ADVANCES IN CLINICAL CHEMISTRY

Volume 7

Harry Sobotka &
C. P. Stewart

ADVANCES IN CLINICAL CHEMISTRY

VOLUME 7

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Advances in CLINICAL CHEMISTRY

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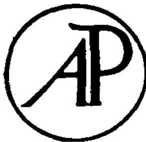
VOLUME 7 • 1964



ACADEMIC PRESS
NEW YORK AND LONDON

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ACADEMIC PRESS INC.
111 Fifth Avenue, New York, New York 10003

United Kingdom Edition published by
ACADEMIC PRESS INC. (LONDON) LTD.
Berkeley Square House, London W.1

LIBRARY OF CONGRESS CATALOG CARD NUMBER: *58-12341*

PRINTED IN THE UNITED STATES OF AMERICA

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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 comparative 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 7

Like its predecessor, this volume of *Advances in Clinical Chemistry* ranges over the whole gamut of the subject as we defined it in our preface to Volume 6. The broad scope presented here is a deliberate act of policy —we wish to emphasize the important role clinical chemistry plays in the progress of medical science and to dispel the view occasionally held that clinical chemistry merely supplies and uses diagnostic tools for the behoof of others who alone can interpret the information supplied by those tools.

Methodology is served particularly in this volume by the review on Absorption Spectrophotometry and by that on Fractionation of Macromolecular Components of Gastric Juice—the alpha and omega of the book. In between are reviews which, while not neglecting the methodological aspects of their subjects, range more widely over the fields of dynamic biochemistry and clinical medicine. But throughout the emphasis is on clinical chemistry, the application of chemistry (or any branch of it) to the study and investigation of disease.

Once again it is a pleasure to thank our contributors and publisher for their splendid cooperation and equally to thank the many readers whose response to previous volumes has encouraged the production of this one, which we dare to hope will be as well received as were its predecessors.

October 1964

HARRY SOBOTKA
C. P. STEWART

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PRINCIPLES AND APPLICATIONS OF ATOMIC ABSORPTION SPECTROSCOPY

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1. Introduction

One decade ago the term atomic absorption spectroscopy was familiar to only a small group of scientists, to a few chemists working on special problems, and to astrophysicists. Within the last few years, however, this analytical technique has found enthusiastic acceptance by science and industry. Several hundred papers have been published in a short time on basic research, instrumentation, development of methods, and practical application of atomic absorption spectroscopy. Several good reviews of the subject stressing various aspects have appeared (A13, D6, E2, G6, L3, L8, M6, M7, R2, R5, W15), and it seems hardly necessary to add to their number. The problems of medical laboratories and clinical

chemists are, however, unique in many respects and it was for this reason that the present article was written.

When new analytical tools become available, more often than not considerations of responsibility to the patient, practicality, and economy will keep the clinical chemist from accepting such newly developed techniques without careful deliberation. It appears that presently atomic absorption spectroscopy is slowly finding entrance into medical research and service laboratories, and there is reason to expect that this technique will find wider use and greater application than emission flame spectroscopy. Virtually all metals, with very few exceptions, can be determined by atomic absorption spectroscopy. It is anticipated that this technique not only will replace currently used analytical methods for metals, but will also make feasible the routine determination of elements now impractical by conventional means. Furthermore, the operational stability of available instruments and the simplicity of actual performance of measurements make this technique well suited for automation, by addition of an automatic sample feed and automatic recording.

Since atomic absorption spectroscopy is based on an atomic property unique for each element, the possibility exists to obtain "true" values, a challenge not to be overlooked by workers attempting to develop atomic absorption methods for a particular metal or material. Some of the original theoretical expectations in regard to freedom of interferences have not been fully substantiated in practice and almost every element has been found to suffer from one type of interference or another.

It is the object of the writer to present in this review the theoretical and instrumental basis of this analytical technique, discuss the practical methods already developed and in use, and consider some of the potentialities for the clinical laboratory.

2. Historical

The first to record a phenomenon caused by atomic absorption was Wollaston in 1802 in England, who observed dark bands in the sun's spectrum, although he was unaware of their cause. The same observation was made in 1814 by Fraunhofer, who, with improved instrumentation, was able to map more than 500 absorption lines in the solar radiation. Not until the days of Kirchhoff and Bunsen, however, was the true nature of the Fraunhofer lines recognized. In a series of papers Kirchhoff and Bunsen laid the foundation for modern spectrochemical analysis (K5–K10).

A typical atomic absorption experiment in the laboratory was carried out by Foucault in 1849 in Paris, as related by Stokes (S9). Foucault

passed a ray of sunlight through a carbon arc and then through a prism to produce a spectrum. In the spectrum he found the expected dark lines, but they were more completely blacked out than in sunlight without traversing the carbon arc. The mysterious power of the carbon arc to enhance these spectral characteristics of sunlight was simply due, of course, to sodium atoms in the arc deriving from sodium contamination of the carbon electrodes, causing absorption on the resonance lines of sodium, the well-known D lines in the sun's radiation.

Following the work of Lundegardh in the twenties, emission flame spectroscopy became established as an analytical tool in almost every branch of science. Although hollow cathode tubes were first studied by Paschen (P2) in 1916, and although atomic absorption spectroscopy had found occasional application, notably in the mercury vapor detector (W20), it remained for Walsh (W2) in Australia in 1955 to recognize the essential advantages inherent in absorption over emission methods and revive general interest in this technique. Shortly thereafter but apparently independently, Alkemade and Milatz (A2, A3) in Holland devised instruments and applied atomic absorption spectroscopy in their laboratory. Walsh and his co-workers have since contributed a remarkable volume of work on instrumentation and application, and patents are held by Walsh on his method in Australia, Europe, and America.

It may be mentioned here that this decade probably will see the soft lunar landing of an atomic absorption spectroscope (M8) intended to analyze surface material of the moon and relay signals back to earth. The system makes use of a solar furnace for sample vaporization and depends on the sun's spectrum as a light source.

3. Theoretical

Atomic absorption can simply be defined as the absorption of light by activated atoms. Such absorption occurs on very narrow spectral lines, the so-called absorption or resonance lines, their theoretical spectral width being of the order of 0.001 Å. The lines are entirely characteristic and specific for each element and to date no two elements have been found to possess an identical resonance line. If monochromatic light of a specific wavelength is provided, it will be absorbed only by atoms of that element whose resonance line is identical with the wavelength of the light source and not by any others. A field of atoms is "opaque" for monochromatic light when resonance line and source wavelength match, but for other wavelengths it is translucent. The degree of opacity is proportional to the total number of absorbing atoms. It follows then that with a beam of specific monochromatic light the concentration of an element can be determined in a mixture of atomic species.

Atomic absorption will take place only in a field of free, neutral, activated atoms. Atomic absorption cannot be brought about by ions, by atoms bound in compounds, or by a molecular gas. When metals are heated to their boiling point, they vaporize as free atoms, provided that interaction with other elements is prevented, and it is for this reason that atomic absorption spectroscopy in its present form has found its most extensive applications in the analysis of the metallic elements.

3.1. Atomic Absorption and Beer's Law

As stated above the degree of atomic absorption depends on the number of absorbing atoms. If the absorbing field is spatially defined, absorption is proportional to concentration of the absorbing species. But this, in essence, is also the content of Beer's Law. The latter holds true under certain ideal conditions (L11):

(1) The analyzing light should be perfectly monochromatic.

(2) The light beam should be strictly parallel.

(3) The light beam should travel in an optically homogeneous medium that does not scatter radiation.

(4) The absorbing units (molecules or atoms) should not be close enough to one another or to other molecules that their structure or their energy levels are affected.

When these points are critically examined, it appears that these conditions theoretically are ideally realized in atomic absorption. First, the light sources used (hollow cathode lamps, discharge lamps) produce extremely sharp lines of 0.001–0.01-Å width, unobtainable by filters or monochromators. Second, parallel light beams can be obtained in most instruments of conventional dimensions and the problem of chromatic aberrations in the optical system is not encountered when working with monochromatic light. Third, under most experimental conditions, flame matrices are transparent to the analyzing light. Only where nonvaporized or unburned particles occur in the flame can light scatter be observed. Fourth, the absorbing units in a gaseous phase are considerably farther apart from one another and from other atoms and molecules than in a liquid phase, as in conventional molecular photometry.

It has been found, however, in practice that a perfectly straight analytical working curve ($-\log T$ plotted against concentration) is seldom obtained in atomic absorption spectroscopy. The reasons for this are usually a combination of instrumental problems; broadening of the emission line of the light source due to self-reversal, Doppler and pressure broadening of the absorption lines of the atoms in the flame, failure to exclude flame emission entirely, use of a focused instead of a parallel

light beam, flame inhomogeneities, and possibly atomic fluorescence (Section 3.2) contribute to interfere with the validity of Beer's Law. The problem is discussed in detail under methodology (Section 5).

3.2. Atomic Activation and Excitation

While mercury evaporates significantly at room temperatures, other metals require a supply of some form of energy to produce an atomic vapor. Most of the activated atoms in such a vapor are present in the ground state. If the supply of energy is sufficient, a small percentage of these atoms can be excited to a higher energy level, at which they remain for a very small period of time and then return to the ground state, thereby giving off their excess energy as light. Several discrete levels of excitation are possible which are reached in a stepwise quantized process. When a certain maximum level of excitation is attained, further excitation gain results in loss of the outer electron and the atom becomes an ion.

The return from higher energy states to the ground state of atoms takes place in the same stepwise transition between energy levels, resulting in emission of radiation of specific wavelengths, the characteristic emission spectrum. Atoms in the ground state can absorb on any of the wavelengths corresponding to transitions between energy levels, but it is usually the first resonance line representing the transition from the ground state to the lowest excited state where absorption is strongest. Exceptions to this are elements (iron, manganese) with complicated emission spectra, where more than one strong resonance line is encountered.

The number of excited atoms in a flame is exponentially dependent on flame temperature (Boltzmann relation). For example, for sodium, one of the most easily excited species, the ratio of excited to unexcited atoms changes at temperatures of 2000 and 4000°C from the order of 10^{-6} to 10^{-3}, respectively. In regard to the population of excited atoms this means an approximate 1000-fold increase, but only an insignificant decrease (less than 0.1%) of atoms in the ground state. The dependence of flame emission on flame temperature is well known (D10, P5, R6), while absorption is much less affected. Other metals, which require much higher levels of energy for excitation, produce such small numbers of excited atoms at ordinary flame temperatures, that their measurement by emission flame photometry is difficult or impossible. Most of these elements, however, at the same temperatures produce significant concentrations of atoms in the ground state which are then amenable to measurement by atomic absorption.

Most of the literature on atomic absorption neglects to mention that the light that is absorbed on atomic resonance lines causes the absorbing atoms themselves to undergo transitions to a higher energy state. Their deactivation in returning to the lower energy states by emitting light on the specific atomic lines is a form of atomic fluorescence, since excitation is from radiational, rather than thermal, energy sources. The use of atomic fluorescence flame spectrometry in the analysis of metals has recently been described (W16, W17) although the principle has been known for many years. One particular advantage of this method should be emphasized, that sensitivity can be altered by changes in the intensity of the exciting radiation.

3.3. Flames

Coincidentally with the revival of atomic absorption methods, renewed interest in flame processes has arisen among many groups of workers (B1, F7, G3, M5). Absorption techniques including the line reversal apparatus have contributed significantly to our understanding of atomic activation, excitation, atomic population densities, and temperature gradients. At the present time, the flame represents the most convenient means to create an atomic vapor under reproducible conditions. For these reasons, a short discussion of processes taking place in the flame is needed. Other means of production of activated atoms will be mentioned under instrumentation and techniques (Section 4.5).

Combustion of a fuel-air or -oxygen mixture progresses with a certain speed, the burning velocity. If the combustible mixture flows against the direction of combustion, and flow velocity and burning velocity equal each other, a stationary flame can be obtained, for instance, at the opening of a tube. When the flow velocity is greater the flame will lift off the tube opening, and when the burning velocity exceeds the former the flame will progress into the tube and cause a back-flash.

Stationary flames can be produced by premixing the fuel and air before they emerge from the burner opening; these are so-called premixed flames. If the fuel and air are fed separately and mixing occurs largely by diffusion of the two gases into each other at the burner opening, such a flame is called diffusion flame.

Stationary flames are composed of different zones: a base cone of nonluminous, unburned gases, corresponding to the transport zone in which preheating up to about 350°C takes place. This is immediately followed by a thin, highly luminous zone, the primary reaction zone, with temperatures between 1500 and 2000°C, depending on the type of

fuel. The large remainder of the flame plume is divided into the secondary reaction zone, where the temperatures reached are highest, and into a large recombination zone. In the latter temperatures generally are somewhat lower, due to entrainment of the surrounding air.

Burning of the fuel is a stepwise degradation leading to many intermediate products and formation of radicals. Some of these are of interest because of their ability to interact with metallic atoms, notably atomic oxygen and the OH radical. If the available oxygen equals the theoretical amount necessary to burn the fuel completely, such a flame is called stoichiometric. Otherwise, depending on the amount of fuel, we speak of a fuel-rich or a lean flame. Where fuel and metallic atoms compete for O and OH radicals, the formation of metal oxides can be kept down by making the flame fuel-rich. Fassel *et al.* (F2, F3) have recently shown that satisfactory atomic vapor concentrations can be produced in fuel rich flames with a variety of metals particularly prone to form refractory oxides in stoichiometric flames.

TABLE 1
FLAME TEMPERATURES OF COMMON FLAMES[a]

Fuel	Temperature (°C)	
	With air	With oxygen
Coal gas	1700–1840	2700–2730
Propane	1925	2800
Hydrogen	2045–2100	2730–2780
Acetylene	2125–2200	3050–3100

[a] Composed from references (D11, M5, W19).

The temperature of flames is dependent on the type of fuel and, of course, on the reactant air or oxygen. A large amount of nonreactant nitrogen in air flames absorbs energy, resulting in lowering of the general flame temperature. The maximum temperatures of some typical flames are given in Table 1. Graphs of isothermic lines (D11, M5) show that the maximum temperature is reached quite close to the top of the base cone, an area usually coinciding with the highest sensitivity in absorption. Maximum temperature is confined to a narrow zone of a few millimeters' cross section in lean or stoichiometric flames, but in fuel-rich flames this zone is spread out over several centimeters.

Under ordinary conditions flames are transparent over a wide range of wavelengths, a fact found with surprise by Foucault in 1849 (S9) and probably by everyone else since with an interest in this subject. Most flame constituents are gases in atomic or molecular form, and whatever

absorption is found of light shone through a stoichiometric flame is confined to atomic (or possibly ionic) lines and molecular bands. Oxygen flames, however, are opaque for wavelengths below 2000 Å because of the strong absorbing power of oxygen in the vacuum ultraviolet. The latter reason appears to be the most serious limitation to the use of absorption techniques for the nonmetals, most of which have resonance lines located below 2000 Å.

The gas velocities in flames are of the order of several meters per second. Consequently, an absorbing atom will pass through the zone monitored by the light beam with an approximate cross section of one centimeter in a few milliseconds. This points toward the relative "wastefulness" of flame procedures, considering that (a) at least 10–20 seconds of time are necessary for one measurement during which a steady stream of activated atoms has to be maintained and (b) that in the ordinary premix-atomizer-burner only a few per cent of the total sprayed sample effectively reach the flame. Clearly, if sensitivity gains are sought, improvements in atomization and atomic activation methods appear to represent the most logical approach.

4. Instrumentation and Techniques

4.1. Commercial Instruments

The early workers in this field built their own apparatus, often assembling these from suitable units available from instruments for other purposes. Emission flame photometers have been converted into atomic absorption spectrophotometers by appropriate attachments consisting of specific light sources, choppers, and lenses, a principle also employed by some manufacturers.

A number of good commercially built atomic absorption spectrophotometers are available now from the following companies: (1) Bausch and Lomb Optical Company, Rochester, New York; (2) Hilger and Watts, Ltd., London, England; (3) Jarrell-Ash, Newtonville, Massachusetts; (4) Messrs. Jobin-Yvon, France; (5) Optica U. K., Ltd., Gateshead, England; (6) Optica S.p.A., Milan, Italy; (7) Perkin-Elmer Corporation, Norwalk, Connecticut; (8) Techtron Pty, Ltd., Melbourne, Australia.

The instruments by Nos. 6 and 7 are double beam spectrophotometers, the others are single beam instruments. Instrument No. 8 is distributed in the United States by Aztec Instruments, Inc., Westport, Connecticut. For detailed information about the various instruments, the reader is referred to the manuals available from the different companies and descriptions in the literature (B7, C2, D1, G2, K1, M7, M12, S3).

4.2. Basic Principles of the Atomic Absorption Spectrophotometer

An atomic absorption spectrophotometer operates on the following principle. Light from special monochromatic sources with the wavelength of the resonance line of the element to be determined is passed through a vapor of neutral atoms. The diminution of the light beam by the activated atoms is monitored by a detector and recorded by a read-out system. Because the wavelength of the light emitted from the excited atoms in the flame is identical with the wavelength of the source line, radiation from both, the lamp and the flame, is passed by the monochromator into the light detector. In order to prevent the undesired flame radiation from interfering with the measurement, the light source is modulated and the currents from the detector are fed into an a.c. amplifier. The latter will amplify only the a.c. components of the output of the detector, while d.c. currents are thus excluded from measurement.

4.3. The Hollow Cathode Tube

Paschen (P2) in 1916 while experimenting with Geissler tubes found that on lowering the pressure of the noble filler gas to 1–2 mm mercury, the light emission would retreat into the hollow cathode, which he had constructed as a small box of sheet aluminum. In the spectrum of the hollow cathode light, he found the expected emission lines of the noble filler gas, but also strong aluminum lines. Modern versions of hollow cathode lamps are sealed vacuum tubes filled with a noble gas (neon, argon) at a pressure of a few millimeters of mercury (J2). The purity of the filler gas and its exact pressure are quite critical. Mounted inside the tube are a rod-like anode and a hollow cathode usually consisting of a cylinder with an open front. The front window of the tube is preferably made of quartz to transmit also light of short wavelengths. When a voltage is applied across the two electrodes, the filler gas becomes partially ionized, but ionization at the low filling pressure remains largely confined to the interior of the cathode. The noble gas ions, traveling toward the cathode, start to strike the interior of the cathode thereby sputtering off atoms of the metal composing it, in sufficient numbers to give rise to a cloud of metallic atoms. These collide with the filler gas and each other and thus become excited to give off light on the atomic lines characteristic for their species. In this way the emission spectrum of any metal can be obtained that composes or lines the inside of the cathode. Tubes with more than one cathode composed of different metals have been constructed (J2).

The harder the cathode is struck by the filler gas ions, the more atoms are sputtered off, resulting in greater intensity of the emitted light.

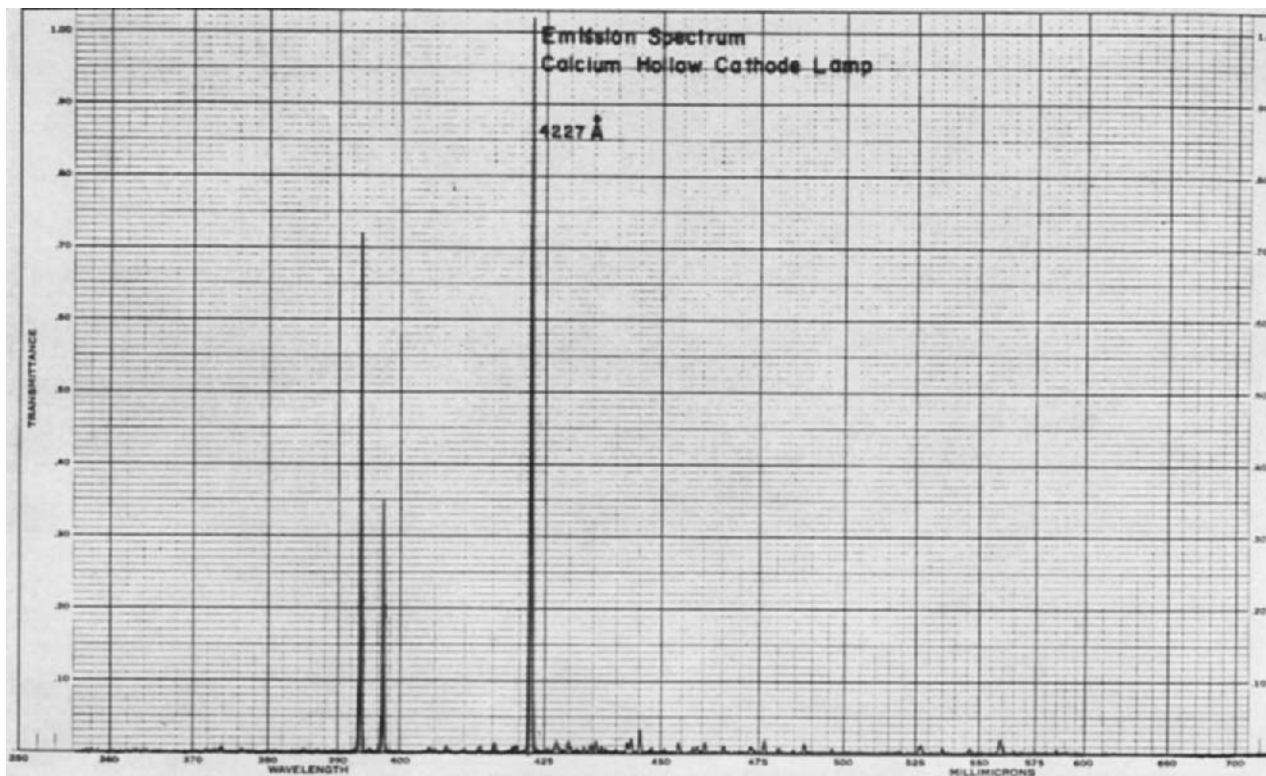

FIG. 1. Spectral scan of the emission of calcium hollow cathode tube (Ransley Glass, Melbourne, Australia). The emission spectrum is dominated by the strong resonance line of calcium at 4227 Å (reproduced from reference (Z3) by permission from the editor of *Clinical Chemistry*).

Therefore, intensity of cathode emission increases with greater cathode current and with higher atomic weights of the filler gas species. Helium, with its low atomic weight and relatively high ionization potential, generally is least suitable (T1).

The lines emitted by hollow cathode tubes run at room temperature generally have a spectral width of less than 0.01 Å. This value, however, is dependent on the tube current. As the current is increased, temperature rises and a denser population of neutral metal atoms is obtained in the cathode. This results in greater width of the emission lines, due to increased Doppler broadening and increased self-absorption, respectively. For optimal results, it is desirable to operate with line widths not greater than one third the width of the resonance line of the particular metal being measured in the flame. The latter generally is of the order of 0.03 Å. A spectral scan of the output of a calcium hollow cathode lamp, as shown in Fig. 1, is indicative of the spectral purity of these light sources (R8). The use of iron hollow cathode lamps in wavelength standardization has been suggested (C3, S7).

In order to determine optimum operating conditions for a lamp, absorption of a given test solution should be measured at various tube

TABLE 2
PERFORMANCE OF CALCIUM HOLLOW CATHODE TUBE AT VARIOUS LAMP CURRENTS[a]

Operating current (mA)	Absorption readings (%)
5	49.5
8	47.6
10	45.8
12	44.0
14	42.5
16	42.0
18	41.5

[a] Absorption readings obtained with 1 mg % aqueous calcium solutions (Z2).

currents. As the tube current is decreased, the line width decreases, but absorption of this light in the flame increases. The line width can be considered satisfactory when further lowering of the tube current does not increase absorption; the current at that point should be chosen as the operating current. Most lamps, however, require a certain minimum operating current to deliver a steady discharge, and for this reason some lamps have to be run at currents higher than those at which maximum sensitivity is observed. In these cases a compromise must be found between reduced sensitivity and improved stability of lamp dis-

charge and meter reading. Table 2 shows absorption obtained with a 10 ppm calcium solution at various lamp currents.

While the first hollow cathode tubes were constructed in such a way that they could be repeatedly flushed with the purified noble gas, the inconvenience connected with such equipment led to the development of permanently sealed tubes. In order to insure a reasonable lifetime of such tubes, they have to be of a certain minimum volume. One of the reasons for the lifetime limits is leakage of air into the tube, but more important seems to be the loss of the filler gas which is slowly absorbed by the metal and the glass surface. Since the lamp operates by the sputtering off of the cathode lining, gradual loss of the latter leads to eventual deterioration of the lamp. Lamps for metals that sputter abundantly, like the alkali metals, or zinc and cadmium, have short lifetimes, mostly well below a hundred hours.

4.4. The Burner-Atomizer

Since atomic absorption spectroscopy in its present form was largely developed on the basis of techniques known from flame emission, many of the original absorption studies were done with means of vaporization available from flame photometry. It was soon realized, however, that these burners had certain shortcomings in absorption and this led to a number of modifications of existing burners or new designs. The flame needed in emission is to concentrate a sample in an area as small as possible, just large enough to illuminate the slit uniformly and as slender as possible to minimize self-absorption. Absorption techniques, on the other hand, require an elongated flame to provide an absorption path as long as possible.

Both types of burner-atomizer, the total consumption type and the discharge type, have been used in absorption. The total consumption type consists essentially of three concentric tubes, all of which end approximately at the same tip level. The sample is aspirated through the central capillary by the shearing-off action of the oxygen passed through the middle tube, and sample spray and oxygen mix into the fuel emerging from the outer tube. Its advantage is the relatively low sample consumption of about one milliliter per minute. Although the whole sample is introduced into the flame, only a fraction of it contributes to emission while larger droplets pass through the flame ineffectively. From the foregoing, it can easily be seen that with this burner an elongated flame is difficult to produce. Nearly everyone employing it in absorption found it necessary to use several burners in series to obtain the desired absorption path length, or to use multiple passes of the light beam

through the flame, or both. The discharge type of atomizer employs two capillaries that open into a spray chamber and are either arranged concentrically or at a right angle to each other. As air or oxygen is forced at high pressure and usually with supersonic speeds through one capillary, the liquid sample is sheared off the other tube and dispersed in a fine mist. Larger droplets settle out and are drained off as waste. Only the smallest droplets are carried by the air stream through a connector into the burner, which in this case usually is of the premix type. The small droplets evaporate readily in the premix area or preheating zone of the flame so that probably all of the sample that reaches the flame is effectively vaporized. The discharge atomizers have a relatively high sample consumption, 3–20 or more ml per minute, but only 3–6% of it effectively reaches the flame. The sample flow rate together with the waste rate permit calculation of the sample volume per minute actually introduced into the flame. The figures obtained are similar to those of the total consumption atomizer, indicating that population densities of activated and excited atoms are of the same order in both. A burner-atomizer with mechanically controlled sample flow has been described by Robinson and Harris (R7). Herrmann and Lang (H2, H4, L2) recently conducted extensive investigations of the efficiency of atomizers and derived formulas for optimal conditions of capillary size, volume of spray chamber, and gas and sample flow rates. The effect of methanol on the efficiency of sample atomization was studied by Winefordner *et al.* (W18).

While it is technically difficult to construct burner-atomizers of the total consumption type to deliver an elongated flame, this is much more readily accomplished by the combination of discharge atomizer and premix burner. When the burner head of the latter is given the shape of a fish tail or modified into a narrow long slot, an elongated, thin, blade-like flame is obtained, satisfying the needs for a long absorption path. A versatile burner-atomizer in one integral unit has been described by Willis (W9) and Slavin (S3). It consists essentially of a bakelite spray chamber mounted on top of which is a burner head of a T-shaped barrel of stainless steel with a narrow slot of 10-cm length. The entire air supply passes through the atomizer capillary and fuel, air, and sample mist are premixed in the spray chamber. From 90 to 97% of the aspirated sample drains through a waste tube. The stainless steel burner head warms up considerably during operation, which effects evaporation of the fine droplets carried forward by the fuel air mixture. This can easily be demonstrated by aspirating a sample without the flame lit; a fine mist is seen emerging from the burner slot of the cold burner, but not

when the flame is extinguished after a few minutes' burning. This is probably reflected in the fact that sensitivity more than doubles after the warming-up period.

An interesting solution to the problem of lengthening the absorption path has been described by Fuwa and Vallee (F10). The flame from a total consumption burner-atomizer (Beckman) is directed into a long narrow tunnel in which the flame is propagated over a distance of about 90 cm. The light beam of the hollow cathode tube is directed through the tunnel which functions as absorption cell. Sensitivity increases of 10–100-fold are stated. The material lining the absorption tunnel and its reflectivity for light were found to be related to the degree of sensitivity gain.

A Beckman total consumption burner was modified into a premix type by Kniseley *et al.* (K11). The burner is mounted into the lower end of a brass tube and centered with aligning screws. A graphite tube is inserted into the brass tube from the top in such a way that a small space remains between the top of the Beckman burner and the bottom of the graphite tube. Premixing occurs in this space and in the bore of the graphite tube. It is maintained that the advantages of the modification are an improvement of sensitivities by an order of one magnitude, probably due to the marked reduction in flame noise. The use of the burner is limited to solutions containing at least 50% of organic solvents.

4.5. Other Means of Sample Vaporization

Although the flame appears to represent the most versatile means to vaporize a sample, it is limited to materials dissolved in a liquid that can be aspirated. Furthermore, some elements fail to produce effective vapors in ordinary flames. Several other methods of vaporization have been described, which are applicable to samples in the solid state and to elements not amenable to measurement in the flame. A short note on the application of atomic absorption to solids has been published (S1) but no details are given.

4.5.1. *Cathode Sputtering*

The principle of the hollow cathode tube, production of a vapor of atoms by cathodic sputtering, has been employed by Gatehouse and Walsh (G1) for sample vaporization. The sample is introduced into a vacuum chamber and is made the cathode which produces a cloud of activated atoms. The light of a separate hollow cathode tube is passed through this vapor and absorption is measured in a spectrophotometer.

The method is said to yield reproducible results, and a straight working curve was obtained with silver.

4.5.2. *Flameless Crucibles*

L'vov (L12) developed a flameless furnace applicable for atomic absorption spectroscopy. A tube-shaped graphite crucible is inserted into a chamber, fitted at both ends with quartz windows. A carbon electrode is installed in the middle of the graphite tube and another one outside. Solid samples weighing less than 0.1 mg are placed at the tip of the electrode inside the tube and the chamber is closed, evacuated, and filled with argon at low pressure. The graphite crucible is heated electrically to 2000°C. Then an arc is struck between the carbon electrodes whereby the small sample is suddenly evaporated and an atomic vapor is created inside the graphite crucible. The concentration of a metal in the vapor is dependent upon the total amount present originally in the sample placed on the carbon electrode. The beam of an appropriate hollow cathode tube is passed through the long axis of the graphite tube and absorption is measured by a spectrophotometer. It is stated that no interference could be found for any of the elements tested, that reproducibility was excellent, and that only 3 minutes were required for one determination.

4.5.3. *Capacitor Discharge Lamp*

Nelson and Kuebler (N1) described a modified capacitor discharge lamp into which samples could be introduced for vaporization. The single, intense light pulse of these lamps results in flash heating of the samples to several thousand degrees Celsius, by which a dense cloud of atomic vapor is produced that persists for a few thousandths of a second. By spectrographic means the authors were able to record the absorption spectra of about 20 metals including copper, iron, lead, calcium, magnesium, and zinc.

4.5.4. *Cool Vapor*

Mercury, the only metallic element with significant volatility at room temperature, has been conventionally determined for many years by atomic absorption spectrometry, as the mercury vapor detector (W20) is based on this principle. Lindström (L7) used a flame to volatilize the mercury in the liquid sample, but determined its concentration in the exhaust gases with the mercury vapor meter after cooling and purification in a filter that removed particulate matter. The method is said to be capable of detecting 0.1 μg % of mercury in the original liquid sample

sprayed into the flame. Urine could be sprayed directly and plant materials were extracted.

4.6. Wavelength Selection

It should be pointed out here that wavelength selection in atomic absorption spectroscopy is largely accomplished by the choice of the monochromatic sharp line source, possessing the wavelength of a resonance line of the element to be determined, a specificity of selection unobtainable by any other means. Any additional wavelength selection can be considered merely secondary and the methods to this end should be examined with this in mind.

If the line source emits radiation on the resonance line only or if the line intensity is so great that any background radiation is rendered negligible, no additional wavelength selection is necessary. This is the case with sodium for which it was possible to construct an atomic absorption photometer without a wavelength selector (B7).

Where only the determination of alkali metals is desired, a simple instrument can be devised in which selection of a resonance line can be obtained with the help of color filters. However, at present such instruments would not appear to have an appreciable advantage over flame emission methods, considering the sensitivity of the latter and the simplicity of presently available flame photometers. Appreciable advantages, on the other hand, are inherent in absorption over emission methods in the determination of the alkaline earths and magnesium. Since these metals have simple emission spectra, the use of filters, notably interference filters, would be feasible in instruments limited to the determination of these elements.

Whenever it is intended to determine a variety of elements with one instrument—and versatility is one of the major advantages of atomic absorption—a high dispersion monochromator of good resolving power and variable wavelength selection is required. It should be capable of transmitting wavelengths from 2000 to 9000 Å and therefore has to consist of either a quartz prism or a diffraction grating. Grating monochromators have linear dispersion but their resolving power suffers from light scattering and from interference from higher diffraction orders. Quartz prisms have the disadvantage that dispersion is nonlinear (smaller at the long wavelengths) and only one third that of glass. The light emitted by hollow cathode tubes is composed of the sharp lines of all excited atoms; the filler gas, the lining of the hollow cathode, and the supportive metals of the cathode, especially when made of an alloy, contribute their own specific emission spectra. Since the monochromator

will have to separate just a few lines and not a continuum, interferences from light scattering and other diffraction orders are minimized when using a grating monochromator. The latter has been found to satisfy most requirements even where complex spectra are involved.

