even further and coupled a microburet and a recorder on the same motor so that true-scale titration curves could be obtained automatically.

Cotlove *et al.* (C7) developed an automatic apparatus for amperometric titration of chloride in biological samples. It registers the time needed for the generation of an equivalent amount of silver ions. When 10 μl serum is used the accuracy is 0.5%.

Although the Dumas method for nitrogen determination is seldom used in clinical chemistry, it is interesting to know that it seems to be well suited for automation. The apparatus described by Gustin (G7) can perform 6 determinations on samples of 1 to 10 mg in 1 hour.

Descriptions of instruments for special manipulations are found widespread in a vast amount of literature. Here only a few will be mentioned.

Herbain and Bertin (H2) describe a microseparator which can be used for the filtration, centrifugation, and concentration of small amounts of radioactive material.

An ultramicro modification of the diffusion technique of Conway is described by Gaberman (G1). A block of transparent plastic contains 6 microdiffusion cells. Titration with an ultramicro buret allows the determination of 0.05 to 10 μ*M* ammonia.

Flame photometric determination of Na and K is widely accepted. However, according to Isard (I2), the determination with alkali-sensitive glass electrodes has a greater sensitivity. Of importance especially for biological systems is the fact that in this way the real activity is measured instead of the total concentration. Microelectrodes based

on this principle are up to now only used in physiological research (e.g., Hinke, H4) but no doubt they will find their way into the clinical laboratory.

Commercial flame photometers are mostly suited for Na and K determinations on an ultramicro scale. Special flame photometers for the determination of these elements in very small samples have been described by Solomon and Caton (S11) and by Ames and Nesbett (A1).

Exley (E1) described a special method to increase the sensitivity of the flame photometric procedure by dissolving the sample in 90% acetone and using an oxygen-hydrogen mixture for the flame.

Polarography has only few applications in clinical chemistry.

Von Sturm (V2) described microcells to be used for polarographic determinations in 50 µl. Using capillary cells this author was able to reduce the volume even to 5 µl.

For pO_2 determinations in blood, Severinghaus and Bradley (S9) used a platinum electrode surrounded by a KCl solution and separated from the sample by a Teflon or polyethylene film. Bartels *et al.* (B1) modified this electrode by applying the separating film directly on the platinum wire. This electrode was used by these authors for pO_2 measurements in 0.5-ml blood. It should be possible to adapt this method for measurement on an ultramicro scale.

Natelson *et al.* (N4, N3) described a method for the ultramicro estimation of potassium, calcium, chlorine, and sulfur by measuring the intensity of the Kα-radiation of these elements; 5–25 µl serum is used. This technique could be adapted to automatic operation.

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3. Applications in Clinical Chemistry

3.1. Methods Described in Literature

Generally, ultramicro methods are used as a system, not for one special test only. Therefore publications in which ultramicro methods are described usually contain advice on and a description of the apparatus which are used by the author. However, in the first publications on the application in the clinical laboratory only special tests were de-

scribed, e.g., a phosphatase determination performed on 5 µl serum (B1) and a serum iron determination for 20 µl serum (B2).

Publications of this kind were followed by a number of articles which are of outstanding importance for everyone who wishes to introduce these techniques in his laboratory.

As it is impossible to deal in detail with these articles only a list will be given. Natelson (N1) published methods which he used for a number of years. He described in detail the construction and use of his apparatus and compared standard deviations of macro and ultramicro modifications of 8 important tests. Sobel and Hanok (S2) give an article on the method of "scaling down" from micro to ultramicro methods in which they also make a comparison of the errors. Caraway and Fanger (C1) give details about their equipment and describe 16 tests, the most of which are performed on 10-µl samples. Here too a comparison is made with conventional methods. Kaplan and del Carmen (K1) give a description and details about equipment and performance of 14 tests. Apart from these publications much valuable advice and methods may be found in several books on ultramicro methods (K3, K4, N2).

The results of these authors prove that ultramicro methods have a distinct advantage over conventional methods and that the majority of the most important tests can be performed with very good results. This no doubt has stimulated the development of complete sets of apparatus with reagents for several tests, which are now commercially available.

The publications on ultramicro methods of recent years generally concern application of newer methods, e.g., enzymatic and catalytic determinations. Some examples will be given.

For instance, the normal method for the determination of protein-bound iodine was transformed to an ultramicro method by Sanz *et al.* (S1). This method is based on the well-known catalytic action of iodine on the reduction of ceric ions by arsenious acid and requires only 50 µl serum.

An ultramicro modification of the enzymatic glucose determination has been described by Kingsley and Getchell (K2). Their method requires 20 µl sample and needs no deproteinization. Fawcet and Scott (F1) developed an ultramicro modification of the urea determination with urease (Sample: 10 µl).

Plasma amylase may be determined in a 20-µl sample of plasma or serum according to Marsters *et al.* (M1) or to Close and Street (C2).

With these examples the literature is not exhaustively reviewed. This is well-nigh impossible as each month new adaptations and developments are published. However, the present authors hope to have given

a selection of papers on ultramicro techniques which is representative for this topic.

3.2. General Remarks

In the latest decennium, methods in clinical chemistry have improved a great deal with regards to the following aspects: accuracy; specificity; sample size; speed; number of determinations per man-hour; costs per determination. The introduction of ultramicro methods offers advantages on most of these aspects.

Sampling and dispensing reagents with polyethylene pipets, for instance, takes much less time, about 10 seconds as against 30 seconds for one delivery with ordinary pipets. Centrifuging erythrocytes or a precipitate takes less than 3 minutes. Taking a reading of a colorimeter with a stationary "flow-cuvette" takes about 15 seconds per determination and a titration with an ultramicro buret about 30 seconds, a complete titrimetric determination (e.g., Ca or Cl) being done within 90 seconds. The amount of chemicals used is roughly 1% of normal consumption, so the use of only analytical grade chemicals is possible and also the use of rather expensive biological and biochemical products.

While specificity of a test is nearly independent of the equipment (not completely, e.g., spectral purity of colorimeter filters!), the introduction of more or less automatic apparatus makes accuracy more and more dependent on the accuracy of these apparatus. Some authors state that ultramicro methods are more accurate than macrotechniques. In our opinion and experience this is not true, the accuracy of most ultramicro methods being the same or somewhat less. The quantity of sample generally used is 10–20 µl. The reproducibility of pipets in this range is very good. It is quite possible to perform determinations on much smaller samples [see, for instance, the outstanding work of Lowry (L1) on enzymes in single nerve cells and also the methods of Ames and Nesbett (A1)], but either the speed or the accuracy diminishes when using pipets of less than 10 µl.

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