4.7. Absorption Measurement and Recording

The measurement which is sought in absorption photometry is the degree of attenuation of the light beam by a sample introduced into the absorption path; this value is compared to the amount of light energy arriving at the detector without the sample. The hollow cathode tube, providing stable operation, emits light of a constant intensity. When passing through the flame, this light is attenuated depending on the presence of atoms of the corresponding species. This reduced light then is transmitted by the monochromator to be measured by the detector. But some of the sample atoms in the flame become excited to emit light themselves on resonance lines or wavelengths identical with those emitted by the hollow cathode tube. Therefore, flame emission together with cathode emission will be received by the detector. This is somewhat similar to the situation in molecular absorption photometry when the sample shows undesired fluorescence. But while fluorescence usually is of different wavelengths than those of the incident light and can therefore be diverted from measurement, the lines emitted by the sample atoms in the flame and by the cathode are the same and cannot be separated from each other by the monochromator. In order to exclude the undesired flame emission from measurement, several possibilities exist. When working with metals possessing absorption lines in the deep ultraviolet, flame emission on these lines is usually small enough to allow for electrical correction with so-called "bucking circuits." Another interesting method (L9) is the use of lenses at both ends of the flame which are positioned in such a way that they focus the light from the sharp line source at the entrance slit of the monochromator, but light emitted from the flame is maximally defocused there.

4.7.1. *Light Beam Modulation*

The most versatile and convenient method to overcome flame emission applicable to all elements is the modulation of the light emitted by the hollow cathode. This can be accomplished by running the lamp on alternating current or by interrupting the light beam mechanically with a rotating disk. The photoelectric current from the photomultiplier tube is then amplified by an a.c. amplifier. The d.c. component of the output of the photomultiplier tube, deriving from the continuous flame emission,

can thus not be amplified and thereby is excluded from measurement. Since the a.c. amplifier is tuned to the frequency of the modulation of the light source (usually 50–120 cycles per second), the random modulation of flame emission due to flame noise will be rejected. Only that component of flame noise possessing the modulation frequency of the lamp theoretically can contribute to the output of the amplifier, but in practice this is entirely negligible.

4.7.2. *Single Beam vs. Double Beam*

Single beam as well as double beam systems have been employed in atomic absorption spectrophotometers. The question of preferability will depend on the applications of the instrument but in general probably can be answered in favor of the double beam. The instruments first built by Alkemade and Milatz (A2, A3) in Holland and Russell *et al.* (R9) in Australia were both based on the double beam principle. Later workers seeking simplification of the equipment were able to do satisfactory work with single beam systems. The problems of reducing lamp drift are considerable and it seems that even with optimal conditions the accuracy of measurements is distinctly below that achieved with double beam instruments, a shortcoming readily admitted by most who have worked with single beam. While drifts and noise levels of single beam instruments are of the order of 1% or more, the double beam system reduces this generally to 0.1%. Gidley and Jones (G4), in order to reduce short term fluctuations, used an integrating system in which the output of the photo cell was fed to a condenser for a certain period of time. The ratio of these signals, integrated in this way between the two voltages obtained with no sample and with the sample sprayed, is measured. Since the sensitivity of an instrument ultimately is limited by the signal to noise ratio, the double beam system with its inherent stability will have to be employed wherever small absorption measurements are sought with high accuracy. High stability further permits the use of electronic scale expansion techniques often with considerable improvements of the limits of detectability.

4.7.3. *Detectors and Read-Out*

Barrier-layer cells and photomultiplier tubes have both been used for photodetection in atomic absorption spectroscopy. The use of barrier-layer cells of course is limited by their sensitivity and the difficulty encountered in amplifying their output. They will suffice where determination of the alkali elements is desired only. For most other work photomultiplier tubes are necessary. These are available for a broad spectral

range from 2000 to 7000 Å. Their sensitivity increases with their operating voltage; when working with low emission levels of the hollow cathode tube, the shot-noise effect of the photomultiplier run under high voltage becomes the limiting factor.

The problems of modulation of two light beams, their optical and electronic separation, and ratio recording have led to interesting technical solutions. In the atomic absorption spectrophotometer of Russell *et al.* (R9) the reference beam was chopped at twice the frequency of the sample beam. The beams were optically recombined, the signals from the detector electronically again separated, and their ratio recorded. In a later developed commercial instrument (Perkin-Elmer Atomic Absorption Spectrophotometer Model 214) the modulated hollow cathode emission was split into sample and reference beams, which were passed into two separate photomultiplier tubes and their amplified signals recorded by a self-nulling digital read-out. In another version, light modulation and beam splitting are elegantly accomplished by a rotating chopper fitted with sectional mirrors. Both beams are recombined and received by one detector, which eliminates the costly process of matching a pair of photomultipliers. The recording is done by a manual nulling slide wire connected to a digital counter. An interesting system of ratio reading without beam splitting (M6) is based on the use of two separate lines emitted by the hollow cathode tube, of which only one line is absorbed. The ratio of the intensities of these two lines is related to sample concentration in the flame. Unfortunately, intensity changes due to lamp drift are not always the same for both lines, so that a steady intensity ratio is not maintained.

5. Methodology

The technique of actually performing measurements is simple and almost identical to that in flame emission photometry. A liquid sample, free of particulate matter and containing the sample metal in solution, is aspirated into the flame. The degree of absorption, usually read out as % transmittance (T), but more conveniently as % absorption ($1 - T$), is converted to absorbance or optical density ($2 - \log T$) in the conventional way. Sample concentrations are obtained from interpolated readings or absorbance-concentration plots of working curves derived from appropriate standard solutions. Even under most ideal instrumental conditions, i.e., low lamp current, homogeneous flame, narrow slit width, optimal monochromator efficiency, etc., analytical curves usually bend toward the concentration axis. The reasons for deviation from a straight line are complex, and maximal correction of one contributing factor

usually does not eliminate the curvature. The effects from Doppler broadening and self-reversal of the source line can be minimized by running the lamp on a low current as discussed above, but the considerable broadening of the resonance lines due to the high temperatures in the flame is difficult to reduce. Flame inhomogeneities, as mentioned by Menzies (M6), should be corrected wherever possible. These inhomogeneities cause variations in absorption in different parts of the

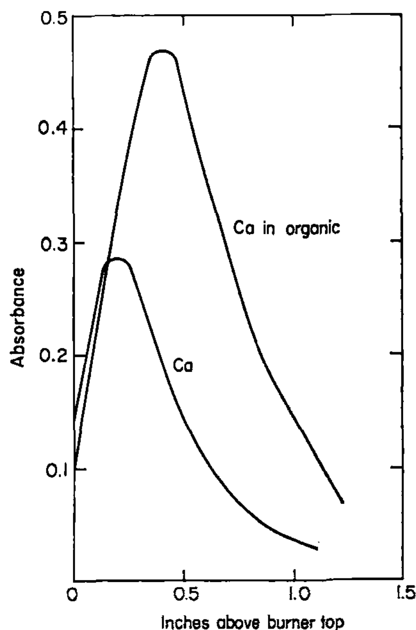

FIG. 2. Vertical absorption profile of 10-cm air-acetylene flame. Lower curve obtained with aqueous calcium solution, upper curve with isopropanol (from reference (S5) by courtesy of Perkin-Elmer Corporation).

elongated flame in regard to its horizontal as well as vertical extension. The sensitivity profiles obtained by scanning the vertical extension of the flame (Fig. 2) indicate considerable absorption differences from the base to the top of the flame and since the sample light beam possesses at least a few millimeters' width, different degrees of absorption will occur in the lower and upper part of the light beam. The problem may be slightly corrected by using fuel-rich flames in which the zone of maximum absorption is widened vertically and by focusing the light beam onto the center of the flame, but in the latter case the lack of a parallel sample beam might worsen the problem. A T-shaped burner

head may lead to higher sample concentrations in the middle third of the flame; (Z3) a baffle of the shape shown in Fig. 3, placed into the burner head below the slot, corrects this problem. When using hollow cathode tubes with simple emission spectra, the monochromator slit width influences neither sensitivity nor curvature of the calibration graph significantly, but for metals with complex spectra (iron, manganese, nickel) the slit setting is critical. Atomic fluorescence (Section 3.2) elicited by the hollow cathode radiation in the flame would also tend to curve the calibration graph toward the concentration axis.

When working with curved calibration graphs, a large number of working standards of varying concentrations would be necessary to

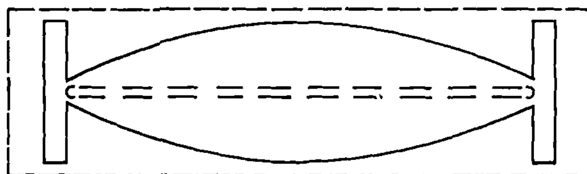

FIG. 3. Shape of the baffle placed into the barrel of the burner head to produce uniform gas and sample flow over the entire slot length. The dotted line indicates the outline of the burner head and slot (from reference (Z3) by permission).

determine the curvature as closely as possible. In practice, four to five calibrating solutions have to suffice but linear interpolation between these few points may lead to considerable error. Since the degree of curvature for a particular metal and a given set of instrumental conditions undergoes less change from day to day than the slope of the curve, it is possible to make a template possessing the "mean" curvature of a number of runs which is then used instead of a straight ruler to connect the points on the analytical curve. Since curves drawn in this way are a much better approximation of the "true" curve than those drawn by straight interpolation, sample values read off the former will be more accurate.

While industrial or agricultural chemists for the most part have large samples available for analysis, the clinical chemist is faced with an ever increasing number of tests to be performed on a small single sample, for instance a few milliliters of serum. The sample size in atomic absorption spectroscopy depends on necessary sample dilutions, aspiration rate, and time of aspiration required to obtain one reading. These are a function of sensitivity and instrumental stability. Since a few seconds (usually 10–30) of sample aspiration suffice for one reading, the total

amount of samples sprayed usually is 1–5 ml. Sodium, potassium, calcium, and magnesium, because of their relatively high concentration in biological materials, require high dilutions and only small samples of the original material, the minimum size of which is restricted only by the impracticality of measuring small volumes rapidly and accurately. Iron, copper, zinc, manganese, and other "trace" metals have to be determined on undiluted or even concentrated materials. In these cases, removal of proteins, ashing, or extraction of the analyte from blood, urine, or tissues will be part of the sample preparation for aspiration.

5.1. Sensitivity

Sensitivity or detection limit in atomic absorption spectroscopy has arbitrarily been defined as that concentration of a particular metal, usually in aqueous solution, to give an absorption signal of 1%. The limits were largely derived from work with single beam instruments where 1% absorption may represent the minimum signal distinguishable from a noisy background. The improvements of instrumental stability with double beam systems make it possible to read much smaller absorptions with certainty, and the limit of detection then is set by the limit of "readability." The possible use of scale expansion may enhance the detectability by one order of magnitude (K1).

The sensitivities for various elements differ considerably from each other, magnesium probably being the most sensitive element (0.01 ppm) (Z4), but with most metals satisfactory work can be done in the range of one to a few parts per million. When determination of a number of elements of varying sensitivities is desired in solutions containing mixtures of the analytes, it may occasionally become necessary to reduce the sensitivity for one or more elements without diluting the sample. It is clear, however, that sensitivity should not be lowered by changes in the optimal instrumental conditions, as such alterations not only would reduce sensitivity but also increase the curvature of working curves. Sensitivity reduction without working curve deterioration, in such cases, is best achieved by shortening the absorbing path of the flame, either by turning the burner head (W11) or by closing the burner opening partially with a suitable piece of metal.

While sensitivity reduction may be desirable only rarely, the necessity for sensitivity increases arises on numerous occasions and methods to this end have been studied by many. Double beam instruments and scale expansion techniques provide optimal experimental conditions and presently little gain can be expected from further instrumental improvements. Elongations of the absorption path for sensitivity increases

have been employed by Fuwa and Vallee (F10) by the use of the burner tunnel.

The effective absorption path length can also be increased by multiple passes of the light beam through the flame with a system of mirrors (W5). This or similar systems have been employed by several workers (B2, R9, Z1) and are incorporated into one commercial instrument (Jarrell-Ash). Actual sensitivity gains, however, seem to fall short of gains expected from a certain number of passes; Russell *et al.* (R9) with 12 passes achieved only a 6-fold sensitivity increase. Lang and Herrmann (L1) recently showed that the use of a mirror system has no significant influence on the signal to noise ratio.

5.1.1. *Organic Solvents*

The first to show the enhancing influence of organic solvents in flame photometry appear to have been Berry *et al.* (B5), who considered it an interference. Since then the use of organic solvents has been studied by many workers in emission flame photometry (B9, D11, D12, K4, W3) and sensitivity increases up to 100-fold (D11) have been reported, depending on the metal and the type of solvent used. Since atomizing conditions in emission and absorption are similar, sensitivity gains by the same means could also be expected in absorption. This was confirmed and extensively studied by Allan (A10), Lockyer *et al.* (L10), and Robinson (R3). Although sensitivity enhancements of emission have been obtained with organic solvents in discharge as well as total consumption burner-atomizers, the mechanism involved is thought to be different. Aspiration of an organic combustible liquid instead of water into the flame of a total consumption burner results in higher flame temperature. If an excitable metal is dissolved in the solvent, considerable emission enhancement can be expected since emission intensity depends exponentially on the flame temperature (Section 3.2). If too much of the solvent reaches the flame, unburned portions of it will lower the flame temperature and even a decrease of intensity might be observed. On the other hand, raising of the flame temperature does not contribute to increasing the population of atoms in the ground state, since many metals are completely atomized from aqueous solutions in most flames. Therefore, little sensitivity gain might be expected in absorption by the use of organic solvents in total consumption burners. The experimental findings of Robinson (R3) do not seem to corroborate this reasoning. Using a Beckman burner (total consumption type) with an oxygen-cyanogen flame, he found absorption enhancements for nickel up to 36-fold with a variety of solvents, while emission enhancement was

considerably smaller. The effect of self-absorption may be responsible for the latter. Since both, emission and absorption, increase, the enhancement cannot be due to a temperature effect only. One is led to conclude that, with organic solvents, a larger sample reaches the flame and atomization in the flame is more complete, due to smaller droplet size and faster vaporization. A scheme of the behavior of aqueous and organic solvent solutions in the flame is discussed by Robinson.

Allan (A10) conducted an extensive study of the effect of organic solvents on absorption in an air-acetylene flame, employing a discharge atomizer. In this system, where only a small fraction of the aspirated sample reaches the flame in the form of the smallest droplets, the concentration of free atoms in the flame can be increased mainly by a larger sample reaching the flame and by facilitating the dissociation of metal compounds into free metallic atoms in the flame. Allan was able to show that the rate of absorption enhancement achieved with one solvent was approximately the same for metals completely atomized in the flame (copper and zinc) and those forming compounds not completely dissociable (iron and manganese). Since the dissociation of these compounds is temperature dependent, flame temperature measurements were carried out concurrently with absorption measurements. While flame temperature, as compared to water aspiration, was found to be slightly lower during solvent aspiration (due to the necessary acetylene flow reduction), absorption increased with all four elements tested. The conclusion that absorption enhancements in the flame by organic solvents are indeed due to a larger sample reaching the flame was confirmed by an experiment in which the spray issuing from the burner slot was collected in glass wool and the eluate analyzed. When methylisobutyl ketone solutions of copper were aspirated, 3.35 times as much copper was found in the eluate as with aspiration of aqueous solutions. The absorption measurements achieved with the same organic solvents were 3.5 times that of aqueous copper solutions. Zettner and Seligson (Z3) obtained a 25–40% increase of sensitivity by the admixture of 7% butanol to aqueous calcium solutions (Fig. 4). Other studies conducted by Lockyer (L8) and Elwell and Gidley (E2), using a system with a separate spray chamber, showed that an admixture of isopropanol to aqueous solutions also was capable of enhancing sensitivity. For a variety of elements tested a sensitivity gain of 2–3-fold was reported, but there was considerable variation from element to element, iron exhibiting an enhancement of up to 10-fold. Allan (A10) states that the increases in sensitivity obtained with the solvents miscible with water would be counterbalanced by the sample dilution incurred in adding the organic solvent. The author feels that Allan's view is not

entirely justified, because in some instances solvent dilution of originally critically small samples would facilitate the analysis by providing sufficient volume for aspiration without loss of sensitivity. Furthermore, the use of solvent dilution would also be of advantage in samples containing a mixture of the test element and interfering substances. By adding the organic solvent the interference might be reduced to a tolerable level due to the additional dilution, while retaining if not increasing the sensitivity for the analyte. Zettner and Seligson (Z3),

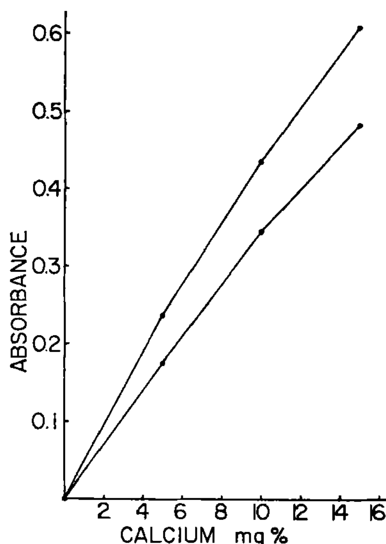

FIG. 4. Enhancement of calcium absorption by admixture of organic solvent to aqueous calcium solutions: —●—●— aqueous calcium standards; —○—○— aqueous calcium standards with 7% *n*-butanol (*upper curve*). Actual calcium concentrations are only one tenth of those given on abscissa (from reference (Z3) by permission).

while studying the effect of organic solvents, found that the addition of *n*-butanol to an aqueous diluent for serum considerably reduced the troublesome clogging of the burner opening by coagulated and half-burned protein. It was actually this elimination of burner clogging that made practical the development of a procedure for serum calcium without deproteinization. This effect may be related to changes in surface tension.

5.1.2. *Solvent Extraction*

The use of water-immiscible solvents for increase of sensitivity led to the development of the selective extraction method by Dean and his

associates (D11, D12). The method is based on the formation of a compound between the metal to be analyzed and a chelating agent added to the original aqueous solution, with subsequent extraction into a suitable organic solvent. The latter is then aspirated for analysis. Several advantages are apparent: (a) the selective extraction makes it possible to concentrate the desired element manyfold; (b) the concentrating effect is enhanced by the sensitivity increase that the solvent yields when aspirated into the flame; (c) interfering substances are left behind in the aqueous phase. Trace quantities far beyond the sensitivity in the original sample can thus be made accessible to rapid determination.

The method was applied by Allan (A10) to the determination of copper, zinc, manganese, and iron, which were quantitatively extracted from aqueous solutions after complexing with ammonium pyrrolidine dithiocarbamate (M1) into ethyl acetate or methylisobutyl ketone. With ethylamyl ketone an extraction ratio of more than 100 was achieved. The same chelator was used by Willis (W14) to extract lead, mercury, bismuth, and nickel from urine into methyl-*n*-amyl ketone with similar concentrating ratios and sensitivity gains. The strict requirements of element selectivity of the extraction methods as necessary for colorimetric work can be considerably relaxed in flame photometry, an advantage fully realized by Dean (D11). The absence of spectral interferences in atomic absorption permits accurate determination of one metal in the presence of many others, and therefore specificity of the extraction procedure is not needed. Furthermore, several metals can possibly be determined in one extract. The substituted dithiocarbamates, as described by Bode and Neumann (B6), permit chelation over a wide range of pH values. Since the sample preparations for extraction of biological materials in most cases entail hydrolysis or oxidation in acid solutions, chelation has to be carried out at very low pH values. Dean (D11) described the extraction of iron as the iron-acetylacetonate chelate at a pH of 0. When working with resonance lines of very short wavelength, the absorptive properties exhibited in the ultraviolet by many organic solvents should be taken into consideration. The enhanced excitation of other metals by organic solvents, which might lead to increased spectral interference in emission flame photometry, is not a limiting factor in absorption spectroscopy.

5.2. Interferences

Interferences can be defined as any physical or chemical agents capable of either increasing or decreasing the degree of absorption usually achieved with the test element in aqueous solution. All interferences in flame emission have one common effect, namely, changing

the state or number of excited atoms. In atomic absorption, interferences can act in two ways, i.e., by changes in the number of activated atoms in the ground state, or by attenuation of the monitoring light beam by processes other than atomic absorption. Fortunately, the latter is rarely observed and it is actually the high transparency of flames over a wide spectral range that makes absorption flame photometry feasible.

Spectral interference, a well-known common difficulty in emission flame photometry, arises from excitation of other metals with emission lines on wavelengths too close to be effectively separated from the line undergoing measurement. In addition, the emission bands of molecular compounds, notably metal oxides and hydroxides, often overlap with the atomic lines. There are no similar effects in atomic absorption, as different elements with common resonance lines are unknown except perhaps in the special case of isotopes (Section 5.4.3). A form of spectral interference is encountered with hollow cathode tubes emitting a complex spectrum, from which it may be difficult to separate the resonance line. This is circumvented by the use of high dispersion monochromators. Light emitted from the flame itself is eliminated from measurement by the modulation of the resonance line source or by chopping of the light beam. The possible interference from atomic fluorescence should also be mentioned here (Section 3.2). This effect, if significantly strong, would be particularly difficult to exclude from measurement, if resonance fluorescence is involved. The latter not only possesses the same wavelength but also the modulation frequency of the hollow cathode emission. Excitation interferences appear to be entirely negligible in absorption, since the fraction of excited atoms of the total population in most flames is insignificantly small (Section 3.2). A significant reduction of the number of atoms in the ground state, however, can be brought about by excessive ionization, as observed with the more readily ionized metals. The degree of ionization is exponentially dependent on flame temperature (Saha equation).

Potassium, rubidium, and cesium possess especially low ionization potentials and at the temperature of the commonly used air-acetylene flame, for instance 30–70% of the total number of these atoms may be ionized (D11, F6). The degree of ionization of an alkali metal, however, is reduced by the presence of other easily ionized elements. The admixture of such elements affords one means of controlling this type of interference.

5.2.1. *Anionic and Anion-like Interferences*

A number of anions forming refractory salts with metals retard the dissociation into free atoms in the flame. The result is a reduction in the

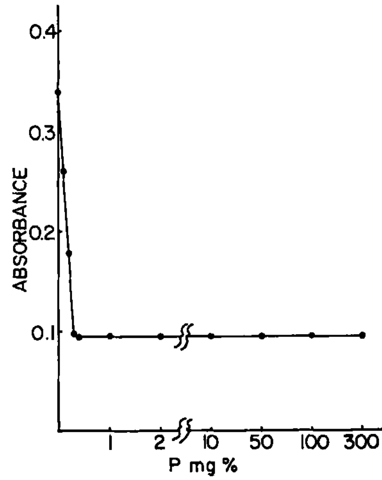

FIG. 5. The depressive effect of phosphate on calcium absorption. All samples contained 1 mg % calcium. Maximum depression is reached at 0.4 mg % phosphate (concentration as phosphorus) (from reference (Z3) by permission).

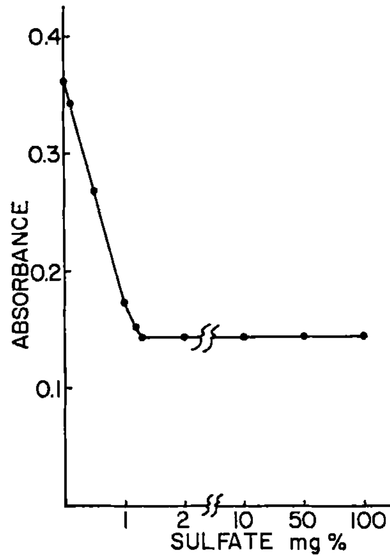

FIG. 6. The depressive effect of sulfate on calcium absorption. All samples contained 1 mg % calcium. Maximum depression is reached at 1.3 mg % sulfuric acid (from reference (Z3) by permission).

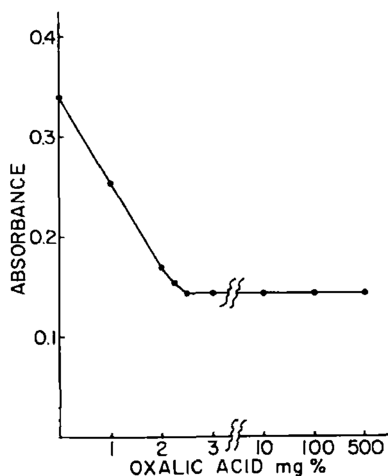

FIG. 7. The depressive effect of oxalic acid on calcium absorption. All samples contained 1 mg % calcium. Maximum depression is reached at 2.5 mg % oxalic acid (from reference (Z3) by permission).

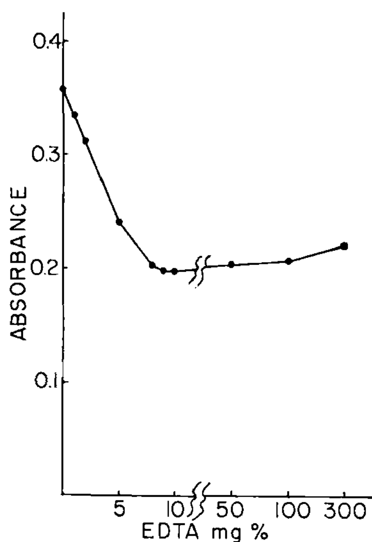

FIG. 8. The depressive effect of EDTA on calcium absorption. All samples contained 1 mg % calcium. Maximum depression is reached at 9 mg % EDTA. At higher concentration a moderate enhancement of calcium absorption is seen (from reference (Z3) by permission).

total number of vaporized atoms of the test element, including atoms in the ground state and in the excited state as well, and therefore a reduction in the emission and absorption intensity. This phenomenon has been known for many years in flame photometry. A crystal of calcium phosphate fails to color an alcohol flame (T2). Among all metals calcium appears to be most severely subjected to this type of anionic interference, and many other anions, i.e., sulfate, nitrate, aluminate, etc., are similarly effective. The phosphate effect is the most pronounced and has been studied by many workers. Dippel *et al.* (D15) based an indirect flame photometric method for the determination of phosphate on this anionic depression, and similar methods for other anions are certainly feasible (M9).

When a series of aqueous calcium chloride solutions is prepared with increasing phosphate content, and the absorbance readings obtained are plotted against phosphate concentrations, a curve results as shown in Fig. 5. Between phosphorus concentrations of 0 and 0.3 mg %, there is a sharp depression of the calcium signal with a linear relationship between phosphorus concentration and depression. Once maximum depression is reached, no further depression can be produced even with a 1000-fold excess of phosphate. Although the depression by phosphate is the strongest of all anions tested, very similar curves can be obtained with sulfate (Fig. 6), nitrate (P1), and oxalate (Fig. 7). Zettner and Seligson (Z3) also investigated the combined action of anions. As anticipated, phosphate-sulfate mixtures did not produce absorption depression beyond the expected maximal phosphate interference; an additive effect, however, was demonstrable with anion concentrations lower than those producing maximum depression with phosphate or sulfate alone. The behavior of phosphate-oxalate and sulfate-oxalate mixtures is similar. Experiments were also carried out with a chelator, disodium ethylenediaminetetraacetic acid (EDTA) (Fig. 8). A sharp depression is seen at low EDTA concentrations, but after the point of maximum depression is reached the curve again rises moderately. An interesting finding is the fact that proteins, made calcium-free by ion exchange, are capable of a similar characteristic absorption depression at surprisingly low concentrations, as shown in Fig. 9. At protein concentrations higher than 100 mg %, however, absorption depression becomes progressively less, and above 1000 mg % protein the calcium signal is even enhanced.

The mechanism of the anionic depression has been the subject of many investigations. It is generally believed that as the aerosol droplet dries up in the preheating zone or in the flame itself, a compound is formed between the metal and the anion which interferes with or retards the

production of the vapor of free, neutral atoms. Since the dissociation of such compounds is heat and time dependent, the magnitude of the depressive effect should be related to these two variables. This reasoning is well supported by experimental evidence. Anionic depression is most pronounced in cooler flames and anions forming salts with higher melting points are the strongest depressors (M6). For instance, calcium phosphate with a melting point of 1670°C, when introduced into a coal gas–air flame of 1800°C, will melt slowly; even at the temperature of the acetylene-air flame, 2200°C, disintegration of this compound will not be rapid enough to be complete near the base of the flame. Dissociation,

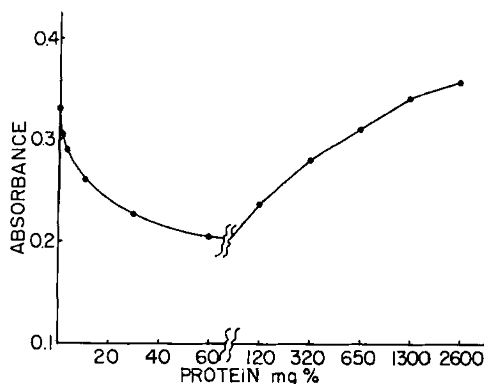

FIG. 9. The depressive effect of protein on calcium absorption. All samples contained 1 mg % calcium. The protein used was made calcium-free by ion exchange. Maximum depression is reached at approximately 80 mg % protein. Higher protein concentrations enhance absorption (from reference (Z3) by permission).

however, progresses as the particle travels upwards through the flame and it has, indeed, been shown, that the anionic depression is smaller or absent in the top of the flame (A4, F8). Vaporization of the aerosol particles is facilitated by decreasing their size. Filcek (F5) was able to abolish the depressive effect of phosphate on calcium almost completely by certain changes in the atomizer design, resulting in greater fineness of the aerosol. The size of the dry particles formed from the drying droplets in the aerosol, however, does not depend only on droplet size but also on the concentration of solutes. By lowering the concentration of calcium phosphate solutions, Gibson *et al.* (G3) were able to show that a minimum concentration can be reached at which no anionic depression is seen. The critical maximum particle weight at which complete and sufficiently fast disintegration in the flame is possible was calculated at 6×10^{-17} gram calcium phosphate.

If the test element and the depressant are sprayed separately into the flame, as was done by Alkemade and Voorhuis (A4, A5) and Fukushima (F8, F9), the depressive effect is not seen. This indicates that the relatively undissociable compound between analyte and depressant must be formed in the aerosol phase or while the droplets dry up. One wonders then, however, about the mechanism of the depressive action of organic substances such as oxalate, EDTA, and proteins that are capable of binding cations (Z3). These substances must certainly disintegrate rapidly in the base of the flame if not already in the preheating zone, certainly below the melting temperature of calcium chloride, 770°C; and yet the chloride anion has no depressive effect while the oxalate, EDTA, and protein are characteristic interfering agents. It may be that the organic particle before complete disintegration in the flame first undergoes some "charring" process during which release of the metal atoms is delayed. It is difficult to explain, however, the seemingly contradictory finding that low protein concentrations are more depressive than high ones. Clearly, further investigations are needed in this area.

5.2.2. *Matrix Interferences*

Various interferences may derive from changes in the composition of solution and flame matrices. The simplest matrix is encountered when an aqueous solution containing the analyte only is aspirated. In biological work this may very rarely be the case.

Relatively large increases of the viscosity of the sample are necessary to lead to reduced vaporization, but the effect is usually combined with changes in the surface tension. If the length of the aspirating capillary of the burner as used by the author (Z3, Z4) is increased, very little change in the absorption signal is seen although sample flow is reduced. For instance, an increase of the capillary length from 3 to 25 inches resulted in a gradual absorption change of only 2%, the longer capillary producing the higher absorption reading (Z2). The results with other atomizers are not necessarily the same (S8). Longer aspirating capillaries may be employed with advantage when working with critically small samples. Absorption readings become lower only when the capillary reaches a length just short of the point when the aspirating pressure is insufficient to aspirate any fluid at all.

The use of organic solvents, a significant change in solution matrix, has been discussed above.

Where discharge atomizers are used, the presence of acids and salts in the sample interferes with the evaporation of the aerosol (P4). The

mild depression of calcium absorption by sodium chloride (Z3) in physiological concentrations may be related to this.

When solutions of high salt content (2% or higher) are aspirated, the salt particles formed from the aerosol are of sufficient size to pass through the flame without disintegrating (W15). These particles are capable of scattering the light from the hollow cathode tube, which will show up in the measurement as erroneously high absorption.

The opacity of flames below 2000 Å, of course, can be considered a matrix effect and should be mentioned here as an obstacle in determining elements with resonance lines in the vacuum ultraviolet. Organic solvents are capable of imparting to the flame additional absorptive properties in the ultraviolet.

5.3. Interference Control

There are several different possibilities to circumvent, compensate for, or suppress interferences.

5.3.1. *Separation of the Analyte from the Solution Matrix*

This method with the attractive advantage that the test element at once can be isolated from all interferences, of course, is fully realized in the solvent extraction technique, although the latter appears to have been employed mainly for reasons of sensitivity problems. Separation by precipitation has been applied in sample preparations for atomic absorption spectroscopy (W9, Z3, Z4), but the presence of an anion in the precipitate capable of binding the cation again poses the problem of anionic suppression. It can be said in general that very accurate results are obtained with this method but where a great number of analyses is involved it may prove too cumbersome. Hinson (H5) described the removal of depressing anions by an ion-exchange technique. Leyton (L6) suggested the use of ion exchange to remove calcium by absorption onto a resin followed by elution for analysis.

5.3.2. *Addition in Excess*

This method used extensively in flame photometry is based on the principle that the interfering agents are added in equal but large amounts to standards and samples alike, and that all determinations are carried out in their presence. The curves obtained with the anionic depressors (Section 5.2.1) indicate that this approach could be used even in cases where the approximate concentration of the interfering agent in the samples is not known. In practice, however, sensitivity is considerably

curtailed and working curves appear to be less linear. Small interferences like that of sodium on calcium absorption, can easily be compensated for by this method.

5.3.3. *Protective Chelation*

If a strong chelator is added to a sample containing the test element and anionic depressors, the metal is preferentially bound by the former and thereby kept from interacting with the anion in solution or in the aerosol phase. When aspirated, the metal is released from the disintegrating chelator in the flame where the anions are incapable of acting upon

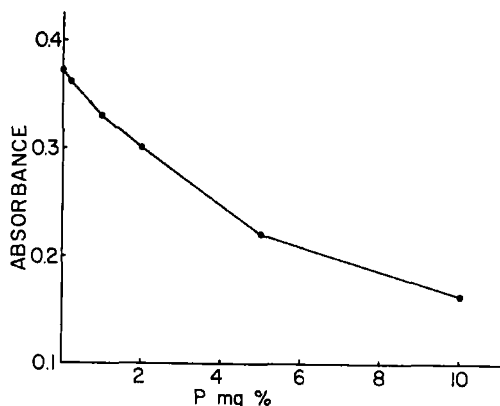

FIG. 10. The addition of 0.65% calcium-free protein to 1 mg % calcium solutions reduces the depressive effect of phosphate on atomic absorption of calcium (compare with Fig. 5). Phosphate concentrations given as phosphorus (from reference (Z3) by permission).

the metal. This method was applied successfully in emission (W4) and absorption (W9, W10, Z3). It should be kept in mind, however, that chelators themselves act as depressants (see Fig. 8). The troublesome clogging of the burner slot with carbonaceous crusts, whenever high concentrations of a large molecular organic compound are aspirated, could make this method impractical for prolonged use.

There is considerable evidence that the concept of protective chelation may not be a correct one. Far greater concentrations of EDTA than molar unity between EDTA and calcium are needed for full protection (W4). Furthermore, the action of EDTA is independent of the pH of the solution but dependent on its cation, the sodium salt being more active than the ammonium salt. Baker and Garton (B2) reasoned that the dry particles deriving from the aerosol consist mainly of a matrix of EDTA

in which calcium or other solution constituents are evenly dispersed. In the flame the organic matrix disintegrates rapidly, and when the metal is released the production of the metallic vapor is greatly accelerated by the high dispersal of the cation in the original particle. Thus, the effect of EDTA in abolishing phosphate depression would not be related to its chelating properties, but simply to the formation of a bulky matrix easily decomposed in the flame. This reasoning is supported by an earlier finding that with very fine aerosols the anionic depression is markedly reduced (A4). In order to prove their assumption, Baker and Garton replaced EDTA by sodium chloride or sucrose in similar concentrations (1–2%), both of which were as effective as the chelator in abolishing phosphate depression. Zettner and Seligson (Z3) showed that the addition of calcium-free protein to calcium phosphate solutions diminished the phosphate effect significantly, although absorption depression was not completely eliminated (Fig. 10).

5.3.4. *Competitive Cation Technique*

Following the observation of Mitchell and Robertson (M9) that the anionic depression of calcium emission in the flame could be abolished by the addition of strontium, many other cations were found to have similar effects. Lanthanum, neodymium, samarium, yttrium, magnesium, beryllium, barium, scandium, iron, and other cations are also capable of releasing calcium and other metals either completely or partially from the depressive action of anions (D14, W6, Y1). It is thought that the releasing or protecting mechanism depends on the competition between the cations for the anionic depressants during salt formation as the droplets of the aerosol evaporate. In order to suppress the anions completely, that is, to keep them from interacting with the test element, the releasing cation has to be added in large excess over the concentration of both, of the metal to be determined and of the interfering anion. Since the depressive action of the anions does not originate from compound formation in the flame, but rather in the aerosol (A4, F8, F9), the protective action of competitive cations cannot be due to the refractoriness of their compounds in the flame, but must depend on other factors such as their concentration in the solution and the solubility of the salts they form with the depressant anions. For instance, if an excess of lanthanum chloride is used to control the depressive effect of phosphate on calcium (Fig. 11), the drying salt particles forming from the aerosol will consist of lanthanum chloride, lanthanum phosphate, and calcium chloride, the latter being readily dissociable in the flame. The true competitive nature of this phenomenon is discussed by Dinnin (D14).

Where applicable, the use of competitive cations for the control of anionic depressors appears to represent the method of choice. No spectral interference arises in atomic absorption from the addition of another cation, an objection often raised in emission. The concentrations of the added salt required for full anion control usually are less than 1%, a salt level well below that at which light scattering is observed. When working with serum, denaturation or precipitation of proteins may occur from the addition of high concentrations of lanthanum chloride or other salts, and the concomitant changes in solution properties should be taken into

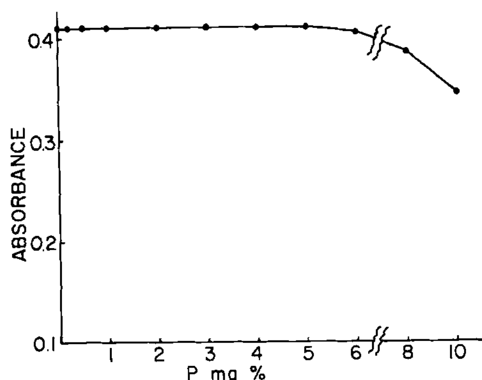

Fig. 11. The protective action of 0.5% lanthanum chloride on atomic absorption of 1 mg % calcium against the depressive effect of phosphate. Compare with Fig. 4 (from reference (Z3) by permission).

consideration (Fig. 12). Lanthanum chloride causes a true enhancement of calcium absorption (Fig. 13), a phenomenon not entirely explained but possibly related to the reduction of calcium oxidation in the flame (Z2).

5.4. Individual Elements

In the following subsections the application of atomic absorption spectroscopy to the determination of the more important elements of biological and clinical interest is presented, and special problems and interferences encountered with individual elements are discussed in detail. The resonance lines given at the beginning of each subsection are those showing greatest absorption, although many elements possess several resonance lines that can be used in analysis. The sensitivity limits quoted are the lowest reported in the literature, usually defined as that concentration of the test element in aqueous solution which produces 1% absorption. The reproducibility of results by most atomic absorption techniques lies

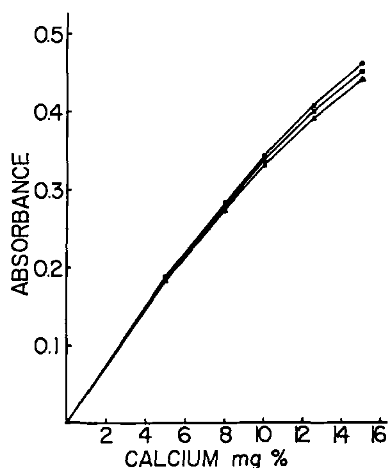

FIG. 12. Protein in the presence of 0.5% lanthanum chloride slightly depresses atomic absorption of calcium: —●—●— 0.1% protein; —■—■— 0.4% protein; —▲—▲— 0.8% protein. Calcium concentrations of samples actually aspirated were only one tenth of those given on the abscissa (from reference (Z3) by permission).

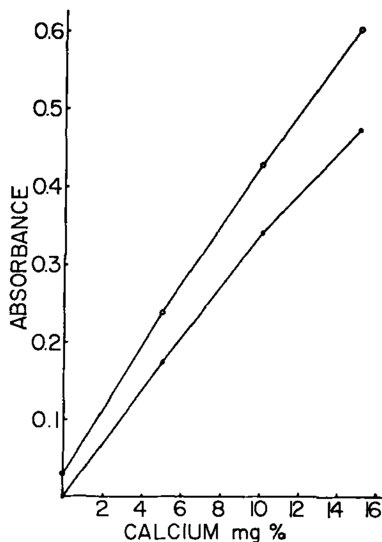

FIG. 13. Enhancement of calcium absorption by lanthanum chloride: —●—●— aqueous calcium standards; —○—○— aqueous calcium standards with 0.5% lanthanum chloride (*upper curve*). (Calcium concentrations of samples actually aspirated were only one tenth of those given on the abscissa (from reference (Z3) by permission).

between 1 and 2% but in many cases is better than 1%. Where available, graphs of typical working curves are shown for some of the individual elements.

5.4.1. *Sodium*

Resonance Line: 5890 Å

Sensitivity Limit: 0.03 ppm (G2)

The determination of sodium by atomic absorption spectroscopy has been applied successfully by several workers using a variety of equipment. The first element to be determined by Alkemade and Milatz (A2), in fact, was sodium. While sensitivity in emission is slightly higher for this metal than sensitivity in absorption, sodium still counts as one of the most sensitive elements in atomic absorption spectroscopy. The absence of any spectral interference (P3) and the relative freedom from other interferences appear to offer promising advantages of absorption over emission also for this element.

Sodium is completely atomized in the flame and since the higher energies of hot flames are not needed for excitation, a cool flame such as the coal gas–air flame is sufficient and perhaps superior because the appreciable ionization of sodium in the hotter flames is avoided.

Most workers used a vapor discharge lamp as the sharp-line source but sodium hollow cathode tubes are also available. The relatively short lifetime of the latter owing to the high volatility of sodium and its rapid loss from the cathode is, however, a limiting factor. Since both light sources at higher currents show considerable line broadening due to self-reversal, most workers recommend a low operating current. Because of the strong emission of these light sources at the yellow sodium doublet and the absence of other strong lines, wavelength selection with filters has been shown to be sufficient (M2). In the preparation of tissues for analysis, the loss of sodium and potassium during dry-ashing has to be considered (G8).

David (D5) employed an air-acetylene flame for the determination of sodium in soil extracts with a detection limit of 0.05 ppm. No effect was seen from the presence of phosphate, aluminum, sulfate, and silicate exceeding the sodium level 100–200-fold.

Malmstadt and Chambers (M2), using an air-propane flame, obtained high accuracy by their standard addition method in the 1–100 ppm range with their nullpoint instrument. It is stated that a small decrease of sodium absorption was found when potassium equaled or exceeded the sodium concentration, and that depression was also seen with high con-

centrations of hydrochloric and sulfuric acids. Samples and standards contained 25% isopropyl alcohol.

Robinson (R1) worked with an oxy-hydrogen flame in which interferences appeared to be less than in other flames, as no interference was seen from a 100–500-fold excess of potassium or lithium.

Willis (W11), employing a sodium hollow cathode lamp, determined sodium in blood serum. With a 10-cm long flame, sensitivity was so high as to make the necessary dilution (500-fold) impractical and subject to contamination. He reduced sensitivity simply in shortening the absorption path by turning the flame 90 degrees, but he also showed that the use of another, much weaker resonance line at 3302 Å reduced dilution requirements to only 10-fold. No interferences were encountered from any of the serum constituents.

Herrmann and Lang (H3) studied various atomizers and recorded best results with a laboratory-built high pressure vaporizer. No ionization interference was seen in an air-propane flame and calibration curves were straight from 1 to 10 mg sodium per liter. Determinations were performed on serum diluted 1:20–1:200 and results agreed well with those concurrently obtained by emission flame photometry.

5.4.2. *Potassium*

Resonance Line: 7665 Å

Sensitivity Limit: 0.03 ppm (G2)

Some of the workers who published absorption methods for sodium also include data on potassium. While certain differences exist between sodium and potassium in regard to sensitivity and interferences, the equipment used by some authors was of the same simplicity for both. Filters and barrier-layer photocells suffice, but when using photomultiplier tubes their reduced sensitivity in the red, where the potassium resonance line is located, has to be taken into account.

Potassium is one of the more easily ionized metals and the type of flame used will be of even greater influence than in sodium work. Since the degree of ionization depends also on other solution constituents, i.e., alkali metals, significant interference from sodium can be expected. The relative enhancement of potassium emission and absorption is shown by Baker and Garton (B2). Atomization of potassium in the flame, however, is not only reduced by ionization but also by compound formation, notably hydroxide.

David (D5) determined potassium in the air-acetylene flame in ammonium chloride extracts of soil and found no interference from a 40-fold excess of phosphate, aluminum, sulfate, and silicate.

Malmstadt and Chambers (M2) with their nullpoint instrument and standard addition method obtained high accuracies; they state that no interference was seen from an excess of sodium. The latter is difficult to understand since the degree of mutual ionization depression between alkali metals at a particular flame temperature can be predicted (F6), and has been experimentally demonstrated in absorption with flames of comparable or even lower temperature (B2, W11). On the other hand, the use of the standard addition method may have minimized the sodium interference and, since the mutual enhancement effect in absorption seems to be smaller than in emission (B2), it may have been negligible in the measurements.

Willis (W11), using a potassium hollow cathode tube instead of the commonly employed discharge lamp, determined potassium in blood serum. At the 1:50 dilution no interference was encountered from calcium, magnesium, and phosphate at serum levels, but sodium gave a small enhancement. The sodium interference was controlled by the addition in excess of sodium chloride or of the disodium salt of EDTA to samples and standards alike.

Herrmann and Lang (H3) also determined potassium in blood serum. Calibration curves were straight from 1 to 100 mg sodium per liter. One wonders about the sensitivity of their technique since it is stated that the serum dilution used was 1:5.

5.4.3. *Lithium*

Resonance Line: 6708 Å

Sensitivity Limit: 0.03 ppm (G2)

Lithium ranks with the easily excited metals and is also distinguished by high sensitivity in absorption. Alkemade and Milatz (A2) determined lithium by an absorption flame photometric method, but instead of employing a lamp as light source, a second flame was used into which lithium was aspirated. The radiation from this exciter flame was passed through the second flame functioning as absorption cell.

Lithium is also one of the very few metals to form isotopes with significantly shifted resonance lines, the difference amounting to 0.15 Å. Zaidel and Korennoi, as quoted by Gilbert (G6), were able to exploit the isotopic shift of the resonance line in the analysis of an isotopic mixture of Li^6 and Li^7. With a Li^7 hollow cathode lamp, Li^7 was determined in solutions containing both isotopes when aspirated into an acetylene flame.

Manning and Slavin (M3) also reported lithium isotope analysis by atomic absorption. The emission of an open, lithium-containing oxygen-

hydrogen flame was used as line source. Despite considerable line broadening in the flame, measurement of Li^6 in the presence of a large amount of Li^7 was possible, although sensitivity was less than that reported for a hollow cathode source (Fig. 14). The spectrum of lithium at 6708 Å consists of a doublet whose lines are separated by 0.15 Å. Since the isotopic spectral shift between Li^6 and Li^7 is also 0.15 Å, one line of the Li^6 doublet is superimposed on one of the doublet of Li^7. This represents true spectral interference, a very rare occurrence in atomic absorption.

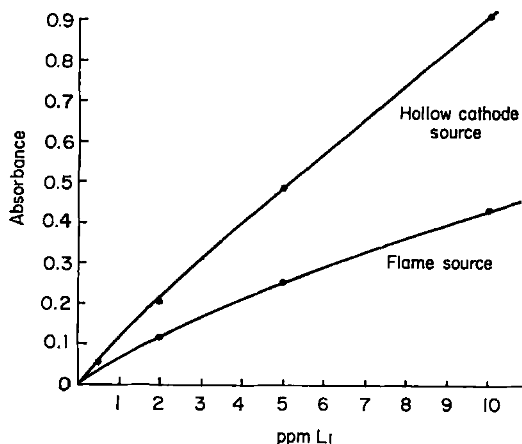

FIG. 14. Absorbance-concentration curves obtained with aqueous lithium solutions. Note the difference in sensitivities depending on the type of line source (from reference (M3) by courtesy Perkin-Elmer Corporation).

The difficulty was overcome by adding Li^7 in excess to the solutions, whereby one line of the Li^6 doublet was virtually completely absorbed and measurements could be carried out on the other line of the doublet.

5.4.4. *Calcium*

Resonance Line: 4227 Å

Sensitivity Limit: 0.06 ppm (Z3)

The development of fast and accurate procedures for the determination of calcium in biological materials represents one of the important early achievements of atomic absorption spectroscopy. The difficulties encountered with calcium in emission flame photometry are well known (D11, L6, S6, S10), but spectral interferences and extreme dependency on flame temperature, serious obstacles in emission, are either nonexistent or of lower importance in absorption. Chemical interferences, however,

are equally severe and it is for this reason that calcium is one of the most difficult elements to deal with in atomic absorption spectroscopy.

The choice of flame appears to be important and best results were obtained with the air-acetylene flame (Fig. 15). For optimal sensitivity, it is necessary to work with a fuel-rich, reducing flame, because of considerable oxide and hydroxide formation in lean flames, an effect even more pronounced with other elements (S4). The concentration of neutral calcium atoms is greatest in a narrow, clear zone of the flame just above

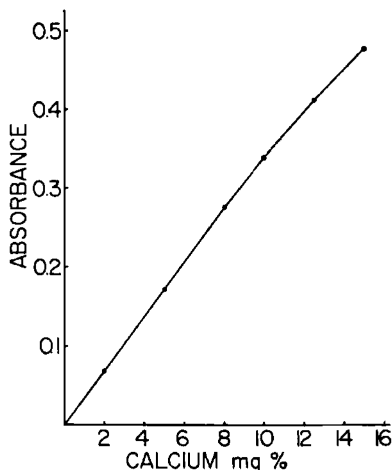

FIG. 15. Typical absorbance-concentration curve obtained with aqueous calcium solutions in 10-cm air-acetylene flame. Note the slight deviation from linearity. Actual calcium concentrations of standards aspirated were only one tenth of those given on abscissa (from reference (Z3) by permission).

the luminous base cone (see Fig. 2), and it is recommended that all measurements be performed in this zone despite the greater interference from anionic depression. A similar distribution of calcium atoms is observed in the air–coal gas flame, but while sensitivity is lower, the anionic effects are even greater and more difficult to overcome than in the air-acetylene flame. If possible, the use of the air–coal gas flame for calcium should be avoided, unless the samples are free of anionic depressors.

David (D3) determined calcium in plant materials, employing an air-acetylene flame. After wet-ashing of the samples, magnesium and sulfuric acid were added to overcome effects of anions. It was demonstrated that high concentrations of phosphate, aluminum, and silicate were completely controlled. In soil samples David (D5) determined calcium by extraction with ammonium chloride, but used strontium and lanthanum

chloride to suppress the effect of phosphate, aluminum, sulfate, and silicate. Methods for calcium in animal feeds and tissues are described by Parker (P1). A strong depressive effect of nitrate on calcium absorption is shown.

Willis (W8, W9) studied the behavior of calcium in various flames and found the air-acetylene flame most suitable for absorption work. He investigated the influence of phosphate, sodium, and protein and found the expected anionic depression by phosphate, but ascribed a mild enhancing effect to sodium and protein, the latter also reducing phosphate depression. Interference control was obtained by dilution of the sample with strontium or lanthanum chloride, or the sodium salt of EDTA. The results were related to those obtained by the Clark-Collip method and to analysis of calcium oxalate precipitates and deproteinized samples by the atomic absorption method. Although acceptable results could be obtained by all modes of sample preparation, deproteinization and dilution with strontium gave the most accurate values. The results by atomic absorption spectroscopy generally were 2% higher than those by the Clark-Collip method, which was related to incomplete precipitation of calcium by oxalate. Willis (W13) also reported the determination of calcium in urine. Because of the occasionally very high phosphate content, high concentrations of lanthanum or strontium chloride had to be used in the diluent. The present writer feels, however, that the composition of urine is too variable in respect to anions, sodium, and protein to permit a simple dilution technique. Separation of calcium by oxalate precipitation is preferable wherever accurate results are desired.

Newbrun (N2) reported the determination of calcium in saliva by atomic absorption. When samples were diluted with water only, results were obviously too low, but with strontium, lanthanum, or EDTA the values agreed with those obtained by oxalate permanganate titration. Calcium was higher in the excretion from the submaxillary glands than in that from the parotid. Depletion of saliva calcium content was not seen after 15-minute stimulation.

Decker *et al.* (D13) analyzed cerebrospinal fluid for calcium by atomic absorption. Excellent results are claimed on samples diluted 1:20 with water only, but anionic depressors are not mentioned.

Zettner and Seligson (Z3) conducted an extensive study of calcium interferences deriving from serum constituents and other substances. In the air-acetylene flame, no effect was seen from excess concentrations of the ions of potassium, ammonium, magnesium, chloride, bicarbonate, and hydroxide. Phosphate, sulfate, oxalate, and EDTA acted as strong anionic depressors. Sodium caused a small but distinct depression of

about 3%. The effect of calcium-free protein was seen to be rather complicated, low concentrations acting like anionic depressors, but concentrations above 1% enhancing calcium absorption. The combined action of interfering agents was also investigated. Mixtures of phosphate and sulfate showed an additive effect only at concentrations lower than those producing maximum depression with phosphate or sulfate alone. Mixing of phosphate and protein resulted in reduction of the expected depression, but the degree of depression progressed with phosphate concentration (see Fig. 10). Interference control was achieved with the competitive cation technique and by incorporating sodium and calcium-free protein into the calibrating solutions. With a diluent consisting of lanthanum chloride, butanol, octanol, and hydrochloric acid, accurate calcium determinations could be carried out on serum samples directly diluted 1:10 or 1:20. Proof of method was obtained from comparisons with the Clark-Collip method, from ashings and recovery experiments, but mainly from oxalate precipitation studies. It was possible to show that calcium precipitation at pH 4.2 in the cold is virtually complete, contrary to statements often repeated in the literature. When the supernatant was evaporated, dry-ashed, and taken up in a small volume of diluent, the amount of calcium found was only slightly higher than that expected from the solubility product of calcium oxalate.

5.4.5. *Magnesium*

Resonance Line: 2852 Å

Sensitivity Limit: 0.005 ppm (Z4)

At least sixteen papers have appeared up to the present time on the determination of magnesium by atomic absorption spectroscopy and nine of these deal with biological materials. This pronounced interest undoubtedly derives from the fact that while wet chemical (H7) and flame emission methods (A1, D11, F4, M10) are unsatisfactory in many respects, atomic absorption allows for rapid and accurate analysis of magnesium with sensitivities unexcelled with other elements (Fig. 16).

Magnesium, like calcium, is subject to the effect of anions, although to a lesser degree. The most serious interference derives from refractory acidic oxides formed in the flame from a number of elements, particularly aluminum and silicon. The effect of phosphate and sulfate is much less marked than with calcium (Fig. 17), but if aluminum or silicon is also present in the solutions, magnesium depression is much more severe than with either of these interfering agents alone. These interferences can be overcome by the addition of strontium, lanthanum, or calcium. Leithe and Hofer (L4, L5) showed that magnesium could be determined

in various industrial materials with a magnesium-aluminum ratio of 1:1000, if calcium was added in excess of 2.5 times the amount of aluminum. No interferences were seen from iron, copper, manganese,

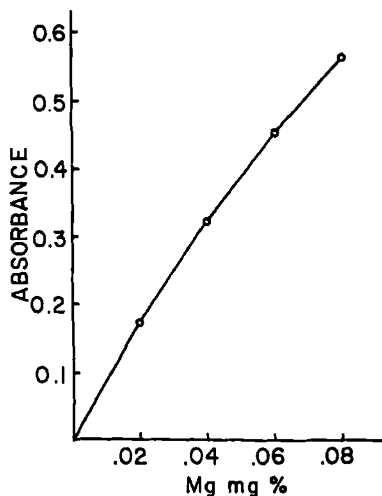

Fig. 16. Typical absorbance-concentration curve obtained with aqueous magnesium solutions in 10-cm air-acetylene flame. Note the slight deviation from linearity (from reference Z4).

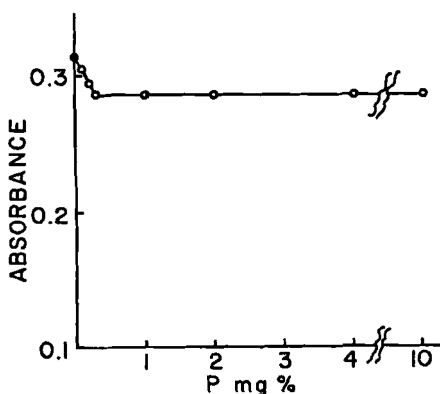

Fig. 17. The depressive effect of phosphate on magnesium absorption (compare with Fig. 4). Phosphate concentrations given as phosphorus (from reference Z4).

chromium, and zinc. Phosphate and sulfate effects were marked in the air–coal gas flame, but negligible in the air-acetylene flame. Andrew and Nichols (A14) demonstrated that the aluminum and silicon interference was overcome by the addition of nickel, and thus were able to analyze

nickel alloys for magnesium. Strontium was used by Belcher and Bray (B3) for the suppression of aluminum interference in the analysis of iron for magnesium. Wallace (W1) was able to investigate aluminum alloys by complexing the magnesium with 8-hydroxyquinoline.

Allan (A6) in determining magnesium in plant materials encountered interference from aluminum. The addition of 10% sodium acetate had no effect on absorption. David analyzed plants (D2) and soils (D5) and used lanthanum and strontium for the suppression of interferences.

Wünsch and Teicher (W21) estimated magnesium in plant substance after dry- and wet-ashing. In the propane flame, depression from phosphate, sulfate, and aluminum was seen, but in the acetylene flame only aluminum had such an effect.

Several papers deal with magnesium determination in blood and urine. Willis (W10) analyzed serum in the air-acetylene flame and found no effect from the presence of sodium, potassium, calcium, or phosphate, but states that an enhancement was seen in serum diluted with water only, probably due to serum proteins. This interference was controlled by addition of strontium or EDTA. Sensitivities were the same in the coal gas–air and air-acetylene flame, indicating complete atomization of magnesium. In urine (W13) no interference was encountered and determinations were performed on samples directly diluted with water.

Dawson and Heaton (D10) determined magnesium in plasma and urine. A water-cooled burner was used with an air-acetylene flame. Good agreement was found with an ammonium phosphate precipitation method when allowance was made for the loss of magnesium during precipitation.

Stewart *et al.* (S8) estimated magnesium in serum and urine. Of four different methods of sample preparation (i.e., wet-ashing, deproteinization, simple dilution with water, and dilution with hydrochloric acid), deproteinization with trichloroacetic acid was found to be most satisfactory. No interference was seen from sodium, potassium, or phosphate, but sulfate produced depression. With protein a 6% decrease in the apparent magnesium concentration was seen. Calcium and sulfate were added to standards and samples to control sulfate depression.

Horn and Latner (H6) analyzed blood and urine, using an air-propane flame. No interferences were encountered from other serum constituents. The fuel mixture of the flame was found to be critical. Decker *et al.* (D13) also include data on magnesium in cerebrospinal fluid.

Zettner and Seligson (Z4) determined magnesium in serum, using an air-acetylene flame. A study of interferences showed that phosphate and

sulfate produced a slight depression at magnesium levels comparable to those in serum diluted 1:50. Lanthanum chloride was used for interference control. Occasional lanthanum samples from commercial sources were inapplicable because of high magnesium contamination.

Cheek *et al.* (C1) used an atomic absorption spectroscopy method to analyze rat skeletal and cardiac muscle for magnesium. Griffith *et al.* (G7) analyzed liver, kidney, muscle, heart, plasma, and bone of growing chicks for magnesium levels following the addition of fluoride to the diet. Only the bone magnesium content was found to be increased. Dietary supplement of magnesium, however, increased magnesium in plasma, bone, liver, and kidney, but magnesium in muscle and heart was constant regardless of dietary intakes. Parker (P1) described methods for the determination of magnesium in animal feeds and tissues. Only minor effects were seen from hydrochloric and nitric acids.

5.4.6. *Copper*

Resonance Line: 3247 Å

Sensitivity Limit: 0.1 ppm (A12)

Copper is distinguished by a complex spectrum and although the 3247-Å line is the most sensitive resonance line, absorption can also be observed on several other lines. Copper is completely atomized in the flame. Allan (A10) made use of this fact in his study of organic solvents, in which he could show that sensitivity increase was not due to better atomization in the flame but related to an increase in the size of the sample reaching the flame. For solvent extraction, the metal was complexed with ammonium pyrrolidine dithiocarbamate. Copper is remarkably free of interferences but the complex spectrum requires high resolution monochromators for adequate wavelength separation. The metal itself may become an interference, as in the instance of Gidley's work on zinc (G4) during which some enhancement was encountered when hydrochloric acid was sprayed. This was later shown (G5) to be due to absorption by copper atoms liberated from the brass burner by the acid. Since a brass hollow cathode tube was used, also emitting a copper spectrum, absorption took place on weak copper lines at 2024, 2165, and 2182 Å, in the vicinity of the zinc resonance line at 2138 Å. This interference was eliminated by neutralizing the samples or by using a steel burner.

Details of copper determination are also discussed by Menzies (M6) and Strasheim *et al.* (S11). Elwell and Gidley (E2) examined the effect of many elements including silicon and found no interference on the absorption of copper.

Allan (A9) described the analysis of soils, fertilizers, and plants by acid extraction, or chelation with ammonium pyrrolidine dithiocarbamate and subsequent solvent extraction. No interferences were encountered. A photographic technique for the selection of the most suitable resonance lines is described.

Berman (B4) reported the clinical application of atomic absorption spectroscopy in the determination of copper in blood, urine, and tissues. After acid digestion of the samples, copper was complexed with EDTA and diethylthiocarbamate and extracted into a ketone. Copper levels in blood and urine from normal persons were compared to those found in patients with neurological disorders.

Zeeman and Butler (Z1) determined copper in wines. Following acid digestion and dry-ashing the samples were taken up in nitric acid and aspirated. None of the elements found in the ash, when tested individually, had any effect on copper absorption, but when synthetic ash solutions were investigated, containing a combination of the ash constituents, absorption depression occurred. This effect had to be compensated for in the calibrating solutions.

5.4.7. *Manganese*

Resonance Line: 2794.8 Å

Sensitivity Limit: 0.05 ppm (A12)

Manganese possesses a spectrum of considerable complexity with multiple ground state lines and, therefore, absorption can be observed on several lines. The fact that the strongest lines in emission are not always the strongest in absorption is well documented for manganese by Allan (A7) in his photographic study of emission and absorption spectra of flames. In order to find the most sensitive line for absorption measurement, Allan positioned a hollow cathode tube and a burner in front of a spectrograph, the light beam traveling through the flame before passing into the entrance slit. In this way photographs of the emission spectrum of the hollow cathode tube could be obtained while distilled water or a series of calibrating solutions of manganese were aspirated into the flame. When the differences in line intensity of the various spectrograms were related to the manganese content of the calibrating solutions, quantification of absorption intensity was possible. Emission spectrograms from the flame alone with the same set of calibrating solutions were used to correct for flame emission on manganese lines. It was found that manganese in the air-acetylene flame emitted at 4030 Å but not at 2800 Å, while the intensity of absorption at 2800 Å exceeded 10-fold that observed at 4030 Å. Absorption at 2800 Å takes place at three lines of a multiplet of varying absorption

intensity. Absorption is strongest at the 2794.8-Å line and for optimal sensitivity and pertinence of Beer's Law, and the next line at 2798.3 Å has to be separated as much as possible by the monochromator.

Manganese is not completely atomized in the flame (A10), which is reflected by the sensitivity differences in air-acetylene and air–coal gas flames. Elwell and Gidley (E2) report that large excesses of sodium, potassium, copper, lead, iron, nickel, tin, and zinc have no effect on manganese absorption while severe depression was seen with silicon. The moderate depression encountered with calcium, magnesium, aluminum, titanium, and zirconium is in contrast to interference studies of Allan (A7), who found no effect from phosphate, calcium, and magnesium. These differences in the influence of calcium and magnesium on manganese absorption appear to be related to the different behavior of these elements in the air–coal gas (E2) and air-acetylene flame (A7).

Only a few reports have appeared up to the present time on the application of atomic absorption spectroscopy to the determination of manganese in biological materials. Allan (A7) analyzed plants after wet-ashing with nitric and perchloric acids, and a similar project including a study of interferences was carried out by David (D9). A manganese recovery experiment from human plasma is reported by Manning (M2a).

Owing to the low manganese content of tissues and biological fluids, considerable concentrating of materials by ashing or solvent extraction will be necessary when determinations of this element by atomic absorption are considered. Hedge *et al.* (H1) studied manganese levels in human heart muscle and blood serum by spectrographic analysis and showed that following myocardial infarction serum manganese rose 2.5 times above the normal level (1 microgram/100 ml serum). In view of renewed biological interest in trace metals, it can be anticipated that the determination of manganese by as fast and accurate a method as atomic absorption spectroscopy may attain new significance.

5.4.8. *Iron*

Resonance Line: 2483 Å

Sensitivity Limit: 0.1 ppm (A12, G2)

Iron also has a very complex spectrum and most other points that have been made in regard to manganese apply to this metal. The choice of the most sensitive among the many absorption lines again was made with the help of the photographic technique by Allan (A7). The strongest line is that at 2483.3 Å at which sensitivity limits of 0.1 ppm have been obtained, but the line at 3720 Å still permits the detection of iron at the 1 ppm level. Especially narrow slit width and high resolution monochromators are necessary for optimal results, because the resonance

line at 2483 Å and the next line at 2488 Å are of different absorption intensity and, therefore, have to be resolved from each other. Emission on these lines from iron in the air-acetylene flame is negligible.

Allan (A7) during his studies of ashed plant materials also investigated interferences. Using an air-acetylene flame, sodium, potassium, calcium, magnesium, and phosphate had no effect. In the air–coal gas flame, as employed by Elwell and Gidley (E2), recoveries of iron were only 80–90% when the test solutions contained an excess of calcium, copper, aluminum, titanium, and zirconium. With silicon added, iron recovery was 26%. Owing to incomplete vaporization of iron in the flame, sensitivities attained are higher in the air-acetylene flame and lower in the air–coal gas flame. Since iron is subject to oxidation in the flame, fuel-rich flames are preferable.

David (D9) determined iron in plants and gives detailed instructions for the treatment of plant samples, entailing acid digestion and filtration before aspiration. The determination of iron in protein solutions is briefly mentioned (M2a).

Clinical uses of atomic absorption spectroscopy in the analysis for iron have not yet been published and the low iron levels in most biological fluids may very well be limiting. An interesting clinical application was demonstrated by Briscoe (B8), who determined by atomic absorption spectroscopy iron excretion in the urine of a patient treated with desferrioxamine for hemochromatosis.

The determination of iron in serum is of great clinical interest and, despite the existence of sensitive colorimetric methods, a direct and accurate method of the reliability of atomic absorption spectroscopy would be desirable. Unfortunately, serum iron levels range near the sensitivity limit of this method, and either very large samples and solvent extraction, or a 100-fold increase of sensitivity, would be required. Useful applications can be expected in the analysis of tissues of normally high iron content and in hemochromatosis. Another important application is anticipated in the determination of iron in hemoglobin, where atomic absorption spectroscopy may be a welcome asset in hemoglobin standardization. Only 0.01 gram of hemoglobin of original sample would be required to yield 5 ml of a solution with an iron content suitable for the sensitivity range of the method.

5.4.9. *Zinc*

Resonance Line: 2139 Å

Sensitivity Limit: 0.03 ppm (A12, G2)

Zinc exhibits very favorable characteristics in its determination by

atomic absorption spectroscopy. Early workers showed its high sensitivity (R9) by this method and the limit of the detection has since been lowered (A12, G2) considerably. This compares with extremely low sensitivity of zinc in emission flame photometry in which the detection limit is 500 ppm in an oxygen-hydrogen flame (D11).

Zinc is completely atomized in the cool air–coal gas flame, but in hotter flames, where a weak emission continuum appears, some compound formation takes place. For this reason highest sensitivities are observed in the air–coal gas flame (A11, G2).

Zinc in atomic absorption spectroscopy is remarkably free from interferences as contrasted to the difficulties encountered in polarography or with colorimetric methods (M4). Gidley and Jones (G4, G5) studied the influence of 27 elements and the only effect seen was a depression with silicon. The absorption enhancement encountered by these authors with haloid acids could be traced back to the attack of the brass burner by the samples and to the use of a brass hollow cathode tube as zinc line source. Methods for the determination of zinc in various metals and alloys are described by these authors.

David (D2) as early as 1958 made use of the high sensitivity of zinc in determining this metal in plants, following acid digestion with a sulfuric and perchloric acid mixture and nitric acid. In the air-acetylene flame he found no interference from other plant constituents, but mild depression from sulfuric acid, which was easily compensated for by treating samples and standards alike. Zinc values obtained by atomic absorption were lower than those by polarography, possibly due to interferences in the latter technique. A similar study was performed by Allan (A11), who estimated zinc in plants, soils, and fertilizers. No interferences were encountered and where zinc concentrations in the original sample were too low to be sufficiently concentrated by ashing, solvent extraction was used.

Willis (W14) was able to determine zinc in untreated urine, the samples being directly aspirated into the flame. The zinc content of normal urine was about 1 mg/liter. Reproducibility was better than that of colorimetric methods.

Zeeman and Butler (Z1) determined zinc in a variety of wines. Twenty-five-ml samples were ashed, and the residue dissolved in nitric acid was aspirated. No interference was seen from any of the ash constituents when investigated individually but, when combined, zinc absorption was depressed, resulting in a change in the slope of the calibration curves.

Parker (P1) described methods of sample preparation for the deter-

mination of zinc in animal tissues. Only slight influence on zinc absorption was seen from hydrochloric and nitric acids.

5.4.10. *Mercury*

Resonance Line: 2537 Å

Sensitivity Limit: 5 ppm (A12)

Mercury is the only metal with a significant vapor pressure at room temperature and the mercury vapor detector, a true atomic absorption spectrophotometer (W20), is based on this property. The sensitivity in the flame is disappointingly low when compared with the detection limits in the mercury vapor meter in which 10,000 times higher sensitivities are attained. The reason for this difference is not entirely clear, but expansion of the flame gases, broadening of the resonance line at the higher temperatures, and the shorter absorption path in the flame may be responsible. The resonance line at 1849 Å shows much stronger absorption than the line at 2537 Å, but owing to the absorptive properties of oxygen below 2000 Å the shorter line can neither be used in the common mercury vapor detector nor in the flame.

Lindström (L7) used a flame to volatilize mercury-containing solutions but determined mercury levels in the exhaust gases which, after filtering and cooling, were passed into a mercury vapor detector. A variety of materials, including urine, were analyzed with the astounding sensitivity limit of 0.001 ppm of mercury in the aspirated sample.

Jacobs *et al.* (J1) developed a method for the determination of mercury in 0.1 ml blood. The specimen is digested in the cold and extracted with dithizone. The extract is decomposed in an electrically heated furnace and the mercury vapor is passed into a mercury vapor meter. The method is said to detect mercury in the nanogram range.

Willis (W14) determined mercury in urine by chelation of the metal with ammonium pyrrolidine dithiocarbamate and extraction into methyl-*n*-amyl ketone. He showed that recovery of mercury added was complete and levels encountered in normal urines were 0.02 mg per liter.

5.4.11. *Lead*

Resonance Line: 2833.1 Å

Sensitivity Limit: 0.3 ppm (A12)

Lead shows atomic resonance on several spectral lines, the strongest absorption line being that at 2833.1 Å. Again, maximum emission is observed on different lines, that at 4058 Å being the most sensitive. If a lead hollow cathode lamp is used, it must be operated on low current

to prevent rapid loss of the metal from the cathode. If the hollow cathode is made of leaded brass, higher currents can be used and Elwell and Gidley (E1) obtained 250 service hours from an argon-filled tube run at 40 milliamperes. Despite broadening of the resonance line, best sensitivity was found at the highest possible operating current, 50 mA. The 2833 line, however, must be separated from lines at 2824 and 2840 Å.

Robinson (R4) described the determination of lead in gasoline and found no interference in the oxy-hydrogen flame from a 90-fold excess of tin, sodium, bismuth, copper, zinc, chromium, iron, nickel. No effect on lead absorption was seen when solutions of lead tetraethyl in isooctane were mixed with equal volumes of carbon disulfide, *o*-thiocresol, diethylamine, and other organic sources of nitrogen and sulfur. Elwell and Gidley (E1) determined lead in alloys and steel and state that no interference, except a slight viscosity effect, was seen from excesses of nitric, hydrochloric, perchloric, and fluoroboric acids, or from a 20-fold excess of sodium, calcium, copper, magnesium, iron, nickel, tin, or zinc. Remarkable is the fact that neither aluminum nor silicon caused absorption depression. An unexplained enhancement, however, was seen when traces of lead were determined in 2% iron solutions in hydrochloric acid; light scattering due to the highly concentrated solutions seems not to have been the cause, since no absorption was found at the neighboring copper lines of the brass hollow cathode lamps.

Willis (W12, W14) reported the determination of lead in urine. Lead was chelated with ammonium pyrrolidine dithiocarbamate and extracted into methyl-*n*-amyl ketone. Urine samples of 50 ml had to be extracted into 1.5 ml of the solvent to obtain sufficient concentration of the metal. Differences in the efficiency of extraction from water and urine at various pH values were noted. The availability of a reliable and simple method such as the one described will be welcomed by medical laboratories, but unfortunately the limited sensitivity requiring large original samples makes the method less applicable for the determination of lead in blood.

Zeeman and Butler (Z1) analyzed wines for lead content. Wine samples of 50 ml were ashed to dryness and the residue taken up in 2 ml nitric acid, effecting a 25-fold concentration. The samples were aspirated into a propane-butane-air flame 8 inches in length, and a simple optical system with two flame traversals was employed. The authors showed that a certain amount of lead was lost in the ashing process, and the loss was smallest with lead sulfate. Lead concentrations in a variety of wines were 0.2–1 ppm.

5.4.12. *Other Elements*

Many other elements can be determined by atomic absorption spectroscopy but little has been published about applications in biological materials.

Noble metals were determined by Lockyer and Hames (L9). Silver shows a sensitivity limit of 0.1 ppm. When working with gold salts, the deposition of gold in the burner may lead to an absorption decrease in the flame.

Cadmium is characterized by high sensitivity (limit 0.03 ppm) (G2) and by complete atomization in the air–coal gas flame. Willis (W14) showed that cadmium when added to urine could be determined by aspirating the urine directly; normal urine levels were at the sensitivity limit of the method, but applications of this technique are certainly feasible in toxicological work. The recent demonstrations of a specific cadmium-containing protein (K2, K3) adds importance to the availability of an atomic absorption method for cadmium.

Chromium was determined by Williams *et al.* (W7) in animal feces samples to study pasture intakes. In the air-acetylene flame the sensitivity limit was 0.15 ppm. Of a variety of substances tested individually, only calcium, silicate, and phosphate depressed chromium absorption. However, when interferences were studied following treatment of solutions with phosphoric acid, manganese sulfate, and potassium bromide, depression was caused by silicon and aluminum, but calcium and magnesium enhanced absorption. Calcium was also capable of abolishing the effect of silicon and aluminum.

The determination of cobalt and nickel is discussed by Allan (A8). Sensitivities are considerably higher than those in emission. Applications of absorption methods to molybdenum and strontium are reported by David (D4, D7, D8).

Atomic absorption spectra of vanadium, titanium, niobium, scandium, yttrium, and rhenium in the fuel-rich oxy-acetylene flame and spectra of the lanthanides were studied by Fassel and Mossotti (F1, M11). The use of the flame as a line source for the study of atomic absorption spectra of europium, thulium, and ytterbium was shown by Skogerboe and Woodriff (S2).

Bismuth added to urine was recovered by Willis (W14) with solvent extraction and determined by atomic absorption spectroscopy. An absorption interference rarely encountered in atomic absorption spectroscopy was seen from the absorption of the 3068-Å line of bismuth by the OH radical in the air–coal gas flame.

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ASPECTS OF DISORDERS OF THE KYNURENINE PATHWAY OF TRYPTOPHAN METABOLISM IN MAN

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1. Introduction

Tryptophan is probably the indole derivative most widely distributed in nature. It is converted into many other substances of important biological significance. The many materials biogenetically related to tryptophan include nicotinic acid (a vitamin), serotonin (a neurohormone), indoleacetic acid (a phytohormone), some pigments found in the eyes of insects, and a number of alkaloids.

Tryptophan quite clearly follows different metabolic routes. Our studies have been devoted to the pathway that, proceeding through the formation of kynurenine (an amino acid but no longer an indole) to the biosynthesis of nicotinic acid, explains the formation of the various intermediates. Kynurenine is the key substance in this process.

The series of steps that take place *in vivo* along this pathway, which we term the kynurenine pathway in order to distinguish it from the others, has now been elucidated through the efforts of many workers.

1.1. Historical

This work began 110 years ago. In 1853 Liebig (L4), in connection with investigations on the urea content of dog urine, isolated small amounts of an unknown acid. From its origin the compound was called

kynurenic acid. It was later observed that a prolonged meat diet is necessary for dogs to excrete kynurenic acid. Even before the exact structure of kynurenic acid (4-hydroxyquinoline-2-carboxylic acid) was elucidated, a remarkable result of biological significance was achieved in 1904 by Ellinger (E2), who demonstrated in the dog that tryptophan is broken down to kynurenic acid.

A genetic relationship between an indole derivative and a quinoline compound was therefore established in animals, analogous to that in the vegetable kingdom in which certain quinoline alkaloids are known to be formed from tryptophan.

In spite of laborious work, knowledge of the mechanism and of the possible intermediates of the pathway tryptophan → kynurenic acid made little progress until the isolation of *kynurenine* (M4). This compound, whose structure (*o*-aminobenzoylalanine) became clear only many years later (B26), is the intermediate (as shown by S. Kotake), no longer an indole, between tryptophan and kynurenic acid (K12).

The second quinoline derivative produced in animal metabolism is *xanthurenic acid,* which was isolated by Musajo (M12). Xanthurenic acid (4,8-dihydroxyquinoline-2-carboxylic acid) also originates from tryptophan through kynurenine (M13).

In 1942 it was shown that the urine of pyridoxine-deficient rats contained large amounts of xanthurenic acid (L2). This was the starting point for studies on the interrelationship among vitamin B_6, tryptophan, and protein metabolism.

Formation of xanthurenic acid is a typical feature of vitamin B_6 deficiency. It is the substance which first drew attention to the possible relationship between pyridoxine and the enzymes connected with protein metabolism. The formation of xanthurenic acid, however, is catalyzed by an enzyme, *kynurenine transaminase,* which requires pyridoxal phosphate as coenzyme. The apparent discrepancy between these two facts will be explained below.

The enzyme cleaving kynurenine to form *anthranilic acid* and alanine (W14) is *kynureninase* which also requires pyridoxal phosphate as coenzyme (B19).

It was furthermore reported (K20) that nicotinic acid-deficient animals would grow only if given tryptophan, thus suggesting the conversion of tryptophan to nicotinic acid. Not only is tryptophan converted to nicotinic acid but also kynurenine and *3-hydroxyanthranilic acid.* The peculiar degradation of the latter to pyridine derivatives gave rise to many interesting investigations. 3-Hydroxyanthranilic acid is derived from *3-hydroxykynurenine,* another important tryptophan metabolite, the his-

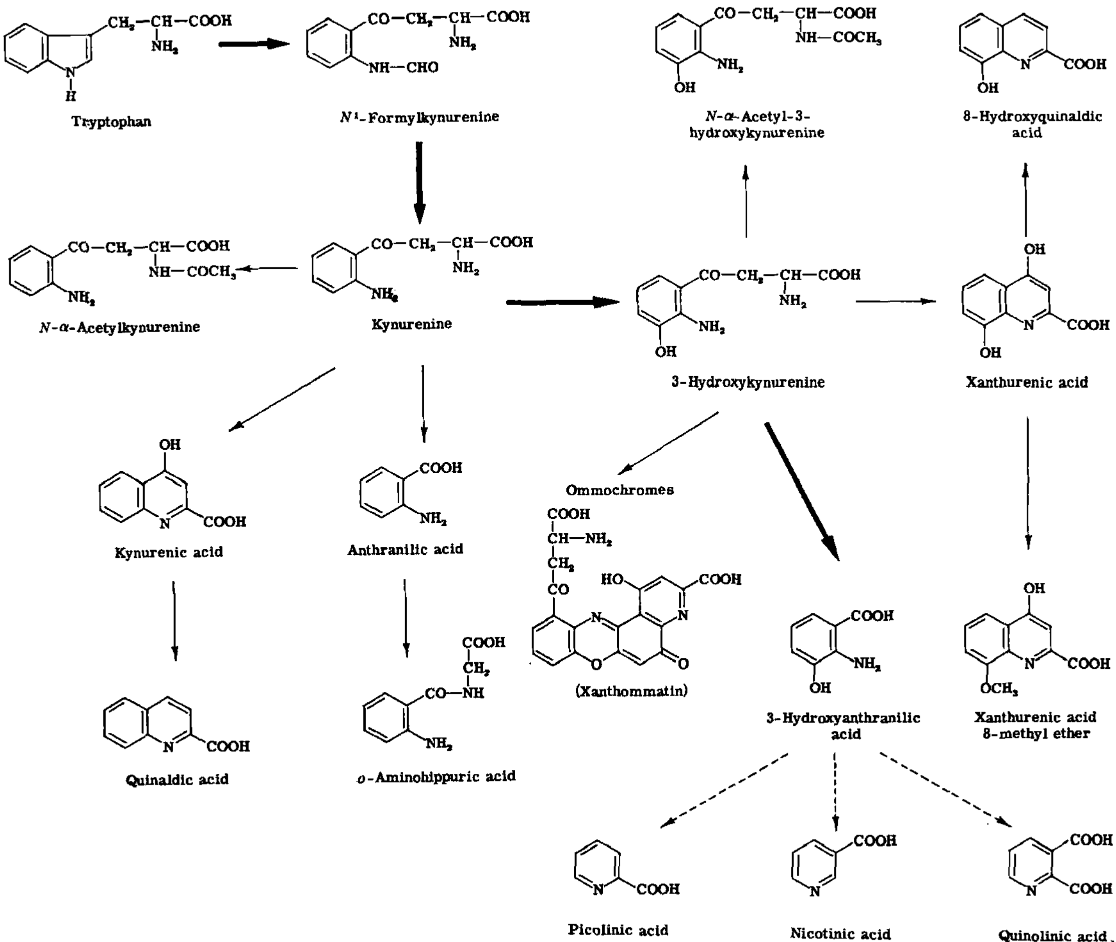

FIG. 1. Kynurenine pathway for metabolic conversion of tryptophan to niacin.

tory of which is more recent, beginning only in 1949. The presence of a hydroxyl group at C-8 of xanthurenic acid as well as the results in this regard led Musajo in 1950 to anticipate the existence of a hydroxykynurenine, precisely 3-hydroxykynurenine (M14), as a direct precursor of xanthurenic acid. The compound was isolated in 1949 by Butenandt, who obtained from 15 kg of pupae of *Calliphora erythrocephala* 100 mg of 3-hydroxykynurenine (B27, B28). It was synthesized by Musajo *et al.* (M15) independently and almost simultaneously. 3-Hydroxykynurenine is the precursor of xanthurenic acid, since it causes immediate excretion of the latter when administered to rats (M16). Furthermore, 3-hydroxykynurenine is cleaved by kynureninase to form 3-hydroxyanthranilic acid and alanine (cf. Fig. 1).

Another aspect, through which the degradation of tryptophan becomes a part of the chapter of the genes, must be remembered for its great biological importance. In 1952 Butenandt (B29) succeeded in clarifying the structure of *ommochromes.* These pigments of the eye of insects, which in the past were considered as pterin derivatives, are today known to be formed directly from kynurenine and 3-hydroxykynurenine.

Ommochromes are acid dyes of red, yellow-brown, and purple color, practically insoluble in all neutral solvents. The biogenesis of ommochromes from tryptophan is influenced by inheritance factors which act at definite steps. Kynurenine is formed under the control of v^+ genes, and from it 3-hydroxykynurenine is formed under control of cn^+ genes.

Kynurenine and 3-hydroxykynurenine are the precursors or chromogens of the pigments. One of them, xanthommatin, was obtained from the eyes of insects: 7800 heads of *Calliphora erythrocephala* gave 19 mg of the pigment (B32). Xanthommatin was also isolated from the molting secretion of *Vanessa urticae*: 10,000 butterflies yielded 100 mg of the substance (B30).

The structure of xanthommatin was established by degradation studies: it is a phenoxazine derivative readily reduced to hydroxanthommatin, a bright red pigment, which is in turn reoxidized by air to yellow-brown xanthommatin (B31). The presence of the alanine side chain was proved by its removal from xanthommatin with kynureninase (B31). Xanthommatin yields, on acid and alkaline degradation, 3-hydroxykynurenine, xanthurenic acid, and 2-amino-3-hydroxyacetophenone (cf. Fig. 2). In addition to dialyzable ommatines, high molecular weight *ommines*, which are sulfur-containing ommochromes, occur in many animal species. They also yield, as ommatines do, xanthurenic acid, which is therefore used to establish the correct structure of these natural pigments.

The actinomycins, a family of chromopeptide antibiotics synthesized

by a number of *Streptomyces* species (B20), are also phenoxazinones in which tryptophan metabolites, such as 4-methyl-3-hydroxyanthranilic acid or 3-hydroxykynurenine, are the immediate precursors of the polycyclic ring (S6).

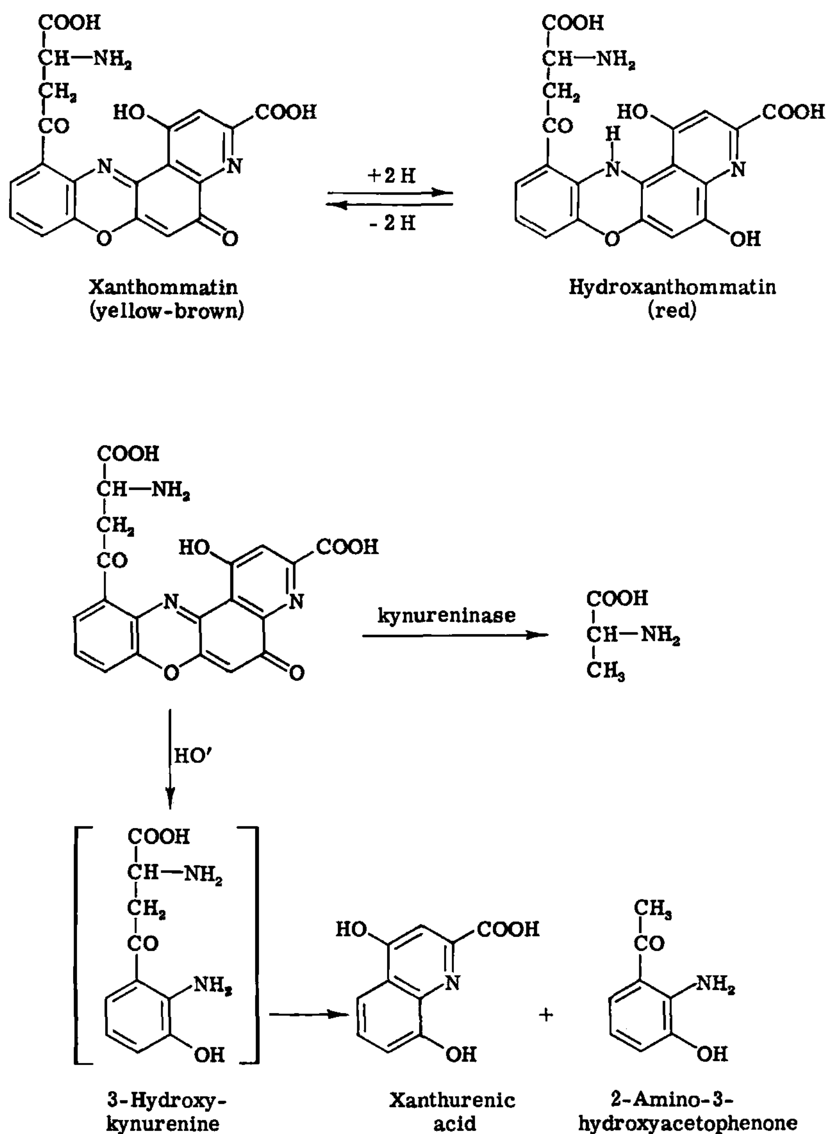

FIG. 2. Xanthommatin; its reduced form and degradation products.

The first observation on the connection of human pathological conditions with abnormal excretion of tryptophan metabolites, through the kynurenine pathway, was made in 1931 (K13).

Kotake and Sakata (K13) reported that the ingestion of 3 g tryptophan or 1 g kynurenine by a human being resulted in an increase of the Ehrlich diazo reaction (i.e., red color with alkaline diazobenzenesulfonic acid). The same authors observed that a patient with pulmonary tuberculosis, when given 2 g tryptophan, showed a strong increase of the urinary diazo reaction. Later it was found that 5 of 19 patients with pulmonary tuberculosis excreted kynurenine when given 0.5 g tryptophan, whereas no excretion was observed in normal controls to whom larger amounts (3 g) of the amino acid (O6) were administered.

The diazo reaction, not given to kynurenine, is clearly produced by xanthurenic acid when present in urine. In view of this, Chiancone in 1935 administered 3 g L-tryptophan to a young man with bilateral tuberculosis and 1.5 g L-kynurenine to another young patient. By the methods available at that time, no xanthurenic acid was found in the urine of either individual (C4).

Musajo *et al.* reinvestigated the problem of the interpretation of the diazo reaction in urine of patients with pulmonary tuberculosis in the light of new information concerning the biological transformations of tryptophan. In fact, among the products of its metabolism are 3-hydroxykynurenine and 3-hydroxyanthranilic acid, both of which give a strong diazo reaction[1] (M18). These authors gave 5 g L-tryptophan to a subject with bilateral pulmonary tuberculosis and were able to isolate from the urine 3-hydroxyanthranilic acid as the methyl ester. Another interesting observation was simultaneously made by means of paper chromatography: 3-hydroxyanthranilic acid was excreted spontaneously by 11 of 13 tuberculous patients without tryptophan load. This result formed the starting point of a systematic investigation on the spontaneous excretion of tryptophan metabolites, which began about 10 years ago in Padua. It is evident that this line of research required suitable analytical methods. The most important contributions to the solution of this problem are reported in the following section.

1.2. Development of Analytical Methods

Although kynurenic acid had been isolated by Liebig more than a century ago and has since then been extensively studied, the history of

[1] In 1956 Wachstein and Lobel (W5) reported the results of analysis of 6350 patients whose first morning specimens of urine were tested with diazo reagent. Of the total, only 24 yielded a positive reaction with the paper and the tube test; this positivity resulted from 3-hydroxykynurenine in the urine of only 11 of these patients.

its determination began in 1872 with the work of Schmiedeberg and Schulzen (S4), followed by that of Hofmeister (H9), Capaldi (C2), and Jaffe (J1). These methods are based on urine purification by means of different reagents and weighing the kynurenic acid precipitated by acidification. They are of course unsuitable for small amounts of material and led to weighing kynurenic acid together with xanthurenic acid, the latter not yet discovered when such a technique was used.

Xanthurenic acid is easily detectable and measurable through the reaction, now well known, of forming a green color with ferrous or ferric salts. In 1951 Musajo and Coppini after many attempts perfected an analytical procedure which allows the quantitative determination of kynurenic and xanthurenic acids, separately or mixed, in amounts of at least 100 μg, also when occurring in urine (M17).

The method is based on the transformation of kynurenic acid into a substance of the type of the quinolinic dyes of Besthorn, but more stable toward light.

By chlorinating kynurenic acid with phosphorus pentachloride and oxychloride and by successive addition of quinoline to the reaction product, the authors obtained a colored substance, soluble in ethanol and giving a brilliant red-violet color with maximum absorption at 556–557 mμ of remarkable stability.

Xanthurenic acid is determined in bicarbonate solution by the green color resulting from addition of small amounts of ferrous salt. Its determination is not influenced by kynurenic acid, whereas the presence of xanthurenic acid can interfere with that of kynurenic acid. To avoid such interference a brief and cautious oxidation with potassium permanganate is necessary to destroy exclusively xanthurenic acid. Afterwards the unchanged kynurenic acid is extracted at pH 2 with isoamyl alcohol, chlorinated, and reacted with quinoline.

In the same year paper chromatography was first attempted by Benassi (B4) for the simultaneous analysis of 8 tryptophan metabolites (kynurenine, 3-hydroxykynurenine, kynurenic acid, xanthurenic acid, anthranilic acid, 3-hydroxyanthranilic acid, 2-aminoacetophenone, and 2-amino-3-hydroxyacetophenone), separated by means of a mixture of methanol, *n*-butanol, benzene, and water and revealed through the fluorescence in ultraviolet light of 3655 Å. Each compound elicits a different fluorescent color (cf. Table 1).

Since one-dimensional chromatography is very often insufficient to permit separation of the many tryptophan or indolic compounds and their degradation products occurring in urine, Dalgliesh (D4) worked out a two-dimensional method suitable for routine analysis and reported a map of spots. Urine extracts were prepared by absorption of aromatic metab-

olites on inactivated charcoal, followed by elution with aqueous phenol, and concentration of the phenol eluate. Extracts were then chromatographed, the organic layer of a freshly prepared *n*-butanol–acetic acid–water mixture (4:1:5) being used as solvent for the first run. The chromatograms were dried and then run in the short dimension with 20% (w/v) aqueous potassium chloride as solvent. With such a technique Dalgliesh studied the chromatographic behavior and located more than 30 substances related to tryptophan metabolism by means of various reactions and fluorescence.

TABLE 1
CHROMATOGRAPHIC BEHAVIOR AND FLUORESCENCE OF METABOLITES EXAMINED

Substance	Mean R_f (Butanol-acetic acid-water)	Mean R_f (water)	Fluorescence (at 3655 Å)
Kynurenine	0.37	0.67	Azure
3-Hydroxykynurenine	0.30	0.56	Yellow-green
N-α-Acetylkynurenine	0.82	0.81	Azure
Kynurenic acid	0.56	0.57	Blue (turning yellowish)
Xanthurenic acid	0.56	0.45	Blue-green
Xanthurenic acid 8-methyl ether	0.56	0.51	Blue
Anthranilic acid	0.88	0.66	Violet
o-Aminohippuric acid	0.76	0.80	Violet
3-Hydroxyanthranilic acid	0.85	0.60	Blue-violet

Furthermore, a colorimetric evaluation of kynurenine, studied by Otani *et al.* (O6), was utilized for urine through a modification introduced by Ginoulhiac (G8).

The technique of Dalgliesh (D4) suggested to Coppini *et al.* (C10) a quantitative determination for tryptophan metabolites in biological fluids. The procedure is based on direct spotting on paper sheets of urine, using butanol–acetic acid–water for the first run and distilled water for the second. Deproteinization is required for blood serum and spinal fluid. The sheets are observed under ultraviolet light (3655 Å); the spots of the considered derivatives show characteristic R_f values and fluorescence; only xanthurenic acid and its methyl ether partially overlap. The chromatographic behavior and fluorescence of the compounds examined are reported in Table 1.

The spots are marked with pencil and cut out. Elution with the sol-

vents indicated below requires 15–16 hours and the resulting solutions are analyzed further. The spots corresponding to kynurenine and anthranilic acid are eluted with 5 ml 1% *p*-dimethylaminobenzaldehyde in 50% acetic acid. The resulting colored solution is read at 450 mμ.

The cuts corresponding to kynurenic acid, *N*-acetylkynurenine, and xanthurenic acid 8-methyl ether are eluted with 5 ml ethanol (spectroscopic grade) and the absorbances are read at 243, 227, and 237 mμ, respectively.

The pieces of paper with 3-hydroxykynurenine, 3-hydroxyanthranilic acid, and xanthurenic acid are each placed in an Erlenmeyer flask fitted with a ground-glass stopper and containing 3.8 ml distilled water. After 15–16 hours, 1 ml diazotized sulfanilic acid (0.5% in 2% hydrochloric acid mixed immediately before using with an equal volume of 0.5% solution of sodium nitrite in water) and 0.2 ml pyridine are added. The temperature must be held constant at 15° to obtain reproducible results. The color resulting from xanthurenic acid is read immediately at 510 mμ, and of the other two derivatives after 60–80 minutes at 450 mμ.

When both xanthurenic acid and its methyl ether are present it is necessary to run chromatograms in duplicate since, as was mentioned, their spots partially overlap. Xanthurenic acid does not interfere in the determination of its methyl ether by measurement of absorbance at 237 mμ.

The determination of *o*-aminohippuric acid was recently checked: its spot is eluted with the same Ehrlich reagent and the color developed is read immediately at 470 mμ (Benassi, unpublished data).

This method is rapid and sufficiently sensitive even when there are great variations in concentration among the nine metabolites studied. As little as 0.25–0.30 μg of anthranilic or xanthurenic acid present on the chromatogram can be measured. Approximately 1 μg of the other derivatives must be present to allow reproducible results; usually not more than 5–6 μg for each metabolite has to be determined. In such a range the method gives linear results within the experimental error, and reproducibility, expressed as a coefficient of variation, averaged less than 4% of metabolites added to urine samples. Its limit depends upon the volume, not exceeding 0.2 ml, of urine used. This fact decreases the sensitivity of the procedure in particular when "spontaneous" excretion is under study, but it allows the observation of any abnormal excretion, i.e., higher than 5 mg daily for each metabolite. The figures will be discussed later in this context after having considered other procedures.

Brown and Price (B22) used ion-exchange chromatography on synthetic resins and gradient elution to separate many metabolites in urine of dog, cat, rat, and man. By means of Dowex 1 (Cl^-) resin and water–

monochloroacetic acid solution of increasing molarity as eluent, kynurenic and quinaldic acids and 4-quinolone and its *N*-methyl derivative are eluted successively, whereas xanthurenic acid remains on the column. All these compounds were then determined spectrophotometrically. Using Dowex 50 (H^+) and hydrochloric acid of increasing concentration as eluent, anthranilic acid and its glucuronide, *o*-aminohippuric acid, kynurenine and *N*-acetylkynurenine were separated, and measured by the Bratton and Marshall technique for diazotizable amines.

Price and Dodge (P8) applied the same technique for separation of kynurenic and xanthurenic acids and xanthurenic acid 8-methyl ether. Dowex 50 (H^+) was employed as the resin with water as eluent. A spectrophotofluorometric method is used for reading: kynurenic acid is made highly fluorescent by concentrated sulfuric acid (cf. Table 1) and xanthurenic acid fluoresces with concentrated alkali, so that both compounds can be read in the presence of each other and of the methyl ether of xanthurenic acid.

This new fluorometric technique makes the determination of kynurenic acid simpler and that of xanthurenic acid more specific: if the ferrous or ferric iron technique is used for the latter, higher results are obtained, probably due to the presence in urine of chelating substances which combine with iron (P8). 3-Hydroxykynurenine is determined by an analogous method (B23).

Tompsett (T3) achieved a separate elution from cation or anion resin columns of several tryptophan metabolites which were then determined colorimetrically. Finally, Boyland and Williams (B18) quantitatively adsorbed on inactivated charcoal anthranilic acid, kynurenine, 3-hydroxyanthranilic acid, 3-hydroxykynurenine, and the sulfuric acid ester derivatives of the two latter compounds from urine of normal controls and of patients with bladder cancer. After elution, the compounds were separated by gradient chromatography on Celite columns and determined colorimetrically or spectrophotometrically.

In spite of the many methods proposed, the problem of simultaneously determining several tryptophan metabolites is not completely solved. The methods utilizing ion-exchange chromatography are considered by the above authors to be very time-consuming and difficult to set up. Paper chromatographic technique, even though relatively simple and rapid, is often limited because of the small volume of urine used. In addition, when metabolites are concentrated through absorption and elution from inactivated columns, quantitative yields are difficult to obtain, especially for the more rapidly oxidizable compounds, e.g., 3-hydroxyanthranilic acid.

These difficulties have been overcome by means of a new method developed in this laboratory and based on the use of a column of ion-exchange resin and elution with volatile buffers at two different molarities and pH's (B10).

A column of Amberlite® IR-120, 0.9 × 28 cm, held constant at 37°C and formic acid–pyridine buffers are employed. With a buffer formic acid–pyridine 0.2 *N*, pH 2.50–2.60, kynurenic, xanthurenic, and *o*-aminohippuric acids are eluted successively from the column. By increasing molarity and pH, respectively, to 0.3 *N* and 4.20 there emerge kynurenine, 3-hydroxyanthranilic acid, and 3-hydroxykynurenine, which are collected automatically in fractions of 2 ml. Figure 3 gives an example of chromatographic separation.

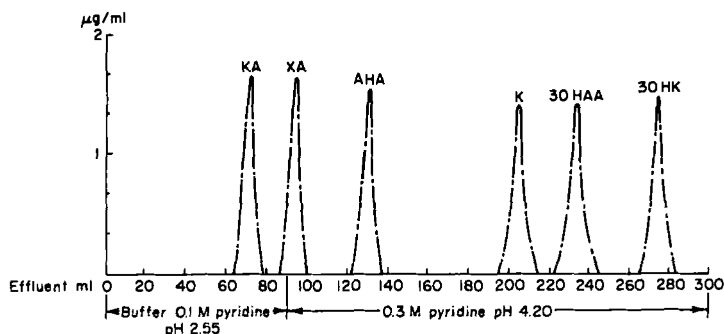

FIG. 3. Chromatographic fractionation of a mixture of tryptophan metabolites on an ion-exchange column of Amberlite® IR-120 (28 × 0.9 cm). The temperature was held at 37°C and the flow rate was adjusted at 12 ml per hour with formic acid–pyridine buffers. The metabolite concentration is given as μg/ml after fluorometric readings. Effluent was collected in 2-ml fractions. The following abbreviations are used: KA, kynurenic acid; XA, xanthurenic acid; AHA, *o*-aminohippuric acid; K, kynurenine; 3OHAA, 3-hydroxyanthranilic acid; 3OHK, 3-hydroxykynurenine.

When pure compounds are chromatographed, the fractions are directly analyzed by an Aminco spectrophotofluorometer. Activation and fluorescence spectra of each metabolite have been determined and concentration-fluorescence curves show that the sensitivity is adequate even at 0.01 μg for at least four compounds. For kynurenine and 3-hydroxykynurenine, which are less fluorescent, colorimetric readings are preferred.

The method is suitable also when a few μg of substances are chromatographed: yields are quantitative even up to 5 μg for each metabolite.

The technique can be applied to biological fluids only with some modifications, since the presence of numerous fluorescent urinary compounds does not allow the direct spectrophotofluorometric reading (B10a).

Volumes of urine of 10–15 ml at pH 2.10 are added directly to the column and elution is carried out with the formic acid–pyridine buffers referred to above. Fractions of 25 ml are collected, rapidly evaporated to dryness *in vacuo*, and after dilution to known volume spotted on paper sheets. At this point the procedure of Coppini *et al.* (C10) is followed; the results obtained by analyzing urine of normal individuals are tabulated below (Section 2.1.1).

Since *o*-aminohippuric acid is partially destroyed with our method during column chromatographic separation, the technique of Brown and Price (B22) has been preferred for its fractionation. The 80 ml 1.0 *N* HCl fraction is evaporated to dryness *in vacuo* and the residue, suitably diluted, is paper chromatographed for a further purification. The spot corresponding to *o*-aminohippuric acid is at this point eluted and spectrofluorometrically or colorimetrically determined.

2. Relationship between Tryptophan Metabolism and Some Pathological States

2.1. Studies on the "Spontaneous" Excretion of Tryptophan Metabolites

As previously mentioned, 3-hydroxyanthranilic acid was found chromatographically by Musajo *et al.* (M18) in the urine of tuberculous patients. This was the starting point for an extensive investigation of tryptophan metabolites excreted spontaneously, i.e., by normal subjects or patients with different diseases all fed a normal diet without added tryptophan.

We began this study in 1953 and, with the collaboration of various clinical departments of the Padua University Medical School, 1170 indi-

TABLE 2
NUMBER OF SUBJECTS EXAMINED

Hemoblastosis		165
Various neoplastic diseases		161
Bladder cancer		201
Urinary diseases		112
Neuropsychopathies		261
Cataract		45
Infectious diseases		83
Tuberculosis		63
Endocrinopathies		36
Pregnancy		43
	Total	1170
Controls		108
	Total	1278

vidual pathological urines have now been examined and those of 108 controls (Table 2).

We shall now discuss the results obtained by means of the different analytical methods cited.

2.1.1. *In Normal Controls*

According to all authors, normal subjects excrete only a few mg daily of the more important tryptophan metabolites.

Price (P11) found in 30 controls the following mean excretions.

	(mg/day)
Kynurenine	2.5
N-Acetylkynurenine	3.0
3-Hydroxykynurenine	0–6.0
Kynurenic acid	2.1
Xanthurenic acid	2.1
o-Aminohippuric acid	4.7
3-Hydroxyanthranilic acid	—

According to Tompsett (T3), 12 controls excreted an average of:

	mg/day (range)
Kynurenine	3.1 (1.8–4.9)
3-Hydroxykynurenine	3.3 (1.6–5.8)
Anthranilic acid	0.9 (0.3–2.2)
3-Hydroxyanthranilic acid	5.2 (3.8–9.8)

Boyland and Williams (B18) found in 10 normal subjects with a mean 24-hour urine volume of 1560 ml:

	mg/liter (range)
Kynurenine	10 (3–12)
3-Hydroxykynurenine	2 (0– 5)
3-Hydroxykynurenine *O*-sulfate	5 (3– 9)
Anthranilic acid	12 (6–23)
3-Hydroxyanthranilic acid	20 (8–32)
3-Hydroxyanthranilic acid *O*-sulfate	9 (5–12)

The method of Coppini *et al.* (C10) displays its limitations from the small volume of urine spotted, so that the mean excretion of over 100 controls can only be evaluated as:

	mg/day
Kynurenine	<5
3-Hydroxykynurenine	<5
Kynurenic acid	<5
Xanthurenic acid	<3
o-Aminohippuric acid	<5
3-Hydroxyanthranilic acid	<5

The following are the last data obtained with our above summarized technique (B10a) for the mean excretion of 20 normal subjects:

	mg/day (range)
Kynurenine	1.14 (0.3–2.6)
3-Hydroxykynurenine	0.49 (0–2.3)
Kynurenic acid	2.83 (1.0–4.2)
Xanthurenic acid	0.66 (0.3–1.8)
o-Aminohippuric acid	1.14 (0.4–1.7)
3-Hydroxyanthranilic acid	0.36 (0.1–1.1)

The figures of Price and Tompsett and of Benassi for kynurenine, 3-hydroxykynurenine, and kynurenic acid agree with each other. Benassi's values for xanthurenic acid are lower and those for *o*-aminohippuric acid are much lower than those of Price. This result seems to be related to a higher specificity of our procedure. They are all lower than the limiting values detectable by Coppini's procedure. On the contrary, the values given by Boyland and Williams (B18) seem to be too high and have not been confirmed by other procedures. Furthermore, only these authors found that anthranilic acid is excreted unconjugated in large amounts.

In conclusion, all human beings fed a free diet excrete daily only small quantities of tryptophan metabolites. However, in some diseases and in vitamin deficiency such a pattern can substantially change.

2.1.2. *In Pathological Conditions*

2.1.2.1. *Hemoblastosis.* Among 300 patients examined in the first screening by Musajo *et al.* (M19, M20), attention was drawn to those with hemoblastotic diseases where the excretion of kynurenine and 3-hydroxykynurenine was found to be well above normal levels.

All 90 hemoblastotic patients, including 30 cases of myeloid leukemia, 30 of lymphoid leukemia, 2 of hemocytoblastotic and 1 of histiocytic leukemia, 21 of Hodgkin's disease, 3 of multiple myeloma, and 1 of lymphosarcomatosis, were followed chromatographically for several days and the increased excretion of the two kynurenines was confirmed. Fever is not a determining factor since the remission of fever did not have a normalizing effect on the metabolite excretion. The investigation was completed with the isolation of kynurenine and 3-hydroxykynurenine in pure form.

From 9 liters of urine from a subject with chronic myeloid leukemia 16.4 mg 3-hydroxykynurenine and 40 mg kynurenine, as the sulfate, were isolated through the mercuric salts, resolution with hydrogen sulfide, preparative paper chromatography of the concentrate, elution of the fluorescent bands, and repeated concentrations and crystallizations. From 5.5 liters of urine from a patient with Hodgkin's disease 34 mg kynurenine and 21.1 mg 3-hydroxykynurenine were obtained. A third patient also with Hodgkin's disease gave 23.8 mg kynurenine and 9 mg 3-hydroxykynurenine

from 7 liters of urine. Finally, 68.5 mg kynurenine was isolated from 24 liters of urine from a lymphatic leukemic patient.

The amounts of the two substances originally present in the urine were higher than those actually isolated. It must be emphasized, however, that tryptophan metabolites have rarely been extracted from urine when neither special diets nor supplementary tryptophan are used. Furthermore, urine of hemoblastotic patients can be considered, together with *Calliphora erythrocephala* pupae, as one of the rare "natural" sources of 3-hydroxykynurenine.

This line of research was pursued in this laboratory by Crepaldi and Parpajola (C12, P1). Table 3 shows the qualitative excretory pattern found by these authors in 68 other patients, who are grouped in four

TABLE 3
EXCRETION OF TRYPTOPHAN METABOLITES[a] IN 68 HEMOBLASTOTIC PATIENTS

Diagnosis		Patients (no.)	Number of cases with urinary excretion at abnormal levels of:					
			K	ACK	3OHK	3OHAA	XA	KA
(A) Malignant lymphoma		13	8	1	1	1	—	—
(B) Leukemia	lymphatic chronic	6	2	1	—	1	—	—
	myeloid chronic	5	3	—	—	1	—	—
	myeloid acute	11	7	—	—	1	—	—
(C) Multiple myeloma		5	3	—	—	—	—	—
(D) Hodgkin's disease		28	28	—	6	8	—	—

[a] The following abbreviations are used: K, kynurenine; ACK, *N*-α-acetylkynurenine; 3OHK, 3-hydroxykynurenine; 3OHAA, 3-hydroxyanthranilic acid; XA, xanthurenic acid; KA, kynurenic acid.

categories according to the nature of the hematological disease. An interesting point immediately stands out: kynurenine excretion is constantly increased above normal levels in all patients with Hodgkin's disease. As appears from Table 4, kynurenine excretion differs among the 28 subjects and is related to the severity of the disease, varying between 10 and 123 mg daily. A close relationship exists between malignant lymphoma and kynurenine excretion.

Excretion of 3-hydroxykynurenine and 3-hydroxyanthranilic acid is found to be increased in some of the patients with Hodgkin's disease (8 of 28), especially when the general conditions of the patients deteriorate. The other metabolites are present in normal quantities.

Ten patients with Hodgkin's disease, whose kynurenine excretion was not influenced by previous treatment, were given vitamin B_6, either as

TABLE 4

SPONTANEOUS EXCRETION OF TRYPTOPHAN METABOLITES[a] IN 28 PATIENTS WITH HODGKIN'S DISEASE

No.	Subject	Age	Sex	Observation days	K (mg/24 hr) Mean	K (mg/24 hr) Range	3OHK (mg/24 hr) Mean	3OHK (mg/24 hr) Range	3OHAA (mg/24 hr) Mean	3OHAA (mg/24 hr) Range	Treatment
1	B.L.	17	f	13	142	(78–123)	—	—	7	—	None[b]
2	B.A.M.	32	f	7	21	(15– 40)	—	—	—	—	None[b]
3	C.N.	13	f	5	38	(30– 64)	25	(20–32)	—	—	None[b]
4	G.E.	34	f	8	00	(63–101)	35	(23–55)	—	—	None[b]
5	S.I.	52	f	4	47	(40– 50)	—	—	—	—	None[b]
6	V.A.	43	f	8	16	(10– 60)	—	—	—	—	None[b]
7	V.D.	28	m	8	54	(27– 95)	49	(29–64)	8	—	None[b]
8	B.L.	24	f	14	15	(10– 35)	—	—	7	—	None[b]
9	T.A.	20	f	6	22	(20– 25)	—	—	—	—	None[b]
10	P.M.	13	f	6	43	(40– 50)	12	(8–15)	9	—	None[b]
11	M.E.	28	f	9	8	—	—	—	—	—	None
12	B.F.	20	m	3	9	—	—	—	—	—	None
13	T.C.	61	m	7	14	(8– 18)	—	—	—	—	None
14	T.D.	21	m	4	9	—	—	—	—	—	None
15	C.I.	14	f	12	12	(5– 18)	9	—	—	—	None
16[c]	D.M.	36	f	2	45	(35– 54)	17	(13–21)	41	(17–65)	None
16*bis*[d]	D.M.	36	f	58	—	(12– 6)	—	(9– 5)	—	(20– 5)	Dichloren®
17	F.M.	23	f	10	15	—	16	—	28	—	Various treatments
18	F.P.	17	m	7	29	—	—	—	8	—	Various treatments
19	C.A.	53	f	16	7	—	—	—	—	—	Various treatments
20	T.G.	34	m	3	8	—	—	—	—	—	Various treatments
21	N.M.	24	m	3	7	—	—	—	—	—	Various treatments
22	D.D.	23	m	14	7	—	—	—	—	—	Various treatments
23	C.F.	49	m	3	7	—	—	—	—	—	Various treatments
24	Z.S.	33	m	42	15	—	—	—	—	—	Various treatments

TABLE 4 (*Continued*)

No.	Subject	Age	Sex	Observation days	K (mg/24 hr) Mean	K (mg/24 hr) Range	3OHK (mg/24 hr) Mean	3OHK (mg/24 hr) Range	3OHAA (mg/24 hr) Mean	3OHAA (mg/24 hr) Range	Treatment
25	G.M.R.	17	f	62	8	—	—	—	—	—	Various treatments
26	M.F.	22	f	51	7	—	—	—	—	—	Various treatments
27	N.N.	56	f	25	17	(15–20)	—	—	—	—	Various treatments
28	A.E.	23	f	45	7	—	—	—	—	—	Various treatments

[a] The abbreviations are the same as those used in Table 3. Values were not reported when excretion was normal (i.e., lower than 5 mg daily for each metabolite).

[b] These patients were subsequently treated with vitamin B_6 for periods varying from 2 to 76 days (30–60 mg/day) to study the effect of this vitamin on the highly abnormal levels of tryptophan metabolites.

[c] Before Dichloren®.

[d] After Dichloren®.

pyridoxine hydrochloride or pyridoxal 5-phosphate. There was a consistent quantitative decrease of kynurenine and 3-hydroxykynurenine levels to normal. It was possible to follow such an effect by observing the simultaneous increase of 3-hydroxyanthranilic acid above normal values.

For the other hemoblastotic diseases, the systemic forms of malignant lymphomas show an excretory pattern similar to that of Hodgkin's disease, but with a lower incidence and lower levels of metabolites.

For leukemic forms no interrelation was found between metabolite abnormality and behavior of the peripheral blood picture. In 4 cases with chronic myeloid leukemia, a tremendous increase in the leucocyte counts (300,000 in one subject) did not correspond to any abnormal excretion. Kynurenine excretion is, however, frequently increased in patients with chronic lymphatic leukemia, which is related to the malignant lymphogranuloma through the same genesis of the lymphoreticular matrix.

In addition, it is difficult to correlate abnormal excretion patterns with the effects of the usual therapeutic agents. In fact the malignancy of the disease does not often allow maintenance or change in the therapeutic treatment of these patients for experimental purposes.

TEM (triethylenemelamine) Myleran or Busulfan (methanesulfonic acid, tetramethylene ester), antimitotic drugs such as Dichloren (mechlorethamine hydrochloride) and Cytoxan (cyclophosphamide), X-rays, and steroid therapy show different effects without consistent results. The question must be further investigated.

In connection with the relationship between tryptophan metabolism and therapeutic effects, a significant variation was noted between the greater amounts of kynurenine excretion observed by Musajo *et al.* (M20) during 1955-56 and the lower quantities now reported by Crepaldi and Parpajola (C12) in patients with the same disease.

The introduction in recent years of new therapeutic agents is modifying the clinical course of leukemias, especially its chronic picture. It cannot therefore be excluded that quantitative changes in kynurenine excretion could be related to the different environment of these patients.

2.1.2.2. *Bladder and Kidney Cancer and Other Urological Diseases.* The excretory pattern in cases of bladder tumor has been studied for many years in our laboratory (B5, B8) after Boyland and Williams (B18) had suspected that *o*-aminophenolic metabolites of tryptophan (i.e., 3-hydroxykynurenine, 3-hydroxyanthranilic acid, and 2-amino-3-hydroxyacetophenone) might be "endogenous" agents of bladder cancer simi-

larly to *ortho*-hydroxylated and methoxylated metabolites of certain aromatic amines (2-naphthylamine, benzidine) known to be agents causing industrial or "exogenous" bladder cancer.

Clayson *et al.* (C8) have suggested that the function of *ortho*-hydroxylation is to activate the region of high electron density (referred to as the KA region, where interaction with the body tissue could take place) of the aromatic amines. Other suitable activating groups such as methoxyl are also able to bring about this activation.

It was thought by Dunning *et al.* (D7, D8) that the combined administration of 2-fluorenylacetamide and tryptophan could be a real factor in the initiation of bladder cancer in rats. It was argued (B18) that 2-fluorenylacetamide acted on tryptophan metabolism by increasing the amount of diazotizable amines and consequently the formed *o*-hydroxyamines might play, in their unconjugated form, a role in inducing bladder tumors. Price *et al.* (P7) first extended the study to human beings by investigating the urine of a group of bladder tumor and control patients. The data, obtained after a test dose of 2 g L-tryptophan, suggested that bladder tumor patients were unable to metabolize ingested tryptophan in a normal manner (P7).

Brown *et al.* (B24) also observed in a few patients, without bladder tumor, that the surgical excision of most of the neoplastic tissue was followed by a return to normal tryptophan metabolism, after the loading test. In cases of bladder cancer, on the other hand, some of the most severe disturbances were observed in patients who had been clinically free of cancer for 1–13 years.

In additional experiments (A3a, B17) tryptophan metabolites were tested by bladder implantation in mice and only 3-hydroxykynurenine, 3-hydroxyanthranilic acid, 2-amino-3-hydroxyacetophenone, and the 8-methyl ether of xanthurenic acid were found to be carcinogenic; the latter is not an *o*-aminophenol derivative, but is sometimes present in human urine (P8).

It was suggested (B15) that a similar essay might account for the occasional tumors found in mice implanted with pellets of paraffin wax or only cholesterol, the pellet acting as a promoting agent for endogenous carcinogens by its presence as a foreign body. It was found (B15) that 2 of 56 mice implanted with paraffin and 5 of 55 implanted with cholesterol developed carcinoma. This induction of tumor with vehicle alone means that relatively large groups of animals must be used to achieve statistical significance.

As many as 368 urological patients (Table 5) were examined by us with regard to their spontaneous excretion of tryptophan metabolites. Table 5

TABLE 5

SPONTANEOUS EXCRETION OF TRYPTOPHAN METABOLITES[a] IN UROLOGICAL DISEASES

Diagnosis	Cases examined			Cases with abnormal urinary excretion of:				
	Total	Normal excretion	Abnormal excretion	K	K + 3OHK	3OHAA	K + 3OHK + 3OHAA	K + 3OHAA
Tumors of the bladder								
Fibroepitheliomas	178	133	45	24	18	2	—	1
Solid carcinomas	20	7	13	2	10	—	—	1
Adenocarcinomas	2	0	2	1	1	—	—	—
Spindle-shaped carcinomas	1	1	0	—	—	—	—	—
	201	141	60	27	29	2	—	2
Extrabladder tumors								
Prostatic carcinomas	18	12	6	5	1	—	—	—
Renal tumors	32	13	19	9	—	6	1	3
Seminomas	2	2	0	—	—	—	—	—
Perineal sarcomas	2	1	1	1	—	—	—	—
Osteosarcomas	1	0	1	1	—	—	—	—
	55	28	27	16	1	6	1	3
Nonneoplastic forms								
Prostatic hypertrophy	34	32	2	2	—	—	—	—
Renal tuberculosis	15	14	1	1	—	—	—	—
Renal lithiasis	8	6	2	2	—	—	—	—
Calculi of the bladder	2	2	0	—	—	—	—	—
Cystic diseases	8	6	2	—	1	1	—	—
Urethral stricture	3	2	1	—	1	—	—	—
Hypospadias	2	2	0	—	—	—	—	—
Neurogenic bladder	2	2	0	—	—	—	—	—
Nephritis	3	3	0	—	—	—	—	—

TABLE 5 (*Continued*)

Diagnosis	Cases examined: Total	Cases examined: Normal excretion	Cases examined: Abnormal excretion	Cases with abnormal urinary excretion of: K	K + 3OHK	3OHAA	K + 3OHK + 3OHAA	K + 3OHAA
Nonneoplastic forms (cont.)								
Bladder diverticulos	4	2	2	1	1	—	—	—
Pyelitis	2	2	0	—	—	—	—	—
Bladder neck sclerosis	6	5	1	1	—	—	—	—
Hydronephrosis	3	2	1	1	—	—	—	—
Various diseases	20	15	5	5	—	—	—	—
	112	95	17	13	3	1	—	—

[a] The abbreviations are the same as those used in Table 3.

shows that more than half of them, i.e., 201 cases, belong to the bladder cancer group, classified according to the nature of the (nonindustrial) tumor.

Of these only 60 excreted abnormal levels of urinary metabolites: high amounts of 3-hydroxykynurenine and 3-hydroxyanthranilic acid were observed, respectively, in 29 and in 4 cases. However, the compound whose

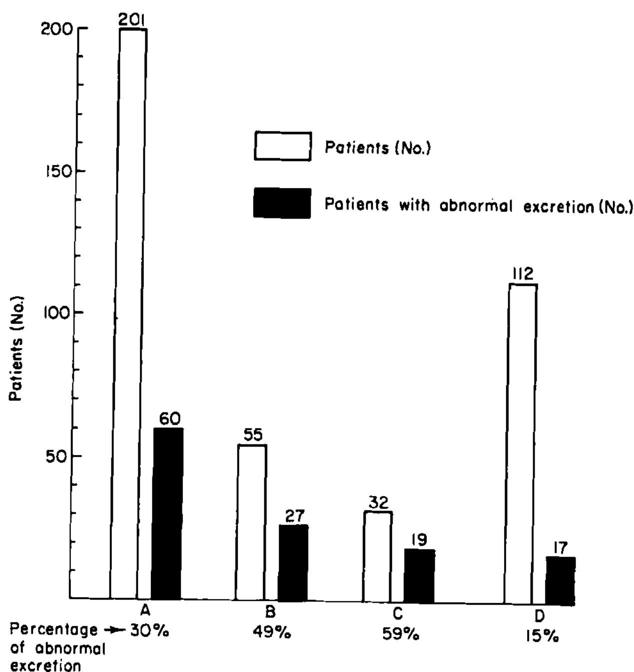

FIG. 4. Number of patients examined, divided according to the urological disease and number of those showing abnormal levels of tryptophan metabolites spontaneously excreted. Percentage of the abnormally excreting patients is given; A = bladder tumors; B = extrabladder tumors; C = kidney tumors; D = urological diseases (non-neoplastic).

excretion was increased in most patients was kynurenine, which is not an *o*-aminophenolic derivative. Its daily excretion ranged from 24 to 233 mg.

Abnormal excretion is not restricted to this group of patients since higher values were found also in the extrabladder tumor[2] patients. In fact 28 of 55 patients of this group were highly positive for urinary kyn-

[2] Extrabladder tumors are tumors of the urinary system, i.e., of kidney, pelvis and ureter, but not of the bladder.

urenine, ranging from 16 to 195 mg daily, and 11 patients for 3-hydroxy-anthranilic acid in amounts of 22–124 mg/24 hours. The highest incidence of abnormal quantities of metabolites was also noted (19 of 32) in the kidney tumor subjects.

Moreover, 17 patients of 112 with various urological diseases (nonneoplastic) excreted abnormal amounts of kynurenine and 3-hydroxykynurenine (Fig. 4).

The excretion of *N*-α-acetylkynurenine and kynurenic, xanthurenic, and *o*-aminohippuric acids was, in all the cases investigated, within the normal range, while 2-amino-3-hydroxyacetophenone and xanthurenic acid 8-methyl ether were always absent.

From our investigation it is evident that abnormal excretion of tryptophan metabolites is not a typical feature of bladder tumor subjects, since human beings with neoplastic and nonneoplastic extrabladder urinary diseases have also been found to excrete spontaneously elevated amounts of tryptophan derivatives. It seems that the metabolic abnormality is not restricted to bladder tumors, but is rather more specific for patients with tumors of the upper urinary tracts and of the renal parenchyma. Actually 59% of these patients (Fig. 4) excreted abnormal amounts of kynurenine, 3-hydroxykynurenine, and 3-hydroxyanthranilic acid.

It is well known that among the factors which may influence tryptophan metabolism, pyridoxine plays an important role. Therefore the study of the effects of pyridoxine was extended to some bladder tumor patients showing a high level of spontaneous kynurenine excretion.

We have observed (Table 6) that the administration of large doses of pyridoxine (120–300 mg/day) to a group of 9 bladder tumor patients, during periods lasting 6–22 days, sometimes decreased but never reduced the daily spontaneous excretion of kynurenine to normal values.

This result differs from that obtained in 10 patients with Hodgkin's disease whose kynurenine and 3-hydroxykynurenine excretion was rapidly brought to normal with vitamin B_6.

As previously stated, quantitative methods agree that all human beings normally excrete a few mg daily of the so-called oncogenic metabolites derived from tryptophan, and increased levels of urinary metabolites are not specific for bladder tumors. Also, normal and increased excretion of tryptophan metabolites is found in individuals of both sexes, whereas bladder tumors are rarer among females.

In the preceding section, the presence of abnormal levels of kynurenine and 3-hydroxykynurenine was demonstrated in urine of patients with different hematological diseases, not only by qualitative and

TABLE 6

EFFECTS OF PROLONGED ADMINISTRATION OF PYRIDOXINE ON DAILY SPONTANEOUS EXCRETION OF KYNURENINE IN 9 BLADDER TUMOR PATIENTS

No.	Bladder tumor patients	Initial kynurenine (mg/day)	Pyridoxine[a] every day (mg)	Urinary kynurenine (mg/day)								
				Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 10	Day 15	Day 22
1	A.P.	107	120	65	98	90	58	61	46	44	41	66
2	A.C.	86	120	71	61	52	35	35	33	40	48	36
3	A.R.	106	120	80	65	50	73	53	56	—	—	—
4	A.B.	80	120	51	—	40	56	—	46	—	—	—
5	A.G.	28	120	57	51	52	42	46	46	—	—	—
6	N.X.	47	120	60	38	—	—	48	46	—	—	—
7	M.L.	44	120[b]	35	83	—	43	31	30	—	—	—
8	A.A.	40	300	68	37	—	50	—	44	—	—	—
9	F.C.	25	300	65	63	48	78	51	31	36	—	—

[a] Divided in 3 oral doses.

[b] Supplemented with vitamin-B complex.

quantitative measurements but also through the isolation of these substances in pure form. Moreover, abnormal spontaneous excretion has been observed in patients with tuberculosis (M18) and after loading tests in patients with porphyria (P12), schizophrenia (B7), lupus erythematosus (P11), scleroderma (P10), pregnancy (B25), mongolism (O1), and hypoplastic anemias and other hematological disorders (M2). All these experimental data raise doubt about the hypothesis of the oncogenic activity of certain tryptophan metabolites. Furthermore, there is no relation, unlike the Hodgkin's disease between excretion of the so-called carcinogenic metabolites and the severity of the neoplastic disease as regards extent, degree of infiltration, histological malignancy, and tumor metastasis.

Quagliariello *et al.* (Q1) followed qualitatively (by means of one-dimensional paper chromatography) the spontaneous excretion of kynurenines and anthranilic acids in 47 bladder tumor patients. They found that 3-hydroxyanthranilic acid was usually excreted and other metabolites only occasionally. Furthermore, Quagliariello (Q4) in an interesting review, reports that in urine of 15 bladder tumor patients 3-hydroxyanthranilic acid is excreted in amounts that are usually accepted as normal, whereas normal controls do not excrete 3-hydroxyanthranilic acid at all.

Brown *et al.* (B24) have published a further study in which the bladder tumor patients are divided into two categories—those with normal and those with abnormal tryptophan metabolism, after a 2-g test load of the amino acid. The latter group excreted levels of kynurenine, kynurenic acid, acetylkynurenine, and 3-hydroxykynurenine several times higher than in the control group. There was essentially no difference between the excretion pattern of patients with normal tryptophan metabolism and control subjects before and after the tryptophan loadings. Administration of large doses of vitamin B_6 had little effect on excretion of metabolites by the bladder tumor patients with normal metabolism, but had a very significant normalizing effect in the group showing abnormal metabolism (B8).

It was recently observed (K2) that a kidney with a small tumor excretes more of the so-called carcinogenic *o*-aminophenols (3-hydroxykynurenine and 3-hydroxyanthranilic acid) than the opposite healthy kidney after a 100 mg/kg loading dose of L-tryptophan. The small size of the hypernephroma encountered at nephrectomy and also the higher excretion of "carcinogenic" metabolites of tryptophan previously thought to be restricted to tumors of transitional-cell epithelium (K2) are emphasized.

This observation is related to that made by us (P4a) that kynurenine alone, or sometimes together with 3-hydroxykynurenine and 3-hydroxyanthranilic acid, was found to be abnormally elevated in 17 of 28 cases with renal tumors, in 3 of 6 cases with pelvic tumors, and in 1 of 2 cases with cystic renal disease.

Schultz (S5) has recently developed the hypothesis that cancer and leukemia are manifestations of a deranged tryptophan metabolism which produces an endogenous primary carcinogen. Its precursors are 3-hydroxykynurenine and an *o*-hydroxyamino compound, probably *o*-aminophenol. The first step in the condensation of 3-hydroxykynurenine with *o*-aminophenol to form an endogenous carcinogen may require the participation of the enzyme phenoxazinone synthetase. The condensation is considered (S5) to be similar to a reaction involved in the endogenous synthesis of insect ommochrome pigments, such as xanthommatin (cf. Fig. 2).

From the data reported here and the considerations presented, it appears difficult to accept the hypothesis that a change in tryptophan metabolism, giving an abnormal excretory pattern of *o*-aminophenolic derivatives of tryptophan, is able to induce bladder tumors.

2.2. Studies on the Excretion of Tryptophan Metabolites after Test Load

Another line of research was initiated by Chiancone (C4a) in 1950, using a tryptophan loading test. The *xanthurenic index* of Chiancone expresses, as a percentage of the administered tryptophan, the amount of xanthurenic acid excreted within 24 hours after tryptophan ingestion.

The improvement in analytical methods has shown the importance of quantitative determination of more than one urinary metabolite which, alone, does not always reveal a metabolic abnormality.

We carried out several experiments by loading with 100 or 50 mg/kg body weight of L-tryptophan and determining kynurenine, *N*-α-acetylkynurenine, 3-hydroxykynurenine, kynurenic acid, xanthurenic acid, 3-hydroxyanthranilic acid, and free anthranilic acid. These tests were performed in a variety of conditions and the results are discussed in the following sections.

2.2.1. *In Normal Controls*

The mean values of single and total excretion of metabolites, as obtained with the method of Coppini *et al.* (C10), are reported in Table 7, both in micromoles and as percentage of the administered tryptophan.

In the first row of Table 7 the data refer (B7) to 6 normal subjects

TABLE 7

AVERAGE URINARY EXCRETION OF TRYPTOPHAN METABOLITES[a] BY 6 (A) AND 5 HEALTHY SUBJECTS (B) ALL GIVEN L-TRYPTOPHAN

	K		ACK		3OHK		KA		XA		3OHAA		Metabolites (sum)	
Subjects	Total	%	Total	%	Total	%	Total	%	Total	%	Total	%	Total	%
(A)[b]	917	2.82	245	0.75	40	0.12	439	1.44	87	0.27	447	1.37	2207	6.67
(B)[c]	210	1.50	105	0.69	34	0.24	176	1.19	41	0.28	89	0.65	651	4.56

[a] The abbreviations are the same as those used in Table 3. Values are reported in micromoles.

[b] Given 100 mg/kg of L-tryptophan (490 μmoles/kg).

[c] Given 50 mg/kg of L-tryptophan (245 μmoles/kg).

loaded with 100 mg/kg of L-tryptophan, whereas the second shows the mean excretion of 5 normal individuals when loaded with 50 mg/kg, according to Crepaldi and Parpajola (C12).

Price (P11) found that when human subjects with no known disease were given 2–4 or 8 g L-tryptophan in a single dose, the increased urinary excretion of the metabolites was not strictly proportional to the amount of amino acid ingested. Thus, when 2 g L-tryptophan was administered, about 1.8% of the amino acid could be recovered in urine in the form of increased excretion of the metabolites. When the dose was 4 or 8 g, 2.7 or 6.8% respectively, could be recovered. These figures are in good agreement with those obtained by us with a different method.

Boyland and Williams (B18) loaded 10 normal controls with 10 g DL-tryptophan. In spite of the fact that it is essential to use L-tryptophan in studies of this type and although data on excretion of kynurenic and xanthurenic acids are lacking, at least 5% was recovered as increased kynurenine, 3-hydroxykynurenine, anthranilic acid, and 3-hydroxyanthranilic acid, free and conjugated. It should be pointed out that in these studies only a low percentage of the ingested dose of tryptophan was found in the form of urinary metabolites. The remainder, approximately 93–94%, of the load could not be recovered.

It is generally accepted that under normal conditions human subjects convert the largest part of an ingested dose of L-tryptophan to nonaromatic products. The last identified benzene derivative on this pathway is 3-hydroxyanthranilic acid; when its ring is opened to form unstable intermediates, apparently only a small percentage of them are converted to niacin (H8).

In fact 60 mg tryptophan are biologically equivalent in man to 1 mg niacin; the conversion ratio of about 60:1 indicates the relative efficiency of tryptophan in substituting for niacin in man (G11, H11).

The remainder is undoubtedly metabolized to aliphatic compounds such as acetate and carbon dioxide to a considerable extent.

However, under special conditions the percentage of aromatic metabolites excreted after tryptophan loading can increase notably.

2.2.2. *Pregnancy*

One of the first applications of the loading test was made in pregnant women for studying vitamin B_6 deficiency which occurs, as is well known, with high levels of urinary xanthurenic acid. In 1951 Vandelli (V1) found that pregnant women excreted greater amounts of xanthurenic acid than nonpregnant women after ingestion of L-tryptophan. The author interpreted this fact as signifying a pyridoxine deficiency in preg-

nancy, inasmuch as supplementation of the diet with vitamin B_6 restored the excretion of xanthurenic acid to control levels (V1). Similar conclusions were afterwards reached by Sprince *et al.* (S8) and Wachstein and Gudaitis (W1).

In 1954 Coppini and Camurri (C9) re-opened the problem by studying the excretion of xanthurenic acid and, at the same time, of kynurenic acid at different stages of pregnancy by means of the method, cited above, of Musajo and Coppini (M17). As Table 8 shows, pregnancy increases the excretion of kynurenic and xanthurenic acids after ingestion of L-tryptophan. The ratio xanthurenic acid/tryptophan increases gradually from the beginning to the end of pregnancy, whereas the ratio kynurenic acid/tryptophan increases in the first trimester, then slowly decreases until, at the end of pregnancy, the same levels are found as in nonpregnant women.

Similar results were observed, using a different amount of DL-tryptophan, in the xanthurenuria of women at the end of normal pregnancy (E3). However, in one investigation, of eight women well advanced in pregnancy, only one excreted excessive xanthurenic acid after a test dose of DL-tryptophan and this pregnancy was complicated by disease (H5). The excessive excretion in this case was reduced with pyridoxine.

According to Sue (S10), blood poisoning during pregnancy placed a stress on the supply of pyridoxine by reason of excess protein metabolism. This fact, together with the presence of elevated amounts of histidine and histamine, inhibited the action of vitamin B_6, and caused excretion of large quantities of xanthurenic acid.

The results of Heller (H6) were in agreement with those of the largest part of the above authors that xanthurenuria was elevated in pregnant women and at the end of pregnancy and was influenced by pyridoxine treatment. This author (H6) did not accept the hypothesis of Wachstein (W3, W4) that vitamin B_6 might be a prophylactic agent against toxemia of pregnancy after examining 200 of these patients in which excretion of xanthurenic acid might be reduced only by therapy with pyridoxal 5-phosphate, together with the reduction of free and bound serum cholesterol (D5).

It was also observed (W6) that at termination of normal pregnancy, vitamin B_6 values were significantly depressed in maternal leucocytes and plasma. In blood of women in the last trimester of pregnancy, however, normal average amounts of the vitamin were found, although these individuals had a distinct abnormality in tryptophan metabolism. Amounts of pyridoxal phosphate are high in cord blood.

The results of Wertz *et al.* (W11) indicated that pregnant subjects

TABLE 8

TOTAL AND % EXCRETION OF KYNURENIC AND XANTHURENIC ACIDS BY 18 PREGNANT WOMEN AT DIFFERENT STAGES OF PREGNANCY AFTER TRYPTOPHAN LOADING[a]

No.	Trimester of pregnancy	Age	Administered tryptophan[b] (g)	KA mg/24 hrs	KA %*	XA mg/24 hrs	XA %*	Observations
1	I	26	5.1	83	1.6	125	2.4	2nd month: normal pregnancy
2	I	18	5.7	201	3.5	271	4.7	2nd month: gravidic hyperemesis
3	I	24	5.5	127	2.3	367	6.6	3rd month: normal pregnancy
4	I	27	6.1	126	2.1	177	2.9	3rd month: normal pregnancy
5	I	23	4.9	118	2.4	156	3.2	3rd month: normal pregnancy
6	I	40	6.5	87	1.3	57	0.9	3rd month: menace of abortion
7	II	35	6.6	121	1.8	397	6.0	4th month: normal pregnancy
8	II	28	6.3	99	1.5	299	4.7	4th month: normal pregnancy
9	II	22	5.6	96	1.7	214	3.8	5th month: normal pregnancy
10	II	42	6.3	85	1.3	360	5.7	5th month: normal pregnancy
11	II	16	6.0	181	3.0	486	8.1	6th month: menace of abortion
12	II	27	7.0	99	1.4	281	4.0	6th month: interruption of pregnancy
13	III	24	5.5	60	1.1	550	10.0	7th month: menace of abortion
14	III	31	7.7	111	1.4	1025	13.2	8th month: normal pregnancy
15	III	24	5.4	61	1.1	224	4.1	8th month: normal pregnancy
16	III	27	4.9	117	2.4	344	7.0	8th month: normal pregnancy
17	III	18	7.1	89	1.2	288	3.2	9th month: normal pregnancy
18	III	31	5.7	70	1.2	1038	18.2	9th month: normal pregnancy

* As tryptophan ingested.

[a] The abbreviations are the same as those used in Table 3. Data from reference (C9).

[b] Tryptophan loads were 100 mg/kg.

excreted increased levels of niacin metabolites. This suggests that the increased efficiency of conversion found in pregnancy is not due to pyridoxine deficiency as such, which had no detectable effect on the efficiency of the tryptophan to niacin conversion, but is probably related to the endocrine changes associated with pregnancy.

Various reports in the literature indicate the influence of endocrine organs on tryptophan metabolism. Chiancone and co-workers (C5, V2) reported that ovariectomy or hypophysectomy of rats caused increased excretion of xanthurenic acid and that adrenalectomy caused a decrease. An adrenal mechanism is suggested for the regulation of 3-hydroxyanthranilic acid conversion to nicotinic and picolinic acids (M7).

Brown *et al.* (B25) confirmed the results of Coppini and Camurri (C9) on excretion of kynurenic and xanthurenic acids and, at the same time, examined the excretion of other tryptophan metabolites. Their data indicate that the high levels of all urinary metabolites excreted by pregnant subjects were lowered by pyridoxine administration. It must be remembered that the requirement for pyridoxine in pregnancy varies in the different animal species (C6). It was also found that the levels of pyridoxine in the fetal blood are elevated whereas those of maternal blood decrease (G10).

According to Brown *et al.* (B25), the pattern of excretion of tryptophan metabolites in pregnancy was different in several respects from the pattern observed in vitamin B_6 deficiency. Those differences were probably a result of both vitamin B_6 deficiency and endocrine changes. The same authors suggested that perhaps hormonal factors were in some way inhibiting the kynureninase activity. Similarly the nonincreased excretion of kynurenic acid suggested that kynurenine transaminase activity was regulated by the hormonal status of pregnancy in such a way that the production of xanthurenic acid is favored over that of kynurenic acid. Brown *et al.* (B25) suggested the possibility that kynurenine transaminase, forming kynurenic acid, and 3-hydroxykynurenine transaminase, catalyzing xanthurenic acid formation, might be different enzymes under different hormonal regulation.

Quite recently Weber and Wiss (W8) studied the influence of vitamin B_6 depletion on various pyridoxal phosphate enzymes and found that rat liver kynureninase is much more affected by vitamin B_6 deficiency than kynurenine transaminase. In fact liver kynureninase of rats on a B_6-deficient diet fell to 17%, whereas kynurenine transaminase was about 58% of the original activity after the same period. The different behavior of these two enzymes offers a way of studying closely the mechanism of the increased excretion of xanthurenic acid in pyridoxine

TABLE 9

URINARY EXCRETION OF TRYPTOPHAN METABOLITES[a] IN 10 AGED INDIVIDUALS 24 HOURS AFTER L-TRYPTOPHAN LOADING

			Administered tryptophan[b]	K		ACK		3OHK		KA		XA		3OHAA		Metabolites (sum)	
Case	Age	Sex	(μmoles)	μmoles	%	μmoles	%	μmoles	%	μmoles	%	μmoles	%	μmoles	%	μmoles	%
A	92	f	21080	1456	6.90	347	1.48	132	0.62	448	2.12	64	0.28	161	0.72	2608	12.12
B	79	f	36765	1930	5.25	407	1.10	408	1.11	750	2.04	333	0.90	520	1.41	4348	11.81
C	72	f	40196	1667	4.15	622	1.55	189	0.47	692	1.72	218	0.54	316	0.78	3704	9.21
D	75	f	29410	5488	18.66	1098	3.73	956	3.25	1779	6.05	511	1.73	542	1.84	10374	35.26
E	76	f	25930	4824	18.60	708	2.73	1015	3.91	592	2.28	638	2.46	483	1.86	8260	31.84
F	82	m	36760	7972	21.68	1262	3.43	664	1.80	3254	8.85	257	0.70	202	0.55	13611	37.01
G	80	m	27450	1956	7.12	324	1.18	354	1.29	596	2.17	153	0.56	298	1.08	3681	13.40
H	73	m	40680	8986	22.02	1650	3.27	726	1.78	2338	5.74	99	0.24	64	0.96	13863	34.01
I	72	m	30340	7307	24.08	760	2.50	1400	4.61	1640	5.40	680	2.24	738	2.43	12525	41.26
J	74	m	24510	6225	25.39	529	2.16	1363	5.56	1253	5.11	708	2.88	516	2.10	10594	43.20

[a] The abbreviations for [a] are the same as those used in Table 3.

[b] 490 μmoles/kg = 100 mg/kg.

deficiency, i.e., the well-known discrepancy by which xanthurenic acid, typical feature of a B_6 deficiency, requires for its formation a B_6-dependent enzyme.

2.2.3. *Old Age*

A preliminary investigation on tryptophan metabolism in aged subjects (over 70 years old) was carried out by Avogaro, Crepaldi, and Parpajola and by Benassi and Allegri, using the tryptophan loading test.

The results, not yet published, are reported in Table 9, and show that 6 of 10 individuals excrete very large amounts of metabolites varying from 31 to 43% of the administered tryptophan.

In this laboratory an excretion elevated to such high levels had never previously been observed in subjects loaded with L-tryptophan. The question is obviously open to further investigation on a larger number of these subjects. Ranke *et al.* (R3) determined, as a measure of the state of vitamin B_6 nutrition, serum glutamic-oxalacetic transaminase levels in 60 young and 62 healthy old individuals. As another indication of the status of this vitamin in the aged, urinary excretion of xanthurenic acid was followed after tryptophan loads of 15 g and found to be significantly higher in older subjects. Daily administration of pyridoxine for 3 weeks greatly reduced the xanthurenic acid excretion in the older group. According to the authors (R3), a mild state of vitamin B_6 deficiency exists in the aged; the increased excretion of tryptophan catabolites is not primarily due to progressive decrease in dietary content.

Weller and Fichtenbaum (W10) followed the urinary excretion of xanthurenic acid in 48 arteriosclerotic patients after an oral dose of 10 g DL-tryptophan. On the basis of urinary xanthurenic acid above the range of normal controls, 29% of these patients showed a deficiency of vitamin B_6. Using the same criterion, pyridoxine deficiency was found in 60% of diabetic patients with arteriosclerotic complications.

2.2.4. *In Pathological States*

2.2.4.1. *Schizophrenia.* In recent years, a relation between the metabolism of aromatic amino acids and neurological diseases, as well as between indole compounds and schizophrenia, has been suggested.

In one experiment, Benassi *et al.* (B7) gave 100 mg/kg of tryptophan to 11 chronic schizophrenic subjects and 24 hours later analyzed the urines in comparison with those of 6 normal controls.

Figure 5 reports the mean values of % excretion for each metabolite and the sum of the compounds. The excretion ratio between schizophrenic patients and controls is also given.

Chronic schizophrenics excrete, on an average, 2.4 times the total amount of the metabolites excreted by normal subjects. The ratio is different for each metabolite: it is highest for 3-hydroxykynurenine, which is excreted in very small amounts by healthy people and in amounts about 9 times as large by schizophrenic subjects. It is lowest for 3-hydroxyanthranilic acid, the mean excretion of which slightly decreases in pathological conditions. Moreover, the schizophrenic patients excrete more kynurenic and xanthurenic acids than the healthy controls.

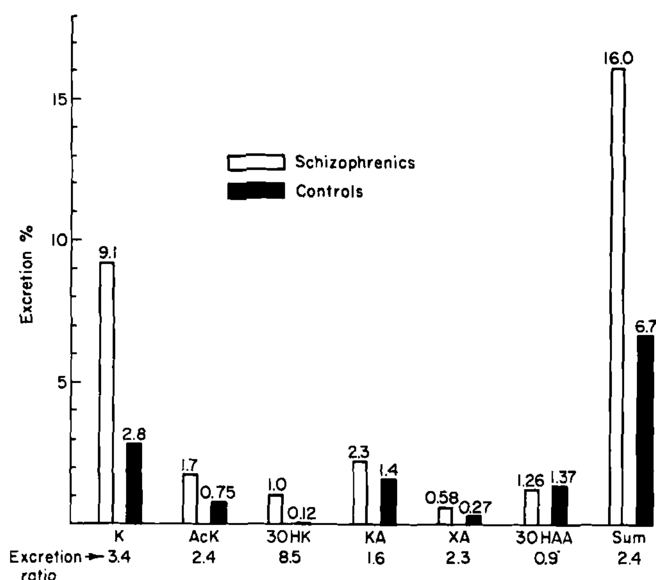

FIG. 5. Mean percentage excretion and excretion ratios for each tryptophan metabolite and total metabolites in 11 chronic schizophrenics and 6 normal controls after a loading test of 100 mg/kg of L-tryptophan (abbreviations in Table 3).

The administration of large doses of pyridoxine (100 mg per day for 30 days) did not change significantly the sum of metabolites excreted by 3 chronic schizophrenic patients after a second load of tryptophan. Only the excretion of 3-hydroxykynurenine is lowered to almost normal levels (B6). Moreover, after a challenging dose of L-tryptophan, chromatographic analysis of the serum of two schizophrenics and one control subject shows that only kynurenine is detectable in blood samples drawn at different intervals of time.

The curves in Fig. 6 represent the kynurenine levels as a function of time and demonstrate that blood levels of kynurenine are higher and

more lasting in schizophrenics than in controls. These values correspond to the amounts of urinary kynurenine, which are notably lower in controls than in schizophrenic patients.

Thus, an anomaly after tryptophan loading exists in these patients in comparison to normal controls. Other results have been reached by Price *et al.* (P12) who, by means of their 2 g loading test, found that 6 patients with schizophrenia had a distinctly abnormal tryptophan metabolism while 13 other patients metabolized tryptophan in a normal manner. The patients with abnormal metabolism excreted significantly

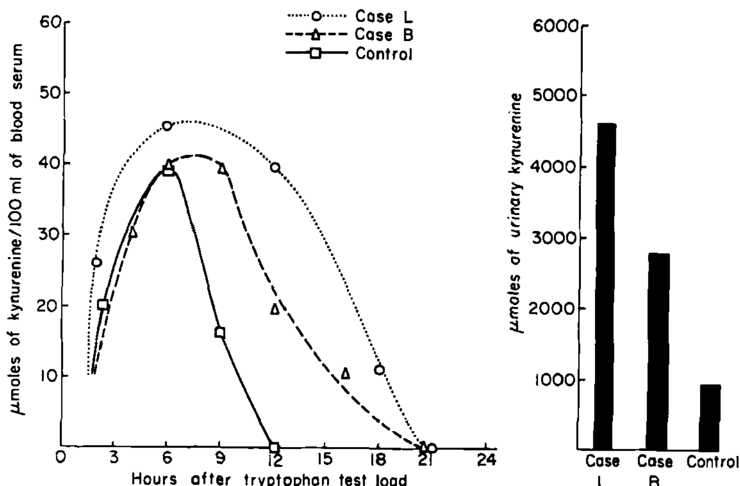

FIG. 6. Comparison between kynurenine content in μmoles per 100 ml blood serum at different times and μmoles of kynurenine excreted after administration of 100 mg/kg L-tryptophan to 2 schizophrenic patients (cases L and B) and 1 healthy control (B7).

more kynurenic acid, *N*-α-acetylkynurenine, kynurenine, 3-hydroxykynurenine, and *o*-aminohippuric acid after 2 g L-tryptophan (8.72% of the dose is excreted) than did the controls (2.12%). Furthermore, other patients with a variety of other psychoses showed a high incidence (7 of 8) of abnormal metabolism when given the same amount of tryptophan (P12).

Tryptophan metabolism was investigated in neurological diseases (V5, C1) in which the xanthurenic acid excretion was measured after ingestion of L-tryptophan (100 mg/kg). The greater amounts of xanthurenic acid excreted by certain patients suggested an alteration of tryptophan metabolism, probably related to pyridoxine deficiency.

Urine from 8 schizophrenic patients and 9 normal controls were analyzed (B21) for metabolites of tryptophan, before and after oral doses of this compound (80 mg/kg). The urinary output of tryptophan metabolites was found by these authors (B21) to be higher in controls than in the patients.

In patients suffering from schizophrenia urinary excretion of both *N*-methyl-nicotinamide and xanthurenic acid was determined (L6) after 10 g DL-tryptophan loadings. Excretion of the former was significantly decreased, in comparison with that of normal controls, whereas an increase was found in that of xanthurenic acid.

Finally, it was found (B7) that the excretion of 5-hydroxyindoleacetic acid, which is taken as an index of tryptophan metabolism by the serotonin pathway, is affected by the administration of tryptophan neither in normal nor in schizophrenic subjects.

The possibility of a genetic relationship between schizophrenia and tryptophan metabolism was investigated by Benassi *et al.* (B9) in four pairs of twins, each consisting of a schizophrenic sibling and a normal sibling. The first pair was, according to complete blood analysis, dizygous or fraternal whereas the other three pairs, by the same criteria, seemed to be of monozygous type. All efforts to extend the investigation to a larger number of these special pairs, which seem to be very rare, were unsuccessful.

Seven of the 8 twins showed, after loading, an abnormal excretion of tryptophan derivatives, higher than that observed in the schizophrenic patients mentioned above. The excretory pattern of all tryptophan metabolites, independent of the monozygous or dizygous character, appears to be unusually elevated, indicating a biochemical lesion in both members of each pair of twins.

The question is obviously open to further studies.

2.2.4.2. *Hodgkin's Disease and Other Hematological Disorders.* A study of the relationship between tryptophan metabolism and Hodgkin's disease was carried out in this laboratory by means of tryptophan loadings which were followed, a week later, by a second test in the same patients with tryptophan and pyridoxine (C12).

L-Tryptophan, in doses of 50 mg/kg body weight, was administered to 8 Hodgkin's disease patients when free from any therapeutic treatment.

As appears from Table 10, the total excretion of metabolites averaged 14.8%, varying from 9.98 to 24.3% (mean control excretion 4.56%), and decreased slightly to 11% in the second test carried out with simultaneous administration of pyridoxine. These results indicate that tryptophan disorders in malignant lymphoma are only partially related to a

TABLE 10

Urinary Excretion of Tryptophan Metabolites[a] in 8 Patients with Hodgkin's Disease 24 Hours after First L-Tryptophan Loading and 24 Hours after Second L-Tryptophan + Pyridoxine Loading

Case no.		Sex	Tryptophan µmoles	K		ACK		3OHK		KA		XA		3OHAA		Metabolites (sum)	
				Total	%	Total	%	Total	%	Total	%	Total	%	Total	%	Total	%
								First tryptophan loading[b]									
(1)	V.D.	m	14700	2431	16.53	302	2.05	464	315	190	1.29	87	0.59	104	0.71	3578	24.34
(2)	Z.S.	m	15930	673	4.22	212	1.33	160	1.00	253	1.60	185	1.16	183	1.14	1666	10.46
(3)	M.E.	f	16105	960	5.96	172	1.07	143	0.88	267	1.65	317	1.97	19	0.12	1878	11.66
(4)	B.A.M.	f	17155	2067	12.04	453	2.64	424	2.47	571	3.32	234	1.36	65	0.38	3814	22.23
(5)	D.R.G.	f	13480	656	4.86	157	1.17	350	2.60	285	2.11	248	1.85	204	1.51	1900	14.09
(6)	N.M.	f	15440	1173	7.60	202	1.31	30	0.19	132	0.85	229	1.48	75	0.48	1841	11.92
(7)	C.I.	f	13480	624	4.63	188	1.40	365	2.71	271	2.01	48	0.35	155	1.55	1651	12.25
(8)	B.L.	f	11400	735	6.44	20	0.17	335	2.94	95	0.83	141	1.23	98	0.86	1424	12.48
		Mean		1165	7.77	213	1.39	284	1.99	258	1.71	182	1.25	113	0.79	2219	14.92
								Second tryptophan loading + pyridoxine hydrochloride[c]									
(1)	V.D.	m	14700	1052	7.15	198	1.35	174	1.18	465	3.15	132	0.89	370	2.52	2391	16.26
(2)	Z.S.	m	15930	360	2.26	16	0.10	20	0.12	174	1.09	89	0.56	620	3.89	1279	8.02
(3)	M.E.	f	16105	804	4.98	14	0.08	13	0.08	158	0.98	102	0.63	133	0.82	1224	7.60
(4)	B.A.M.	f	17155	1221	7.12	273	1.59	161	0.94	253	1.47	171	0.99	686	4.00	2765	16.12
(5)	D.R.G.	f	13480	506	3.75	12	0.09	110	0.81	254	1.88	07	0.72	574	4.26	1553	11.52
(6)	N.M.	f	15440	936	6.06	15	0.09	16	0.10	132	0.85	167	1.08	240	1.55	1506	9.75
(8)	B.L.[d]		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		Mean		635	4.99	91	0.60	72	0.48	229	1.52	111	0.71	417	2.74	1717	11.06
								Excretion ratio between second and first loading test									
				0.64		0.31		0.24		0.89		0.57		3.47		0.74	

[a] The abbreviations are the same as those used in Table III. Values are reported in micromoles.

[b] 245 µmoles/kg = 50 mg/kg.

[c] 180 mg pyridoxine in 3 parenteral doses.

[d] Not performed because of the death of the patient.

vitamin B_6 deficiency. The observation that large doses of pyridoxine, in a relatively short time, are able to return abnormal levels of metabolites to normality is valid only for the spontaneous excretion. In fact, simultaneous loads of pyridoxine and tryptophan cause only a slight decrease, but never lower the percentage of metabolite excreted to control levels. Very likely other enzymatic disturbances, closely related to the disease, contribute to the excretion of abnormal amounts of tryptophan metabolites by many of these patients.

One of the rare studies of the excretion of tryptophan metabolites, both spontaneous and after load, by patients with hematological disorders, is the work of Altman and Miller (A4). They reported an elevated urinary excretion of anthranilic acid in 9 children with an unusual congenital anemia referred to as "erythrogenesis imperfecta" (A4). Oral administration of 1.6 g L-tryptophan to one patient led to increased urinary excretion of anthranilic acid as well as other intermediary metabolites of tryptophan. Massive doses of riboflavin *per os* during 30 days caused no change in the hematological status, but there appeared to be a decrease in the amount of anthranilic acid excreted.

5-Hydroxyanthranilic acid was shown to be present in the urine of patients suffering from the same disease (F3).

Harris *et al.* (H4) reported a case of an adult patient with hematological abnormalities unresponsive to the usual hematopoietic agents and characterized by a hypochromic anemia, leucocytosis, high serum iron, and high percent iron-binding protein saturation. Measurements of the urinary excretion of kynurenine, kynurenic acid, acetylkynurenine, xanthurenic acid, *o*-aminohippuric acid before and after an oral dose of 4 g L-tryptophan indicated abnormalities of tryptophan metabolism. This alteration was partially normalized on a 1-mg pyridoxine dose and completely normalized at the 10-mg level. Also the clinical and laboratory abnormalities disappeared and hematological remission followed the pyridoxine administration.

Aksenova and Messineva (A2) investigated the excretion of 4-pyridoxic acid in 63 patients affected by different forms of leukemia and hypoplastic anemia. Since all pyridoxine derivatives are oxidized to 4-pyridoxic acid, such a compound was considered an index of vitamin B_6 balance. These patients showed a deficiency of pyridoxine, which increased with simultaneous loadings of tryptophan and pyridoxine (A2).

According to Terent'eva *et al.*, disturbed tryptophan metabolism was not restricted to leukemia and cancer patients. Nevertheless, change in tryptophan metabolism played a definite role in the pathogenesis of leukemia and cancer (T1).

A leukemogenic effect of 3-hydroxyanthranilic acid was observed in RFH strain mice 132 days after 40 subcutaneous doses of 5 mg 3-hydroxyanthranilic acid (E1).

By determining only xanthurenic acid, Gehrmann (G2) found that patients with neoplastic anemias excreted large amounts of this metabolite after a 10-g load of DL-tryptophan. Such a xanthurenuria was slightly positive in some cases of leukemia and multiple myeloma and fairly normal in patients with Hodgkin's disease.

Xanthurenic acid excretion was followed in a group of 20 patients with different forms of anemia after a 10-g load of DL-tryptophan (R2). Abnormal increasing of urinary xanthurenic acid was considered by Rademaker and Verloop an early diagnostic signal of a pyridoxine deficiency. A case of hypochromic anemia, unreactive to any therapy but pyridoxine, showed a normal excretion of xanthurenic acid. On the contrary, an increase on excretion of xanthurenic acid was observed in some iron-deficiency anemia patients whose anemia was not B_6-dependent (R2).

The investigation of Marver (M2) indicated that the urinary tryptophan metabolites were elevated in 2 children and 3 adults with erythrogenesis imperfecta after a 2-g load of L-tryptophan. Also anthranilic acid was excreted in abnormally large amounts, thus confirming the findings of Altman and Miller (A4). The repeated elevation of urinary tryptophan metabolites after a load dose in the mother of one child with aplastic anemia suggested that the tryptophan abnormality noted in at least this case may be genetically determined.

Sporadic elevations of tryptophan metabolites, particularly kynurenine and 3-hydroxykynurenine, in 11 subjects with neoplastic hematological disorders were also observed (M2), confirming the observation of Musajo and co-workers (M20). The fact that these patients were afebrile and yet excreted increased quantities of urinary tryptophan metabolites before and after loading argues against Dalgliesh's hypothesis (D3) that increased tissue breakdown associated with fever was responsible for this phenomenon.

Tryptophan metabolism was also evaluated in 17 patients with congenital aplastic anemia, aged 3 months to 12 years, in comparison with 29 normal controls (S7). At the time of the experiments 13 patients were being sustained only by repeated blood transfusions while others were maintained by administration of corticoids. Oral doses of 0.5–2.0 g L-tryptophan were given. Although an increased excretion of anthranilic acid, both free and conjugated with glycine and glucuronic acid, was not demonstrated, some patients showed an altered excretion of other tryptophan metabolites (S7).

Finally, it should be remembered that a statistically significant decrease of coenzymatically active B_6 in plasma was found only in patients with nutritional anemia and various forms of leukemia. Moreover, B_6 values in leucocytes were consistently decreased in patients with various forms of leukemia [Wachstein *et al.* (W7) used a modification of the manometric method of Umbreit (U1) for the determination of pyridoxine levels].

2.2.4.3. *Bladder Tumor.* In addition to the study of the spontaneous excretion of tryptophan metabolites by bladder tumor patients, a comparison was attempted between the excretion of normal subjects and that of a group of 4 bladder cancer patients after a loading dose of 100 mg/kg of L-tryptophan (B8).

These patients appear to excrete a slightly higher amount (8.9–12.3%) of tryptophan derivatives than normals (average 6.7%). The excretion ratios are different for each metabolite: the highest ratio is found for 3-hydroxykynurenine and the lowest for 3-hydroxyanthranilic acid.

2.3. Studies on the Excretion of Tryptophan Metabolites in Other Pathological Conditions

The metabolism of tryptophan, usually after loading with different doses of the amino acid, has also been investigated in a less homogeneous group of pathological states.

2.3.1. *Pediatrics*

2.3.1.1. *Newborn.* It is known that some mammalian enzymes are absent in fetal life or may display a low activity only at the end of gestation whereas after birth their activity is enhanced to adult levels more or less rapidly. This is the case with tryptophan pyrrolase, a liver enzyme catalyzing the conversion of tryptophan to N^1-formylkynurenine, the first step of the kynurenine pathway.

Tryptophan pyrrolase activity is lacking in fetal liver of rat and rabbit and appears late in gestation in the guinea pig. In the fetus and newborn of these animals the metabolism of tryptophan to nicotinic acid is decreased or completely absent (N1).

Auricchio *et al.* (A9–A11) analyzed the excretory pattern of tryptophan metabolites in urine of 10 normal newborn children from the first to 30th day of life. During the period 1st day to 4th–11th day no tryptophan metabolites were detected. During a second phase, 5th–12th day until 7th–28th day, several metabolites together with their conjugates were

present in urine. A third period was characterized by a progressive disappearance of the urinary derivatives. In addition Auricchio *et al.* (A11) fed L-tryptophan to neonates on the second day of life and found that urine contained only tryptophan and none of the metabolites of the kynurenine pathway. The authors' conclusion is that tryptophan pyrrolase activity is absent in the first days of life.

Sartori and Nico (S1) found the excretion of xanthurenic acid, after oral L-tryptophan (100 mg/kg), to be directly proportional to the age. It was negative in 10 healthy breast-fed infants and elevated in their nursing mothers, whereas 10 children, aged 4–6 years, showed a mild xanthurenuria.

According to Careddu *et al.* (C3), the human tryptophan pyrrolase is, on the contrary, very active during the first days of life and the inability to synthesize nicotinic acid is due to a metabolic block of the kynurenine pathway. They administered 800 mg L-tryptophan to 21 premature and 5 full-term nursing infants aged 1–30 days. The presence of kynurenine, *N*-α-acetylkynurenine, 3-hydroxykynurenine, kynurenic and *o*-aminohippuric acids was demonstrated by paper chromatography and that of xanthurenic acid colorimetrically. Hence, tryptophan pyrrolase was active a few hours after birth. That the urinary excretion of these tryptophan metabolites was high in the first 10 days of life was ascribed to pyridoxine deficiency. Vitamin B_6 therapy resulted in the cases examined in a decreased excretion of metabolites after a loading test. The authors postulate that pyridoxine deficiency frequently observed in the last trimester of pregnancy is carried over into the newborn or the demands for vitamin B_6 are higher in neonatal life than in later periods.

Vassella and Hellström (V3) investigated the fetal urinary excretion of tryptophan metabolites at the end of pregnancy by means of paper chromatography in urine samples obtained at birth from 50 neonates before clamping of the umbilical cord. They found that these "fetal" urines revealed trace amounts of tryptophan, kynurenine, and very often kynurenic and xanthurenic acids. In addition urine samples collected during the third day after birth from 20 newborn of the same group showed in all cases an increased excretion of the derivatives already present at birth and the presence of additional metabolites. These findings lead to the view that in most infants some pyrrolase activity, although weak, is already present at the end of fetal life (V3).

A chromatographic investigation was carried out in 10 weaned infants given tryptophan loads, before and after pyridoxine treatment (P2). The consistent excretion of high levels of kynurenine and kynurenic and xanthurenic acids is decreased for the former compound and normalized

for the latters following pyridoxine administration. On the contrary, biotin administration caused an increase of excreted kynurenine and no change for the other metabolites upon tryptophan loading (P3).

Dahler (D1) examined xanthurenic acid excretion after a tryptophan load in 44 infants aged 3 days to 18 weeks and that of kynurenine in 12 newborns. An increase of xanthurenic acid excretion above normal levels was encountered in 4 of 25 full-term and in 9 of 19 premature infants. Administration of vitamin B_6 to 5 cases reduced the output of xanthurenic acid to normal levels.

No evidence was found (D1) for immaturity of the enzyme systems involved in tryptophan metabolism, since kynurenine and xanthurenic acid could be detected in the first weeks of life in urine of infants loaded with tryptophan.

The disappearance of xanthurenic acid after pyridoxine therapy suggested the possibility of a latent B_6 deficiency (D1). Dahler-Vollenweider (D2) screened 159 children in respect to the urinary picture of tryptophan metabolites and found an abnormal excretion of xanthurenic acid in 17, whereas 142 fell within normal limits after tryptophan loading. There seems to be an increased rate of excretion with age. In the newborn the excretion was the lowest while the maximal values were found in the age group 10–16 years. During periods of rapid growth there was a high excretion rate of xanthurenic acid, which was not appreciably affected by daily oral administration of 100 mg pyridoxine.

Paper chromatographic analysis indicated that the abnormal excretion of xanthurenic acid always corresponded with higher amounts of other tryptophan metabolites such as kynurenine, acetylkynurenine, 3-hydroxykynurenine, and kynurenic and 3-hydroxyanthranilic acids after the usual load of tryptophan.

The urinary excretion of tryptophan, either spontaneous or after tryptophan loading (100 mg/kg), was recently studied in 50 healthy infants 1–20 months of age (V4). The results indicated that 94% of healthy infants excreted spontaneously higher amounts of kynurenine, on the basis of body weight, than do adults. This high value of kynurenine was confirmed by quantitative determination after a tryptophan load and was considered on an average to be 20 times larger than that of healthy adults.

This fact has been explained (V4) in different ways. One possibility is that tryptophan pyrrolase is more "active" in the growing organism than in the adult. An accumulation of kynurenine can also be the result of relative insufficiency of the later steps of enzyme activity in the kynurenine–nicotinic acid chain.

2.3.1.2. *Mongolism.* Several authors have pointed out that children affected by mongolism show a defect in tryptophan metabolism which is characterized primarily by a decreased urinary excretion of xanthurenic acid.

Gershoff *et al.* (G3) observed no significant differences, following the ingestion of 5 g DL-tryptophan, in N^1-methylnicotinamide and creatinine excretion, but xanthurenic acid was lower in mongoloids than in non-mongoloid mentally retarded children.

Jérôme *et al.* (J2) found that urinary excretion of 5-hydroxyindole-acetic acid, indoleacetic acid, and xanthurenic acid was less in 15 mongoloids than in 16 normal children. An oral dose of 30 mg/kg of L-tryptophan produced an increased excretion of these compounds, the increase being in the same proportion in the mongoloids as in the normal. Excretion of kynurenine is at the same level in both groups and is increased after the loading test.

The study of O'Brien and Groshek (O1) confirmed the previous observations (G3, J2) that in children with mongolism there was a decreased urinary excretion of xanthurenic acid following an oral dose of L-tryptophan. They administered a tryptophan load of 100 mg/kg to 21 mongoloid children 9–16 years old and to 19 comparable nonmongoloid mental defectives, and measured the urinary excretion (μmoles/kg/7-hour period) of 5-hydroxyindoleacetic acid, kynurenic, xanthurenic, 3-hydroxyanthranilic, and 4-pyridoxic acids, kynurenine, and 3-hydroxykynurenine. The results suggest that in children with mongolism there is a specific abnormality of 3-hydroxykynurenine transaminase resulting in a diminished conversion of 3-hydroxykynurenine to xanthurenic acid. According to the authors (O1) it would be necessary for a final proof to measure urinary xanthurenic acid after the intravenous administration of its precursor 3-hydroxykynurenine, and to demonstrate a normal renal clearance of xanthurenic acid per unit of glomerular filtrate.

2.3.1.3. *Hematological Disorders.* Seventeen boys under 4 years of age were studied by Rabe and Plonko (R1) for evidence of pyridoxine need. Four of the children had hypochromic microcytic anemia, 4 had convulsions, and 9 suffered from miscellaneous acute infectious illnesses.

Xanthurenic acid and N^1-methylnicotinamide were measured before and after a standard DL-tryptophan load of 0.54 mg/kg, whereas xanthurenic acid and 4-pyridoxic acid were determined before and after parenteral pyridoxine hydrochloride (25 mg) plus the standard DL-tryptophan load.

It was suggested that the tests may indicate pyridoxine requirement

in the following order of decreasing sensitivity: xanthurenic acid, N^1-methylnicotinamide, and 4-pyridoxic acid excretion tests (R1).

Auricchio *et al.* (A12) analyzed the excretory pattern of 6 children with leukemia and 2 with Hodgkin's disease. No definite relationship could be observed between the disease and the abnormally elevated amounts of tryptophan metabolites. After niacin therapy the urinary excretion of kynurenines and kynurenic and xanthurenic acids was normalized in one case of Hodgkin's disease and in one leukemic subject after pyridoxine administration.

Chizhova and Ivanova (C7) studied 20 children, aged 1–12 years, under therapy for leukemia and 10 healthy children as control. A total of 15–20 g of tryptophan was administered during 5–10 days (1.5–3 g/day) to 7 children whereas 13 were given a single dose of 2–3 g. Daily determinations of urinary metabolites by paper chromatography demonstrated a disturbance of tryptophan metabolism in 19 of the 20 leukemic children before and after tryptophan loading. Kynurenine, 3-hydroxykynurenine, and anthranilic and 3-hydroxyanthranilic acids appeared in urine, whereas 5-hydroxyindoleacetic acid was absent in the majority of the young patients. The disturbances of tryptophan metabolism were similar in all of them. Administration of vitamin B_6 restored tryptophan metabolism to normal in the majority of the patients.

Interrelations among vitamin B_6, hemoblastotic diseases, and tryptophan metabolite excretion have been investigated by Anderson (private communication to Professor L. Musajo) in a case of infantile leukemia showing spontaneous excretion of urinary kynurenine and 3-hydroxykynurenine, whose levels were normalized by daily administration of 50 mg pyridoxine.

The studies of Altman and Miller (A4), Marver (M2), and Smith *et al.* (S7) of several cases of congenital aplastic anemia in children have been mentioned in the section 2.2.4.2.

2.3.1.4. *Erythroderma Desquamativum and Acrodynia.* Studies on erythroderma desquamativum at different stages in several nursing infants were carried out in 1953 by some authors (B13, S2, S3).

Peressini (P4) followed xanthurenic acid excretion in two children with acrodynia loaded with L-tryptophan. Xanthurenuria was influenced by administration of both deoxypyridoxine and pyridoxine which, respectively, increased and normalized the levels.

2.3.1.5. *Convulsions and Spasms.* The experimental production of vitamin B_6 deficiency in man and many data on animal experimentation have suggested that convulsions might well be a critical manifestation of this deficiency in infancy.

Bessey *et al.* (B11) studied 11 infants, 1–12 months of age, with convulsions without apparent cause, who have remained free of seizures after a change to a diet providing an increased amount of pyridoxine. Seven of the infants became free of convulsions when given an evaporated milk mixture supplying an average of 0.26 mg vitamin B_6 (as pyridoxine, pyridoxal, or pyridoxamine hydrochloride) daily. Four of these seven infants were studied for biochemical evidence of deficiency of pyridoxine by the tryptophan loading test. All four excreted large amounts of xanthurenic acid after receiving 0.54 g/kg of DL-tryptophan; 1–1.4 mg pyridoxine daily was required to prevent the excretion of xanthurenic acid. In contrast, 10 of 11 control infants receiving 0.2–0.5 mg vitamin B_6 daily failed to excrete xanthurenic acid when similarly tested. Moreover, two of these controls infants, who began to excrete xanthurenic acid after receiving for 1 month a diet low in vitamin B_6, ceased to do so after receiving 0.3–0.4 mg pyridoxine daily. Hence the requirement for vitamin B_6 of the 7 infants with convulsions who developed the deficiency is, for some reason, greater than that of the average infant.

Two infants whose convulsions appeared in the neonatal period required 2–5 mg vitamin B_6 daily to eliminate the seizures. However, like the controls, these two infants required only 0.3–0.4 mg vitamin B_6 daily to prevent excretion of xanthurenic acid after receiving a dose of tryptophan. It seemed probable that these two infants have an anomalous metabolism of pyridoxine rather than simple deficiency (B11). They appear to have a condition very similar to that of pyridoxine dependency described by Hunt *et al.* (H12). The mother of their patient had been treated with large amounts of pyridoxine parenterally during pregnancy and the authors (H12) suggested that this treatment might in some way have influenced the development of pyridoxine dependency in their patient. She had seizures within 48 hours when she was not receiving 2 mg pyridoxine hydrochloride daily. Xanthurenic acid excretion following a tryptophan loading was not increased in this patient and the electroencephalogram was abnormal only during the seizure episodes.

The authors (B11) suggest that the requirement for vitamin B_6 of the 7 infants who developed the clinical manifestations of deficiency was for some reason greater than that of the average infant. Their cases, however, were not born to mothers who had received massive doses of pyridoxine during pregnancy.

The discrepancies between the amount of pyridoxine required to suppress abnormal xanthurenic acid excretion and to eliminate the convulsions in these infants suggests that an aberrant metabolism of trypto-

phan, rather than dietary deficiencies, is most likely the cause of this syndrome.

Increased excretion of xanthurenic acid was found (H7) in 13 cases of infantile spasm as compared with a control group of healthy infants, before and after tryptophan loadings. This finding is interpreted as a sign of relative vitamin B_6 deficiency in tissue concerned with tryptophan metabolism caused by an increased demand for pyridoxine as a coenzyme in the brain tissue. In most cases the disturbed tryptophan metabolism should have no direct relation to the cerebral symptoms, as these were not relieved by parenteral administration of high doses of vitamin B_6.

Despite normal plasma pyridoxal 5-phosphate values, the urinary excretion of xanthurenic acid was found abnormally elevated in 3 epileptic children with disturbed tryptophan metabolism (H0). Administration of pyridoxine restored xanthurenuria to normal and raised plasma pyridoxal 5-phosphate levels.

2.3.1.6. *Various Diseases.* Abbassy *et al.* (A1) observed in 12 cases of malnutrition (including kwashiorkor), toxic dyspepsia, 8 cases of acute nephritis, 8 cases of infective hepatitis, and muscular dystrophy an increased spontaneous excretion of xanthurenic acid, the amount of which was found to depend on the severity of the case. In all these cases, with the exception of acute nephritis and hepatitis, the amount of xanthurenic acid was restored to normal levels after vitamin B_6 therapy. In 8 children with mental retardation, cerebral palsy, recurrent convulsions, 5 with nephrotic syndrome, and 5 with pellagra the amount of xanthurenic acid spontaneously excreted was found to be within the normal range, indicating that pyridoxine is probably not concerned in these cases.

Morales and Lincoln (M11) studied pyridoxine deficiency in 26 tuberculous children. Of these 20 received isoniazid therapy for various periods and clinical signs of B_6 deficiency were not observed. The ability to convert tryptophan to N^1-methylnicotinamide was used as a test for pyridoxine deficiency. Except for 1 case, all patients showed an increase in urinary N^1-methylnicotinamide excretion after tryptophan loading, and the authors admit the absence of pyridoxine deficiency.

Cotte and Plantier (C11) also applied tryptophan loading (0.54 mg/kg) as a tool for detecting pyridoxine deficiency in children by means of xanthurenic acid determination. The investigation was extended to normal controls, tuberculous children treated with isoniazid, 9 with convulsive syndrome and infantile seizures, 2 with Wilson's disease, and 2 with Fanconi's anemia.

Urines with positive diazo reactions were found by Ijiri (I1) in

infants with measles. Such a reaction was given by three substances, separated by paper chromatography, one of which was identified as 3-hydroxykynurenine by its ultraviolet absorption spectrum.

In 48 children with diabetes mellitus the urinary xanthurenic acid excretion was studied. In patients with a well-managed diabetes no difference was found in comparison to normal controls (B3).

The data collected in the pediatric field, although by different methods, are many, sometimes contradictory, but interesting. However, for their interpretation and correlation other information is evidently necessary.

2.3.2. *Diabetes*

In 1953 Kotake and Inada (K14, K15) described a diabetic syndrome in rats fed a diet containing tryptophan and sodium butyrate. They found also abnormal urinary excretion of 3-hydroxykynurenine and xanthurenic acid in patients with diabetes.

The diabetogenic effect of xanthurenic acid was not confirmed in rats fed the same diet as that of Kotake (H10, K1) with or without pyridoxine and riboflavine added (H1). Moreover single and repeated intraperitoneal injections of xanthurenic acid (200 mg/kg) failed to induce diabetes in rats (H10, W9) and dogs (M1). Rats receiving a diet devoid in pyridoxine, but rich in tryptophan and fatty acids, show an increase of their blood sugar levels but no histological change (H10). It has been surmised that the rats used by the Japanese authors belonged to a strain particularly predisposed to diabetes (H1, H10).

In human diabetes Rosen *et al.* (R5) found that 20 diabetic patients with and without retinopathy excreted on the average significantly greater quantities of xanthurenic acid after an oral test load of 10 g DL-tryptophan than did nondiabetic controls. No significant difference was found in the xanthurenic acid output of diabetic patients with and without retinopathy (R5).

According to Wachstein and Lobel (W5), 4 patients with diabetes mellitus excreted low amounts of xanthurenic acid and showed a negative diazo reaction in urine after a 10-g load of DL-tryptophan.

Dalgliesh and Tekman (D3) examined a large number (44) of insulin-treated diabetes patients and in no case observed a spontaneous 3-hydroxykynurenine excretion. In view of the marked tendency for diabetes to coexist with other disorders it seemed probable to Dalgliesh and Teckman (D3) that the excretion observed by Kotake and Tani (K16) may be ascribed to fever due to complications, and not to diabetes as such.

Wiseman *et al.* (W13) found that the excretory picture of tryptophan

metabolites in diabetic patients did not differ from that of normal controls, except for a lower level of kynurenine. After ingestion of 4 g L-tryptophan there was observed a decreased excretion of tryptophan, kynurenine, and anthranilic and xanthurenic acids in the urine of 12 diabetic patients in comparison with that of 17 normal controls.

Montenero (M10) screened more than 1000 diabetic patients, not hospitalized, and found abnormal presence of spontaneous xanthurenic acid in the urine of 13.5% of subjects. This excretion was followed qualitatively in 119 patients at least thrice in a period of several months and was confirmed in almost all cases. It seems that no direct relationship exists among spontaneous xanthurenuria, glycemic response, severity, and duration of the disease. The data obtained were examined (M10) in relation to age and sex, and a higher degree of xanthurenic acid excretion was noted in subjects aged 30–39 years and among women. Parenteral administration for a week of 500–600 mg per day of pyridoxine caused almost complete disappearance of urinary xanthurenic acid. If the excretion of xanthurenic acid is considered an index of pyridoxine deficiency, its normalized levels after pyridoxine therapy may be a confirmation that in some diabetic patients there exists a metabolic derangement of tryptophan due to a possible deficiency of vitamin B_6.

Wohl *et al.* (W15) found that 4 diabetic patients excreted, before and after tryptophan loading, low levels of xanthurenic acid and that this is not modified by administration of pyridoxine or by a second test with 10 g DL-tryptophan. Kojecky and Telupinova (K11) also found no increase in xanthurenic acid and 3-hydroxykynurenine excretion in diabetic patients loaded with 100 mg/kg of DL-tryptophan, whereas increased amounts of kynurenine were observed in the same patients. By determining urinary pyridoxine after a 1-g vitamin B_6 load and urinary xanthurenic acid after a 10-g tryptophan load, Lebon *et al.* (L1) demonstrated pyridoxine deficiency in about half of 144 diabetic patients. This defect is more frequent in patients with juvenile diabetes and is curable by treatment with 0.5–1 g pyridoxine daily.

It has been observed (D6) that addition of vitamin B_6 to insulin therapy allowed the employment of lower doses of insulin and, in one subject, the total cessation of insulin administration. Finally, Oka and Leppänen (O4) studied the tryptophan metabolism in 10 patients with diabetes mellitus and in 12 control subjects by determining the urinary excretion of 5-hydroxyindoleacetic acid, kynurenine, and anthranilic, 3-hydroxyanthranilic, and xanthurenic acids before and after a load of 2 g L-tryptophan. The authors noted a markedly increased excretion of

xanthurenic acid both before and after the loading dose in diabetic subjects, whereas the excretion of kynurenine was lower and that of 5-hydroxyindoleacetic acid a little higher in patients than in normal controls after the test. According to Oka and Leppänen (O4), the metabolic derangement is partly due to B_6 disturbance.

The findings of Kotake (K15) of an abnormal presence of xanthurenic acid in urine of diabetic patients could not be confirmed also by Pelikan *et al.* (P1a) in 131 of these patients. They observed, however, after a 5-g tryptophan loading in 15 diabetics an increased excretion of xanthurenic acid with a simultaneous glycemic rise. Furthermore, insulin and pyridoxine showed favorable effects on xanthurenuria.

The problem was further investigated (W8a) in 120 diabetic patients without other diseases. The authors concluded that pyridoxine deficiency is not a metabolic peculiarity in diabetics as the levels of xanthurenic acid in the patients group are comparable to that of the controls.

Other tryptophan metabolites would be of interest in the diabetic syndrome in both experimental animals and human beings. In regard to this the investigations of Kotake (K17, K18) and Ayad (A13) have to be mentioned.

Alloxan-diabetic animals, in which the conversion of tryptophan to methylnicotinamide was greatly impaired, excreted increased amounts of methylnicotinamide when very large doses of tryptophan were given, indicating that the defect may be due to changes in the kynurenine pathway of tryptophan (M5). Moreover, diabetic rats excreted much more xanthurenic acid and less anthranilic and 3-hydroxyanthranilic acids than did nondiabetics, following large doses (200–400 mg) of tryptophan.

Study of the interrelationship between insulinase, diabetes mellitus, and tryptophan (M9) revealed that L-tryptophan, administered either subcutaneously or by stomach tube in the amount 1–4 mM/kg, produces a statistically highly significant hypoglycemia in the rat, whereas D-tryptophan and 16 other amino acids were ineffective when given by mouth in similar doses. In contrast to the effect produced in normal rats, there is no hypoglycemic response in severely diabetic alloxanized rats given L-tryptophan.

In view of the hypoglycemic and insulinase-inhibitory action of L-tryptophan, it seemed pertinent to Mirsky to study the effect of various metabolic products of tryptophan on the blood sugar of normal and diabetic rats (M9). A statistically significant hypoglycemic response was produced after oral administration of anthranilic acid, niacin, indole-3-acetic acid, 5-hydroxytryptophan, and serotonin. A hypoglycemic fol-

lowed by a hyperglycemic response was produced by kynurenine, whereas kynurenic acid and nicotinamide gave a significant hyperglycemic response. The hypoglycemic and insulinase-inhibitory effect of tryptophan was, therefore, not due to the products of any one step of its metabolism.

It was also observed (B33) that the hypoglycemic response after L-tryptophan plus tolbutamide was greater than that with L-tryptophan alone and less evident than that with tolbutamide alone.

From such a complex of data one can draw conclusions which permit us to either accept or reject the evidence for a direct connection of tryptophan and its metabolism with diabetes. Further investigations are therefore necessary.

2.3.3. *Hyperthyroidism*

Wohl *et al.* (W15) determined the occurrence of pyridoxine deficiency in 14 patients with hyperthyroidism and in 14 control euthyroid patients by loading with 10 g DL-tryptophan. Urinary xanthurenic acid excretion following the tryptophan dose was significantly greater in hyperthyroid patients than in controls. Mean xanthurenic acid excretion after pyridoxine administration was not significantly different in the two groups. It is suggested that it is not unreasonable to state that in hyperthyroidism the availability of pyridoxine is limited.

Wachstein and Lobel (W2) had already pointed out an elevated xanthurenuria, after tryptophan loading, in 4 hyperthyroid subjects, which was normalized with administration of pyridoxine. Kotake *et al.* (K19) observed disappearance of urinary xanthurenic acid after thyroidectomy in 3 patients with Grave's disease. It is certain that, even though only a small number of these patients has been studied, a relation between thyroid activity and tryptophan metabolism has to be admitted. The nature and significance of the biochemical lesion for thyroid pathology must, however, be elucidated. It should be recalled that recent evidence (L5) has shown that inhibition of the effect of thyroxine on oxygen consumption of rat kidney *in vitro* was produced by the addition of tryptophan to the incubation medium. A study of the effect of tryptophan metabolites, such as kynurenic, xanthurenic, and anthranilic acids, nicotinic and quinolinic acids, kynurenine and 3-hydroxykynurenine, and analogs such as *N*-α-acetyl-L- and DL-tryptophan, tryptamine, serotonin, 5-hydroxy- and 5-methyltryptophan, and indoleacetic acid also showed a similar inhibition by many of these compounds. Little relationship was apparent between this effect and the ability of the compound to inhibit thyroxine activity (L5). Galton and Ingbar (G1) attempted to determine the

nature of this inhibitory effect of tryptophan on thyroid function *in vitro*. Their experiments showed that the degradation of thyroxine to inorganic iodide and that of several of its analogs are markedly depressed by the presence of several metabolites of tryptophan.

Among the active compounds were xanthurenic and 3-hydroxyanthranilic acids, whereas tryptophan, kynurenine, and kynurenic, anthranilic, and nicotinic acids were unable to cause deiodination. These findings support, according to the authors, the view that the deiodination of the thyroid hormone may be closely associated with its biological action.

2.3.4. *Liver Diseases*

Metabolic modifications in the chain of reactions involved in tryptophan metabolism were studied by Quagliariello *et al.* (Q2, Q3) and Piazza and Tancredi (P5) by qualitative analysis of urinary metabolites in 24 patients with viral hepatitis in an acute stage and in 34 subjects at least 2 months after recovery in comparison with normal controls.

The spontaneous urinary pattern during viral hepatitis in an acute phase seems to be abnormal, showing kynurenine in a high percentage of cases together with 3-hydroxykynurenine, 3-hydroxyanthranilic acid, and in a few instances anthranilic acid. Some discrepancies appear for the excretion in clinically recovered subjects, since Quagliariello (Q3) found a normalized output of metabolites, whereas Piazza and Tancredi (P5) found that about 60% showed an abnormal excretion of the same substances.

Raskin (R4) tried the DL-tryptophan (10 g) loading test on 12 patients with infectious hepatitis, 72 with chronic hepatitis following the infectious phase, and 5 with Parkinson's disease. It was shown, by the xanthurenic acid test, that the chronic form was accompanied by a disturbance of tryptophan metabolism, and that xanthurenuria persisted even when amounts of pyridoxine were excreted, i.e., in a state of hypervitaminosis. Vitamin B_6 was therefore well above the minimal daily requirement of the patients, thus showing that other factors are involved in the abnormal excretion of xanthurenic acid.

Alferova and Raskin (A3) gave DL-tryptophan to 22 patients with chronic hepatitis and determined the xanthurenic acid excretion in urine and pyridoxine levels in blood serum. A decreased pyridoxemia was demonstrated in patients excreting xanthurenic acid after tryptophan load, when compared with that of patients excreting less xanthurenic acid or with that of healthy persons.

It was observed in viral hepatitis (B16) that the production of kynurenine from tryptophan is disturbed owing to a cellular deficit in en-

zymes which act upon kynurenine. In two cases of severe viral hepatitis, L-tryptophan was given orally, followed by 24-hour collection of urine. A marked excretion of kynurenine plus smaller amounts of *N*-α-acetylkynurenine and xanthurenic acid was noted. At this time transaminases were also high, indicating that the disease was in an active phase. In a second tryptophan test after administration of vitamin B_6, no urinary kynurenine was found although some *N*-α-acetylkynurenine and xanthurenic acid were excreted. The results suggest (B16) that in this disease the administration of pyridoxine should have therapeutic value.

When the tryptophan loading test was applied in 2 cases of cirrhosis of the liver, the results were the same as in normal persons, with a relatively slight urinary excretion of kynurenine and its acetyl derivative.

Certainly, the altered excretory pattern of tryptophan metabolism could be related, according to most authors, to an acute lesion of the hepatic cells and subsequently to its enzymatic activities.

Increased xanthurenic acid excretion after 10 g DL-tryptophan was demonstrated by Lerner *et al.* (L3) in 3 of 5 patients with rum fits. This metabolic defect was corrected by pyridoxine administration, as observed in a second tryptophan load test. Using the same xanthurenic acid test, significant vitamin B_6 deficiency was not observed in patients with alcoholism and associated epilepsy, acute and chronic alcoholism, cirrhosis, acute hallucinosis-tremulousness, acute peripheral neuropathy, Wernicke-Korsakoff syndrome, and nonalcoholic, healthy individuals. It is postulated that pyridoxine deficiency is etiologically related to rum fits (L3).

More recently, Olson *et al.* (O5) reported that in 34 patients with chronic alcoholism the excretion of 5-hydroxyindoleacetic acid was significantly lower than in normal controls, after oral ingestion of 10 g DL-tryptophan. Such lowered excretion was unaffected by improved nutritional status or abstinence from ethyl alcohol. The urinary excretion of xanthurenic acid was similar in the alcoholic and control groups. The conclusions indicate that in chronic alcoholism the conversion of tryptophan to 5-hydroxyindoleacetic acid is preferentially depressed, while the kynurenine pathway is normal (O5).

2.3.5. *Renal Calculi*

The inorganic part of a majority of renal stones is composed of either pure calcium oxalate or calcium oxalate mixed with apatite.

Studies of human beings, cats, and rats have shown that endogenous oxalate excretion is inversely related to the amount of vitamin B_6 in the diet (G4–G6). It was shown that the amount of pyridoxine apparently adequate to sustain normal health and growth does not necessarily ensure

minimal excretion of endogenous oxalate in cat and man. Andrus *et al.* (A5) have observed the development of renal calcium oxalate calculi in vitamin B_6-deficient rats accompanied by obstructive sequelae similar to those in man.

Gershoff and Prien (G6) found that normal subjects excrete significantly less xanthurenic acid and 4-pyridoxic acid and more citric acid than patients with chronic formation of calcium oxalate. A marked rise in excretion of calcium oxalate followed administration of tryptophan in these patients, whereas ingestion of pyridoxine was followed by a decrease in urinary oxalate.

Faber *et al.* (F1) studied the effects of induced pyridoxine and pantothenic acid deficiency, obtained by use of a semisynthetic formula and deoxypyridoxine and ω-methyl pantothenic acid supplements for six weeks, by determining in 5 men nitrogen retention and the urinary excretions of xanthurenic and oxalic acids during deficiency and recovery. They postulated that tissue catabolism releases sufficient pyridoxine to partially metabolize a tryptophan load, after which the amounts of urinary oxalic acid were sharply increased for 1–2 days.

Gershoff and Andrus (G7) found that less xanthurenic acid was excreted spontaneously by vitamin B_6-deficient rats receiving high levels of magnesium. Just how magnesium functioned biochemically in this investigation is not clear according to the authors (G6). Magnesium is essential, however, for many of the reactions of intermediary metabolism, and it appears possible that B_6-dependent enzyme systems may be more sensitive to the dietary level of magnesium.

These studies indicate an intimate relation between the magnesium level of the diet and the pyridoxine requirement. It is suggested (A6) that, insofar as growth and urinary excretion of citrates, oxalates, and xanthurenic acid are concerned, high levels of magnesium appear to have a sparing effect on very low dietary levels of pyridoxine.

Interesting results were reported when C^{14}-oxalate appeared in rat urine after administration of C^{14}-tryptophan (F2). The data showed that tryptophan is a precursor of oxalate in rats and that approximately 16 times more labelled oxalate was excreted when tryptophan labelled in the 2-C than 3-C position was used. Although more oxalates were excreted by pyridoxine-deficient rats than their controls, the deficient animal did not convert more of the injected tryptophan to oxalate than the control rats. Thus, B_6-deficiency did not appear to affect the percentage of administered C^{14}-tryptophan which appeared in urine as oxalate. This may have been a reflection of the often reported defect in

the conversion of 3-hydroxykynurenine to 3-hydroxyanthranilic acid and alanine in B_6-deficiency.

2.3.6. *Rheumatoid Arthritis*

McMillan (M6) has recently reported the presence of increased amounts of spontaneous 3-hydroxyanthranilic acid in urine of patients with rheumatoid arthritis. This author also measured 3-hydroxyanthranilic acid excretion in 3 cases of osteoarthritis, 18 cases of other orthopedic conditions, and 25 cases of miscellaneous diseases.

Oka and Leppänen (O3) also have suggested that the metabolism of tryptophan may be abnormal in rheumatoid arthritis. From chromatographic investigations of urinary indole excretion they concluded that, in such patients, there might be some alteration in the metabolism of the amino acid by the kynurenine pathway.

Bett (B12) has confirmed McMillan's finding and has shown a raised excretion of 3-hydroxyanthranilic acid in 39 patients with rheumatoid arthritis. Moreover, kynurenine excretion has been measured and found to be higher in the rheumatoid patients than in the controls.

The effect of loading doses of DL-, L-, and D-tryptophan on the excretion of 3-hydroxyanthranilic acid and kynurenine has been studied (B12) in a series of 25 controls and 15 rheumatoid patients. D-Tryptophan (1 g) was not converted to 3-hydroxyanthranilic acid by any of the subjects. After ingestion of 3 g L-tryptophan the level of 3-hydroxyanthranilic acid excretion was raised in both groups and there was no difference between controls and rheumatoid patients. Kynurenine excretion was increased in all subjects after ingestion of all three forms of tryptophan (D, L, and DL). With D-tryptophan the rise was moderate and there was no difference in excretion between the two groups. After ingestion of L-tryptophan there was a large increase in kynurenine excretion in the patients with rheumatoid arthritis, but only a moderate one in the control group.

A spontaneous increased excretion of 3-hydroxyanthranilic acid was confirmed in a group of 26 patients with rheumatoid arthritis (S7a). The amount excreted did not correlate with any other clinical or laboratory parameters.

Sudakova and Ryvkin (S9) studied 52 patients of different ages with active rheumatism and determined their pyridoxine deficiency through the degree of disturbed tryptophan metabolism in the form of increased rates of xanthurenic acid excretion in urine. After a load of tryptophan the concentration of xanthurenic acid in urine of 40 patients rose to higher levels, in some cases as high as 300 mg, with a total average of 97 mg. Urinary xanthurenuria remained normal in only 9 patients

after loading and in 3 cases its presence could not be detected. Pyridoxine deficiency, caused by administration of antibiotics, was considerably reduced and in some cases completely eliminated with vitamin B_6.

In conclusion, all these experimental data agree that a deranged tryptophan metabolism is observable in patients with rheumatoid arthritis.

2.3.7. *Scleroderma*

Price *et al.* (P10) found in 3 female patients with scleroderma (acrosclerosis) an abnormal tryptophan metabolism characterized by an abnormally large urinary excretion of kynurenine, 3-hydroxykynurenine, kynurenic acid, and *N*-α-acetylkynurenine. One of them excreted normal amounts of xanthurenic acid, before and after the ingestion of 2-g L-tryptophan. During therapy with sodium ethylendiaminetetraacetic acid (Na_2EDTA) the tryptophan metabolism became nearly normal in one patient and normal in a second. The simultaneous administration of Na_2EDTA and pyridoxine considerably improved the tryptophan metabolism in the subject less responsive to Na_2EDTA alone. During a second course of Na_2EDTA, both subjects responded as in the first clinical trial. The third patient was found to have normal tryptophan metabolism after treatment with Na_2EDTA or pyridoxine.

For the authors (P10) the simplest explanation of the data on tryptophan metabolism in these 3 patients would be as follows: in scleroderma (acrosclerosis) there was an abnormal urinary excretion of kynurenine and its metabolites after oral ingestion of tryptophan. The administration of pyridoxine or pyridoxine plus nicotinamide partially corrected the metabolic abnormality. The efficacy of pyridoxine plus Na_2EDTA could be explained on the basis of a decrease in tissue calcium and zinc (and possibly other cations), enabling the metal ions, normally functioning with pyridoxal phosphate, as magnesium ions, to be utilized more advantageously.

2.3.8. *Skin Disorders*

Knapp (K5) investigated the possibility of reduced vitamin B_6 supplies in people with skin diseases by using a 10-g DL-tryptophan loading test. Excessive xanthurenic acid excretion occurred in 18 of 43 cases, 8 with allergic dermatitis, 2 with eczema from exposure to light, 2 with exudative multiform erythema, and the remainder with various afflictions.

Studies on skin tuberculosis cases indicated that isoniazid may act as a B_6 antivitamin. The relation of pyridoxine deficiency to niacin deficiency was investigated by determining both urinary xanthurenic acid and

niacin metabolites after tryptophan loading in healthy and sick subjects given isoniazid.

Furthermore, Knapp and Gassmann (G1a, K3, K8) loaded normal subjects and patients having various cutaneous disorders with 10 g DL-tryptophan orally, and determined the 24-hour excretion of xanthurenic acid. Values indicating an abnormal excretion of xanthurenic acid were observed in 22 of 55 patients, predominantly those with cutaneous allergic or light sensitivity manifestations. In many instances these abnormal results were normalized following therapy with vitamin B_6.

2.3.9. *Pellagra*

Another disease of prominent dermatological interest is pellagra, in which pyridoxine deficiency seems to represent one of the pathogenetic factors even though of less importance than the fundamental niacin deficiency. For this reason Csermely and Zardi (C13) examined 12 patients with this disease in an attempt to demonstrate a pyridoxine deficiency by determining xanthurenic acid after loading with L-tryptophan (100 mg/kg). The results obtained (C13) show that an abnormal excretion of xanthurenic acid occurred in 5 of 12 patients. Furthermore, the clinical picture of the disease does not differ in patients with normal or abnormal xanthurenic acid output. These data provide no definite information in regard to this disease, in which more than one metabolite would have to be measured.

Urinary excretions of nicotinic acid metabolites and 2-pyridone, as well as of 4-pyridoxic and xanthurenic acids were determined in 15 South African Bantu pellagrins before and after tryptophan administration (P13). Red blood cell riboflavine levels and serum glutamic-oxalacetic transaminase levels were also measured. The authors discussed the apparent inability of the pellagra patients to convert tryptophan to nicotinic acid as indicated by their low excretion of nicotinic acid metabolites before and after tryptophan load. The possibility that the subjects were also suffering from a riboflavine deficiency was also discussed.

2.3.10. *Cataract*

Ogino and Ichihara (O2) reported in 1957 the isolation in pure form of 5-hydroxyanthranilic acid and anthranilic acid from urine of patients with senile cataract. Bromine oxidation of 5-hydroxyanthranilic yielded quinoniminecarboxylic acid, which showed cataractogenic activity when injected in scorbutic guinea pigs.

Having examined by two-dimensional paper chromatography the urine

of 45 subjects with incipient or mature senile cataract, we (Benassi, unpublished data) were unable to confirm the results of the Japanese authors since no 5-hydroxyanthranilic acid was excreted by these patients.

Also Tojo and Uenoyama (T2) by means of paper chromatography detected neither 5-hydroxyanthranilic acid nor its conjugate forms in urine of senile cataract patients. They found, however, an ethanol-extractable substance giving a positive ninhydrin reaction and R_f value coinciding with that of tryptophan. The oral administration of a dose of anthranilic acid did not alter their findings.

No other investigation has been recorded for cataract.

2.3.11. *Wilson's Disease*

A number of inherited conditions are now known in which some degree of renal aciduria has been found. These include diseases as diverse as cystinuria, galactosemia, and Wilson's disease. In the last of these the aminoaciduria is probably a consequence of renal tubular damage secondary to excessive copper deposition (H2).

The disease is a rare inherited disorder characterized by a progressive degeneration of the lenticular nucleus in the brain and by cirrhosis of the liver. Barbeau *et al.* (B1) studied a case of Wilson's disease which presented normal ceruloplasmin and serum copper values but increased excretion of kynurenine, 3-hydroxykynurenine, and conjugated anthranilic acid in urine after an oral dose of 2 g L-tryptophan. This defect in tryptophan metabolism could be related to that of other amino acids and to the actual content of ceruloplasmin in Wilson's disease (B1). These findings corroborated Marver's (M2) investigations demonstrating a definite excretion of kynurenine and 3-hydroxykynurenine in abnormal proportions after a tryptophan load in a case of Wilson's disease.

In independent studies, Aprison *et al.* (A7) found among tryptophan metabolites that 3-hydroxyanthranilic acid was capable of inhibiting the oxidation of *N,N*-dimethyl-*p*-phenylendiamine by purified ceruloplasmin and serum oxidase.

2.3.12. *Hartnup Disease*

Metabolic studies by Milne *et al.* (M8) showed that in Hartnup disease the renal aminoaciduria is more constant than the excessive excretion of indican and indolic acids (indoleacetic acid, indolelactic acid, and indoleacetylglutamine). After ingestion of L-tryptophan in this disease there is usually delayed and incomplete absorption from the gut of the amino acid which is partly converted, by intestinal bacteria, to indole

and other products of bacterial activity. After absorption, less L-tryptophan than in normal subjects is metabolized to kynurenine. A greater amount is converted to indoleacetic acid and indoleacetylglutamine. Also excessive excretion of indoleacetic acids, after tryptophan ingestion, persists longer in subjects with Hartnup disease than in normal. After loading with DL-tryptophan, normal subjects excrete higher amounts of indolic acids than after ingestion of the L-amino acid, and a large part of the excess is 3-indolelactic acid.

The results obtained (M8) suggest that a defective transport of tryptophan is established across the cells of the jejunum and across the proximal renal tubules. The reduced conversion of tryptophan to kynurenine is probably due to a deficiency of tryptophan pyrrolase or to a lower transport of substrate to the enzyme. According to the same authors (M8), the cerebellar ataxia may be ascribable to intoxication by retained indolic acids.

The Hartnup disease described in 1956 (B2) under the title "hereditary pellagra-like skin rash with temporary cerebellar ataxia, constant renal aminoaciduria, and other bizarre biochemical features" evidently belongs to the diseases related to inborn errors of metabolism due to inherited differences. As was pointed out by Harris (H3), very often the detection of urinary amino acid metabolites has represented the starting point of the investigation of different genetic biochemical disorders. A recent study (A8) showed that the excess production of indole by colon bacteria in 15 cases of Hartnup disease was due entirely to an increased amount of tryptophan contained in the large bowel, and not to an abnormality of the bacteria themselves.

It is to be hoped that further investigations of tryptophan metabolism in this disease will give more knowledge of the problem.

2.3.13. *Hereditary Disturbance*

The observation of a chronic urticaria in two sisters who excreted abnormal amounts of xanthurenic acid after a 10-g DL-tryptophan load indicated to Knapp *et al.* (K6) that constitutional genetic factors may be involved in vitamin B_6-deficiency symptoms. This was borne out by a very high xanthurenic acid level during a similar test on another family.

The peculiarities of tryptophan metabolism in the 15 individual members of this family were demonstrated by oral ingestion of 10 g DL-tryptophan per test case and quantitative determination of the urinary content of kynurenine, 3-hydroxykynurenine, xanthurenic acid, nicotinic acid amide and its N^1-methyl derivative, and 4-pyridoxic acid. Of the 15 members of the family examined 6 showed, repeatedly, abnormal levels of

xanthurenic acid; its excretion was elevated along with that of kynurenine and 3-hydroxykynurenine. It is suggested (K6) that this is probably a situation involving a genetically conditioned partial block of tryptophan catabolism, at the step of kynurenine and 3-hydroxykynurenine.

In subsequent studies (K7), 9 members of 3 different families were loaded with 10 g DL-tryptophan which resulted in a 10–20-fold increase in the 24-hour urinary excretion of kynurenine, 3-hydroxykynurenine, and xanthurenic acid. It appears to be a genetically conditioned disturbance, with dominant inheritance, involving metabolic reactions dependent upon pyridoxine. In most subjects the urinary changes after tryptophan loading could be corrected by vitamin B_6 therapy. The following diseases were found in this order of frequency in these subjects and their families: bronchial asthma, chronic urticaria, anemia, diabetes, varices, and crural ulcers. Knapp's (K7) conclusion is that these disorders may be partially attributable to metabolic disturbances.

The problem was further investigated by the same author (K9) who, examining 9 families, found an inborn error in homozygous or heterozygous form of tryptophan metabolism, resulting in incomplete catabolism of tryptophan and mimicking a vitamin B_6 deficiency. Only minimal increase of urinary xanthurenic acid and kynurenine was found in heterozygous members after tryptophan load. Such a metabolic error may arise, according to Knapp (K10), from a defect in apokynureninase, resulting in an altered dissociation constant and requirement for higher levels of coenzyme to establish normal activity. There may also be diminished potency of enzymes required for phosphorylation of pyridoxine. The metabolic error may be a predisposing factor in the frequency of apparently unrelated illness in the affected families.

2.3.14. *"Induced" B_6 Deficiency*

The administration of the pyridoxine antagonist, deoxypyridoxine, to man (G9) and rats (P6) has been shown to produce a severe disturbance of tryptophan metabolism in these species as manifested by the excretion of large amounts of xanthurenic acid following the administration of a tryptophan load.

Peripheral neuropathy has been observed as a complication in tuberculosis therapy with isonicotinic acid hydrazide (isoniazid), especially when large doses have been employed (B14). This complication of isoniazid therapy has been largely eliminated by simultaneous administration of pyridoxine. These observations have prompted studies on the urinary excretion of xanthurenic acid as an index of the antipyridoxine activity of isoniazid. The excretion of an excess of vitamin B_6 as such

has been demonstrated in 12 patients receiving isoniazid. Xanthurenic acid excretion following a loading of 10 g DL-tryptophan was greatly increased, but in an irregular fashon, in the same tuberculous patients. Xanthurenuria was roughly proportional to isoniazid doses and fell to normal when pyridoxine was given (B14).

In a group of 10 tuberculous subjects, no modification of tryptophan metabolism was observed at the beginning of isoniazid therapy, whereas abnormal excretion of xanthurenic acid and kynurenine appeared after prolonged isoniazid treatment following a tryptophan load (R6).

The effect of isoniazid and deoxypyridoxine on the urinary excretion of several tryptophan metabolites by 6 tuberculous patients was studied by Price *et al.* (P9). The differences in pattern of the urinary metabolites produced by isoniazid and deoxypyridoxine were striking after loading with 2 g L-tryptophan. Xanthurenic and kynurenic acids were both excreted in abnormally large quantities during ingestion of deoxypyridoxine, while less than normal amounts of kynurenic acid and only moderately increased levels of xanthurenic acid were excreted under the influence of isoniazid. Since kynurenine and 3-hydroxykynurenine were produced in large amounts in the presence of each drug, it would appear that *in vivo* isoniazid was a better inhibitor of kynurenine transaminase than deoxypyridoxine. Pyridoxine administration resulted in a return to normal tryptophan metabolism even when the ingestion of the two drugs was continued, whereas supplementary thiamine, riboflavin, or niacin had little or no effect.

Williams and Wiegand (W12), using tryptophan-loaded dogs, confirmed Biehl and Vilter's (B14) findings in man that isoniazid increases urinary xanthurenuria. In addition they found that three other convulsant hydrazides (thiocarbohydrazide, thiosemicarbazide, and semicarbazide) produced xanthurenuria in dogs somewhat similar to that produced by isoniazid.

The effect of isoniazid was studied also in pregnancy (K4), both normal and with complications, indicating a deficiency of vitamin B_6 in these subjects before and after loading with 10 g DL-tryptophan.

According to Zini (Z1), prolonged oral treatment with isonicotinic acid hydrazide interfered with tryptophan metabolism, probably by inhibiting kynureninase activity, which catalyzes the synthesis of anthranilic acid from kynurenine.

3. Conclusion

It appears from this survey that more than 12,000 subjects have been studied by investigating the excretion of tryptophan metabolites, partic-

ularly of the compound more typically related to vitamin B_6, xanthurenic acid, which is also the most easily measurable.

Investigations so extensive will certainly be utilized in the near future when the improvement of biochemical and chemical knowledge provides a clearer vision.

Enzyme studies are still necessary, but one cannot exclude the role that new factors may play here. In this connection we (M21) have described the presence in human urine of substances interfering with pyridoxal-5-phosphate-dependent enzymes.

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THE CLINICAL BIOCHEMISTRY OF THE MUSCULAR DYSTROPHIES

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I. Introduction

Muscular tissue comprises some 40% of the total body weight in man, and skeletal muscle, its major component by far, consists of about 75% water, 20% protein and 5% carbohydrate, organic phosphates, inorganic salts, and other solids.

The commonest affliction of skeletal muscle is weakness and wasting due to a variety of causes, many of which may be extrinsic to the muscle itself. Throughout history these have been known simply as the palsies. The rapid advance of neurological knowledge, however, has led to more precise understanding of the commoner extrinsic causes.

Skeletal muscle depends for its vitality on innervation by long motor fibers integral with and proceeding from the anterior horn cells of the spinal cord, or lower motor neurons, which are in turn controlled by the pyramidal fibers descending in the cord from the upper motor neurons of the precentral gyrus of the cerebral cortex. Damage to the latter, as in a stroke, removes cortical control without destroying the lower motor neuron, so that the muscles affected are thereafter subject only to an essentially disuse atrophy. Damage to the lower motor neuron, however, causes rapid and profound wasting of the muscle fibers supplied by it, and is one of the commonest causes of weakness. These latter causes can be classified as myelopathies, or damage within the spinal cord by such processes as poliomyelitis, motor neuron disease, or tumor growth; as radiculopathies affecting the motor root in the spinal canal, like acute Guillain-Barré polyneuritis or mechanical compression by tumor or anatomical distortion of the spine; as a wide variety of peripheral neuropathies that may additionally involve sensory fibers; and as the myoneural junction disorder of myasthenia gravis.

While these disorders were being defined, about the middle of the nineteenth century it became recognized that progressive weakness and wasting of the skeletal musculature could occur in the absence of neurological disease, and with a distribution unrelated to motor innervation, thus implying intrinsic disease of the muscles. The original descriptions, by Duchenne in 1868 (D20) and Gowers in 1879 (G8), of progressive weakness in childhood at first accompanied by apparent muscular enlargement (pseudohypertrophy) were followed by observations, by Ley-

den in 1876 (L6) and Möbius in 1879 (M12), on familial atrophy of the muscles of the pelvic girdle and then, by Erb (E3, E4), on juvenile scapulo-humeral weakness and on the absence of neurological disease in what he termed "progressive muscular dystrophy." About the same time, Landouzy and Dejerine in 1884 (L2) described the facio-scapulo-humeral form of the disease, Thomsen in 1876 (T7) his own familial affliction of myotonia congenita, and both Steinert (S31) and Batten and Gibb (B5) in 1909 simultaneously described dystrophia myotonica.

It became plain that these were true intrinsic diseases of skeletal muscle without neurological disturbance, and many clinical surveys have appeared, with repeated attempts to achieve a satisfactory classification without which no systematic investigation could succeed. That of Walton and Nattrass (W6), recently revised and fortified (B17a, C8a, M17a, P7a, W4, W4a), has achieved some recognition, and has been adopted throughout this text.

2. Clinical Definitions

Myopathy is the term used broadly for affections of the skeletal musculature, in which the muscular symptoms in no way arise from disordered function of the central or peripheral nervous system. Within this definition are included, therefore, such widely differing conditions as the polymyositis syndrome, endocrine myopathies associated with thyroid gland disorders, and the muscular dystrophies.

Muscular dystrophy itself, however, is strictly defined as a genetically determined degenerative primary myopathy (W4). The condition is progressive, painless, almost always widespread, and frequently disastrous to the sufferer. The three main clinical forms are now described, together with the myotonic syndrome, but omitting those rare varieties mentioned elsewhere (W4).

2.1. Duchenne Type

Genetic: *Transmission*—by female carriers as a sex-linked recessive condition, with a mutation rate so high that 1 in 3 cases is a mutant (M17, R12); occasionally as a more benign autosomal recessive character (B15, D19, J1, K8b).

Manifestation—almost always complete, rarely and debatably of such low apparent expressivity as to be symptomless and detectable only by serum enzymology (Section 8.3.6); usually in males, but occasionally in either sex as the milder autosomal recessive form or as an infrequent manifestation in heterozygous females (Section 8.3.6).

Clinical: *Onset*—possibly at birth (A1, A2, P6, P7a, P7b, P8, R12); usually clinically apparent in the first 3 years of life; rarely as late as the second or even third decades with correspondingly slower evolution (W4a).

Evolution—rapid and relentlessly progressive symmetrical weakness of thigh and pelvic girdle muscles (tendency to fall, difficulty in rising, rolling gait, lumbar lordosis) and soon of muscles of shoulder girdle, trunk, and upper limbs; 80% of cases show initial pseudohypertrophy of muscles, notably the calves, due possibly to fatty replacement (P7b).

Prognosis—inability to walk 10 years after onset, with progressive weakness, atrophy, and contracture of muscles leading to skeletal distortion, complete helplessness, and death in the second decade due to inanition or infection.

2.2. Limb-Girdle Type

Genetic: *Transmission*—59% by autosomal recessive inheritance, thus often appearing in sibs without previous family history; remainder sporadic (C8a, M17).

Manifestation—apparently complete penetrance but variable expressivity; both sexes affected equally.

Clinical: *Onset*—usually late in the first or in the second or third decades.

Evolution—variable severity and rate of progress, appearing first in either shoulder or pelvic girdle muscles, and spreading to the other limb girdle after a variable period; pseudohypertrophy uncommon; tendency to spare for many years calves and forearms compared with proximal muscle wasting.

Prognosis—contractures and skeletal deformities occur late in the disease, but severe disability is usual 20 years after onset, with death before the normal age.

2.3. Facio-scapulo-humeral Type

Genetic: *Transmission*—by autosomal dominant inheritance.

Manifestation—apparently complete penetrance with very variable expressivity; both sexes affected equally.

Clinical: *Onset*—from childhood to late adult life, but commonly in adolescence or early adult life.

Evolution—very variable severity, usually slow with long periods of arrest, but may be very mild or even abortive; appears first in muscles of face (inability to close eyes

fully or to whistle, characteristic loose pout), upper arms, and shoulder girdle (difficulty in raising arms), with late spread to pelvic girdle muscles; wasting usual, pseudohypertrophy very rare.

Prognosis—contractures and skeletal deformity rare; condition compatible with active survival to normal age.

2.4. The Myotonic Syndrome

The syndrome is characterized by myotonia, i.e., failure of immediate relaxation of muscle after voluntary contraction, usually made worse by cold and relieved by repeated movement.

2.4.1. *Dystrophia Myotonica*

Genetic: *Transmission*—by autosomal dominant inheritance as the commonest form of the myotonic syndrome.

Manifestation—complete penetrance but very variable expressivity; both sexes equally affected. Anticipation common, with minimal signs in one generation often followed by florid declaration in the next.

Clinical: *Onset*—from childhood to old age, but usually in third or fourth decades.

Evolution—exceedingly variable severity but constant progress; complete syndrome includes myotonia of tongue (dysarthria), hand, and forearm muscles, symmetrical wasting of facial and masticatory muscles (ptosis, narrow face), sternomastoids, and muscles of hands, forearms, and lower legs (foot-drop), with later spread to proximal muscles of limbs and trunk; pseudohypertrophy rare. Cataracts, frontal baldness, gonadal atrophy, and mental defect occur additionally.

Prognosis—severe disability in 20 years, with death in middle life.

2.4.2. *Myotonia Congenita*

The other chief form of the myotonic syndrome, myotonia congenita, is transmited equally to both sexes by an autosomal dominant gene solely as lifelong myotonia, with generalized muscular hypertrophy but occasional minimal dystrophic signs late in life, and is benign, only mildly disabling, and compatible with long life.

Because of these dystrophic possibilities, the occasional transition of myotonia congenita to dystrophia myotonica (W6), and the extreme clinical variability of the latter, Maas and Paterson (M1) consider both conditions variants of the same disease, though Bell (B11) re-

garded them as separate entities, while Penrose (P10) suggested that the variable manifestations of dystrophia myotonica might arise by modifications exerted by an allele on a dominant gene.

Progressive muscular dystrophy is an affliction of all mankind. In the United States alone there are said to be some 200,000 cases (probably an overestimate), of whom almost two thirds are children feebly awaiting gross distortion and early death by the rapid Duchenne type.

3. Structure of Muscle

3.1. Normal Muscle

Skeletal muscle is surrounded by a connective tissue sheath (epimysium), from which subdividing septa (perimysium), carrying blood vessels and nerves, pass inwards to surround each bundle of muscle fibers (fasciculus), and from which in turn pass very delicate extensions (endomysium) to invest each muscle fiber and convey the capillaries and finest nerve branches. In coarse muscles, required to provide more power than precision (e.g., glutei), both fasciculi and fibers tend to be thicker, and longer in long muscles (e.g., sartorius) acting through freely moving joints. Microscopic cross section of normal skeletal muscle shows that most muscle fibers are similar in area, and are so neatly packed together in each fasciculus that they appear polyhedral.

Each muscle fiber, or cell, is enveloped by an elastic membrane (sarcolemma), immediately beneath which lie large numbers of ovoid, longitudinally oriented nuclei, leaving the interior of the fiber occupied by the sarcoplasm and myofibrils. In embryonic muscle, the nuclei occupy a central position.

The motor unit consists of the motor neuron in the anterior horn of the spinal cord and the muscle fibers supplied by its branched axon closely applied at the motor end-plates. In this way a single motor neuron may supply a very large number of fibers, which tend to be grouped together but may be dispersed between different fasciculi.

Sustained contraction of muscle fibers produces repetitive electrical potentials in them that increase in rapidity with strength of contraction and in number as more motor units take part. These potentials may be conducted from a coaxial needle electrode in the muscle, amplified, and their amplitude and frequency analyzed and recorded on a cathode-ray oscilloscope as the very typical full interference pattern of normal voluntary contraction. Characteristic departures from this normal electromyogram are of very great importance in the diagnosis of muscle disease (B21a).

3.2. Diseased Muscle

The microscopic appearances of diseased muscle may sometimes be the only means by which a diagnosis is possible.

In established neurogenic weakness and wasting due to lower motor neuron damage the characteristic appearances are those of groups of atrophic fibers, lately supplied by the damaged neuron, lying beside healthy fibers with intact innervation. This is in full accord with the structure of the motor unit, described above. In peripheral nerve disease, clinical evidence of sensory involvement, such as paraesthesias, may be present as well.

In polymyositis, which seems similar in origin to the collagen diseases, and which may occur with persistent muscle pain and sometimes with skin lesions (dermatomyositis), the appearances are typically patches of segmental necrosis of muscle fibers in which the parts of the fiber on either side of the necrotic area remain normal, together with interstitial infiltration of inflammatory cells to greater or less degree, and sometimes attempts at regeneration by the damaged fibers.

In all types of progressive muscular dystrophy, however, in contrast with the typically discrete lesions of polymyositis, every muscle fiber is affected to give extreme variability in diameter, with fibers of all sizes from the grossly hypertrophic to those showing every degree of atrophy, scattered irregularly throughout the whole field (P3, P6, P7b). Early in the disease the muscle-fiber cross section loses its polyhedral appearance and becomes rounded instead. The subsarcolemmal nuclei tend to migrate toward the center of the fiber where they may lie in chains, especially in dystrophia myotonica. In developing cases, inflammatory cells and abortive fiber regeneration are not infrequent (W1, P6, P7b). As the disease progresses, however, interstitial fibrous tissue with some fat surrounds the shrinking fibers and replaces them. This fibro-fatty overgrowth is extremely slight in neurogenic atrophy and in polymyositis, but is characteristic of all forms of muscular dystrophy. In the Duchenne type the fatty change may be so voluminous as actually to replace the entire muscle without much loss of bulk, so that eventually it may be difficult to find a single muscle fiber in a given microscopic field (B15a). The biochemical implications of this unusual characteristic are mentioned later (Section 6.6).

In these conditions the electromyogram shows typical appearances (B21a, R2a). In neuropathy, the individual denervated muscle fibers are free to discharge spontaneously, and thus at rest partially denervated muscle shows repeated single "spike" (fibrillation) potentials, or some-

times a spontaneous chain of "saw-toothed" potentials, while on voluntary contraction the remaining healthy fibers give a sparser interference pattern, though still showing normal motor potentials. In the myotonias, mere insertion of the needle electrode often starts a chain of very characteristic bizarre repetitive potentials that persists for a few seconds. In muscular dystrophy and in polymyositis, however, since degeneration is universal and unrelated to motor units, the interference pattern is still full but complex and broken up due to polyphasic action potentials of very short duration and low amplitude, and these appearances can be diagnostic.

4. Biochemistry of Muscular Contraction

Each muscle fiber appears cross-striated due to the presence in its sarcoplasm of alternate longitudinal bundles of actin filaments (50-Å diameter; isotropic) interdigitating with layers of myosin rods (110-Å diameter; anisotropic), in such a way that each myosin rod is surrounded by six actin filaments of which each is thus shared with three myosin rods. From the myosin rods regular cross-linkages extend to the actin filaments (H10) and in the absence of adenosine triphosphate (ATP) these remain locked in rigor. In the continued presence of ATP the linkages relax between these proteins, and the muscle may be extended. Muscular contraction is initiated by acetylcholine liberated at the motor end-plate, propagating membrane depolarization as an action potential along the muscle fiber. ATP is then rapidly hydrolyzed to ADP, with loss of high energy phosphate, by the specific myosin and actomyosin ATPases (N1); the linkages at once reform in numbers proportional to the rate of ATP hydrolysis, and each actin bundle approaches its neighbor by sliding into and progressively cross-linking with the myosin layers. Muscle in man can thus shorten to 57% of its length when fully stretched (W2). Cessation of hydrolysis with maintenance of a supply of ATP once more brings about relaxation. That this can be sustained may be due to inhibition of ATPase by the soluble Marsh factor or other inhibitor (M13a, N1), and, since Ca^{++} and Mg^{++} are important in ATPase activity, some alteration in their state may be material. It must be noted that, although ATP is most effective, other nucleotide triphosphates, notably cytidine triphosphate (CTP), have a similar though lesser action *in vitro* and are present in small amounts in skeletal muscle, though more accurate modern analyses might now disclose greater amounts (N1).

Sarcoplasmic glycolysis and especially sarcosomal oxidative phosphorylation, not only of the products of glycolysis but also of free fatty

acids, provide the essential ATP (G3), of which some of the high energy phosphate is equilibrated with and stored in the sarcoplasm as creatine phosphate for rapid transfer by ADP and expression as actomyosin contraction (M13, M14). Equilibration and transfer are accomplished by the soluble enzyme creatine kinase.

In muscular dystrophy profound alterations of these normal processes occur, with consequent changes in the composition of the body fluids, many of which are now partially understood and are predictable. Their measurement gives very valuable information in support of diagnosis and in the further elucidation of the disease process.

5. Preliminary Observations

5.1. Aminoaciduria

Abnormal excretion of urinary amino acids in muscular dystrophy has been recorded on several occasions. A comparative study of 52 dystrophic patients and a corresponding number of normal individuals showed in the patients an increase in the number of urinary amino acids and certain related unidentified substances, and, moreover, a similar and as extensive abnormal excretion of amino acids by normal siblings and maternal parents of these patients (B14). The evidence of other investigators supports these findings in dystrophic patients (D9, K9), though a decrease in urinary amino acid excretion has been recorded (C4). Recently, however, it has been pointed out that, as in normal individuals, dystrophic aminoaciduria is liable to daily variations, and that urinalysis over many days is obligatory for accurate assessment. Further, though at some point in the course of the disease a partial or universal hyperaminoaciduria occurs, with the excretion of glycine and glutamic acid most often increased, in advanced cases with only a small residual muscle mass there may be hypoaminoaciduria (B13). While these results accord with the protein destruction of muscle wasting, the real cause of dystrophic hyperaminoaciduria is still far from clear.

5.2. Ribosuria

The urine of dystrophic patients was reported in 1949 by Minot *et al.* (M11) to contain quantities of organic ribose phosphate esters, which could be detected for diagnostic purposes by a modified qualitative Benedict's test (O3). Walton and Latner (W5), however, found this proposed test to have no diagnostic value. Determined and thorough biochemical investigations in large numbers of patients with all types of muscular dystrophy (P11, R10) have since shown that any difference

in pentosuria between normal and dystrophic subjects, if such exists, is so very slight that present techniques do not permit sufficiently accurate study for any but negative conclusions to be drawn.

5.3. Endocrine Studies

The only useful contribution of endocrine studies to diagnosis has been in the condition dystrophia myotonica, with its 80% incidence of eventual primary testicular atrophy in males. Many workers (C1, D1, G7, K15) have reported the urinary excretion of 17-ketosteroids in this condition to be subnormal in males, and even in females to be in the low normal range. Recent thorough investigations, however (B22, D18), have disclosed that, apart from an unexplained frequently low basal metabolic rate and the consequences of eventual testicular atrophy, there seems to be no reason to suppose that adrenal cortical, thyroidal, ovarian, and pituitary functions are other than normal in dystrophia myotonica. A majority of males examined had been fertile, and most women had brought one or more normal full-term pregnancies to spontaneous delivery.

5.4. Hepatic Function

Some conflict in minor detail has arisen in the interpretation of liver function tests in muscular dystrophy. Using the standard series of liver function tests, Morell in 1959 (M16) reported a significant incidence of abnormal results, and signs of fatty infiltration at liver biopsy in 15 patients. Other workers (B9), using similar tests in a series of 67 patients, obtained substantially normal results, though some patients showed a longer Weltmann coagulation band than normal, while a few exhibited a diminished ability to excrete hippuric acid; again, 21 autopsies showed fatty infiltration of the liver. Hepatic pathology of this kind, arising perhaps because of the terminal years of increasing immobility and inanition, could well account for some abnormality of liver function. Moreover, in patients at this stage of the disease it has been noted that severe and intractable gastrointestinal symptoms become common, sometimes with disordered motility of the gut (P2), which by defective nutrition may impair hepatic function. Recently, however, in a series of 11 patients with Duchenne-type dystrophy (T9), of whom only three were confined to wheel chairs, urinary urobilin and bilirubin, serum bilirubin, thymol and zinc sulfate turbidities, and alkaline phosphatase were examined. Though all other tests were normal, 4 patients had a slight hyperbilirubinemia of 1.1–1.6 mg per 100 ml, three of whom had urobilinuria but only one of whom (on a single occasion) had also bilirubinuria and

serum giving a positive direct van den Bergh test, indicating some hepatic regurgitation. These findings, quite unrelated to the stage of disability, suggest a hemolytic origin, perhaps due to erythrocyte fragility in turn suggested by the sallow appearance of many patients with Duchenne-type dystrophy. An unusual finding, for which no obvious explanation presents itself, was the presence of a persistent marked halitosis, often with a lifelong dislike for sweets, in most of the 11 patients. Four patients had no halitosis and enjoyed sweets, but seven had halitosis and three of these disliked sugar in any form. Again, no relationship was apparent between this condition, the results of liver function tests, or stage of physical disability. After treatment with a parenteral nucleoside/nucleotide mixture (Section 8.3.4) the halitosis always disappeared, but the dislike for sweets was more invincible.

5.5. Creatine Metabolism

In man creatine is produced in the liver at a constant rate (S2) by the methylating action of methionine on glycocyamine derived from the transfer of an amidine group from arginine to glycine. Normal skeletal muscle contains some 400 mg % of creatine absorbed from the circulation (C15) and equilibrated with ATP as creatine phosphate by the action of creatine kinase; on giving up its high energy phosphate for contraction a proportion is irreversibly cyclized to creatinine, which, like ingested creatinine, is promptly and completely excreted as waste in the urine (B16) without tubular reabsorption. Renal tubular reabsorption of creatine, however, is such that, with its steady acceptance from the circulation by a normal muscle mass, the renal threshold is not exceeded and little creatine appears in the urine.

Dystrophic muscle may contain only a tenth as much creatine as normal muscle (R1), despite a sufficient content of ADP and ATP (R9), due to its very low content of creatine kinase on which muscle creatine depends for its acceptance, retention, and use as creatine phosphate, since the total creatine content of dystrophic muscle is known to be directly proportional to the activity of the creatine kinase within it (R8). Excess creatine from the liver thus exceeds its renal threshold and is excreted in large amounts, while the low creatinine excretion indicates how slight is the remaining muscle function.

These processes have been illustrated by the administration of glycine-N^{15} to a dystrophic patient (B12, R7), when the isotope content of the daily urinary creatinine excretion quickly reached a low, constant level, while that of creatine at the same time rose to a value some 10 times higher and took 11 days to sink again to the same level. The inter-

pretation of these findings was that, even if the surviving dystrophic muscle were removing creatine from the blood stream at the normal rate per unit weight of muscle—which now seems extremely unlikely because of its reduced content of creatine kinase—the residual muscle mass was now too small to accept the normal liver production of the isotopic creatine, so that the excess was simply excreted.

Creatinuria, diminished tolerance of ingested creatine, and hypocreatininuria are thus established features of muscular dystrophy. Healthy, well-developed men on normal diets excrete practically no creatine but up to 2 g daily of creatinine, while women, with their smaller muscle bulk, may excrete small amounts of creatine, especially in pregnancy, but only about 1.5 g of creatinine daily. Small children, probably for similar reasons, excrete more creatine than creatinine until about the age of 3 years (C10), so that in a single urine specimen the ratio of creatine to creatinine is unity or greater; thereafter, as the muscle bulk increases with age, the respective values steadily approach those of adults until by 6 years of age the ratio is about 0.4, becoming exceedingly small by the age of 16. In Duchenne-type muscular dystrophy, however, this ratio increases with age instead of diminishing (H1, M3) for the reasons just described, and has thus been used for many years as a useful confirmation of clinical diagnosis. Similarly, while orally administered creatine is rapidly absorbed and retained by normal muscle far in excess of its immediate needs (M10), that of the dystrophic patient cannot do so, and this inability is the basis of the creatine tolerance test described in laboratory manuals.

While these measurable disorders of creatine metabolism have been in the past of great service in diagnosis, the tests dependent on them have the serious disadvantage of being nonspecific since similar findings occur in any condition causing substantial loss of muscle bulk (C8, M10). Thus no distinction can be made by them between the weakness and wasting of muscular dystrophy and those of neurogenic origin, as in poliomyelitis or progressive muscular atrophy due to motor neuron disease. This lack of specificity is irremediable, but the applications of serum enzymology to this problem are exceedingly precise and rewarding, and in them are found most of the recent rapid advances in the clinical chemistry of muscular dystrophy.

6. Muscle Enzymes

6.1. Introduction

In 1943 Warburg and Christian (W7) reported that five of the glycolytic enzymes are present in the blood serum of rats, and of these the activities of aldolase and triose isomerase are usually increased in the

serum of rats carrying large tumors. A few years later Sibley and Lehninger (S23) announced a colorimetric method for the determination of aldolase activity, and in the same year (S24) confirmed these findings and in the course of clinical investigations discovered elevated serum aldolase activities in two patients with muscular dystrophy. Five years later Sibley and Fleisher (S22) announced the results of an extensive investigation into the clinical significance of serum aldolase activity in 880 patients with a wide variety of diseases. These included five with primary myopathies, two of whom showed raised serum aldolase activity consistent with the authors' comment on the particularly high aldolase content of skeletal muscle; in no case of neurogenic muscular atrophy, however, was elevation of serum aldolase activity observed. Meanwhile Schapira and his co-workers, on the basis of Sibley's earlier report, had begun a systematic study of several serum enzymes in progressive muscular dystrophy, including both aldolase (S16) and transaminases (D11), and found considerable elevations in the serum of patients with myopathies, but none in those with neurogenic muscular weakness and wasting. Confirmatory reports from other workers followed immediately, and an important diagnostic advance seemed apparent.

6.2. Origin of Serum Enzymes in Myopathies

Since the work of Sibley and Fleisher (S22) made it plain that elevation of serum aldolase activity occurred quite characteristically in other diseases besides myopathy, such as in hemolytic anemia and in acute hepatitis, it would be most useful to know that in muscular dystrophy the increased serum aldolase was indeed derived from the diseased muscle. Direct demonstration of this origin has been provided (D14) by showing that in 5 of 10 patients with muscular dystrophy the femoral venous return had a higher serum aldolase activity than the femoral arterial supply to the diseased muscles of the lower limb. Further strong support is given by the discovery that serum contains two aldolases (S8) with different substrate requirements (H5) whereby colorimetric methods have been devised for the separate assay of each (S5). These are 1,6-diphosphofructoaldolase ("muscle" aldolase) and 1-phosphofructoaldolase ("liver" aldolase). The ratio in mammalian tissues of muscle to liver aldolase activity is 40 in skeletal and cardiac muscle, 12–25 in spleen, lung, and red cells, and only unity in liver and kidney (S6, S7). The serum activities of both are equally elevated in hepatitis, but in muscular dystrophy and in muscle crush injury only that of muscle aldolase is raised (S4, S6); indeed, the ratio of serum activity of muscle to liver aldolase has been reported as about unity in healthy individuals and in patients with virus hepatitis, but as about 26 in a series of 14

patients with muscular dystrophy (C12). Again, the enzyme creatine kinase, which is very abundant in skeletal muscle, much less so in brain and cardiac muscle, and practically absent elsewhere (C13), may display in Duchenne-type muscular dystrophy a serum activity some 50 times the upper limit of normal (S14), while in virus hepatitis its serum activity remains normal though that of aldolase may be grossly elevated (C13, D17, H7, O1, S33).

Taken together, these findings are formidable evidence in favor of a muscular origin of the elevated serum enzyme activities in muscular dystrophy. In the various neurogenic muscular atrophies, however, normal activities of serum aldolase (A3, A5, E5, G2, H7, R14, S1, S14, S16, S17, S22, T8, W12) and serum glutamic-oxalacetic transaminase (SGOT) (A3, D11, K1, M18, P4, S1, S14, S26, T8, W12) are found even when the rate of muscular wasting, as in early convalescent poliomyelitis, is proceeding at a rate much greater than is seen in any form of muscular dystrophy. Likewise, normal serum enzyme values occur in the slow muscle atrophy of polyarthritis (G1), essentially a disuse phenomenon. During the acute phase of neurogenic muscular atrophy of sudden onset there may be an initial trivial rise in the serum activities of these enzymes, but this is always transient and rapidly reverts to normal. Experimental confirmation of these findings has been forthcoming for aldolase by surgical nerve section in dogs, when even after dividing the cauda equina only modest and transient serum elevations were observed (A6).

Much information about the skeletal musculature is thus conveyed in the serum by variations in the activities of serum enzymes, and for the first time rapid and convenient biochemical tests become available to distinguish myopathic from neurogenic weakness and wasting.

6.3. Serum Enzyme Clearance

The maintenance within a recognizable range of the serum enzyme activities implies some similarity between their rate of discharge from the tissues into the blood stream and the rapidity of their clearance from it. This clearance can be exceedingly swift, though confronted by skeletal muscle of which 300 mg contains as much aldolase as the entire adult circulation (S22). After the intravenous injection of crystalline aldolase in rats the activity of the serum aldolase rises 7-fold in the first 15 minutes, but 12 hours later has fallen to only 1.5 times the normal value (S24). Further, the intravenous injection in rabbits of pure crystalline aldolase labeled with I^{131} and the measurement of the rate of disappearance of radioactivity in successive serum specimens indicate

that the "half life" of the serum aldolase is about 3 hours, i.e., the time taken to register a 50% decrease of the original radioactivity, and that therefore the aldolase is not simply inactivated in the serum but is actually removed from it (S9).

Similarly, intravenous injection in dogs of purified glutamic-oxalacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT) concentrates causes a rapid and considerable rise in the serum activities of both enzymes; 24 hours later 97% of the administered GOT has disappeared from the serum and all of it within 72 hours, while in 24 hours only 58% of the administered GPT has been eliminated and after 3 days 18% of it still remains in the serum (F2). Comparable findings on SGOT have been reported in a series of dogs after experimental subjection to extensive severe muscle compression (B4). The disparity in serum clearance rates between the two transaminases may perhaps be related to the molecular weight of GOT being much less than that of GPT (G10, J2), and may help to explain the later elevation and return to normal of SGPT compared with SGOT activity in cases of myocardial infarction (R13).

Again, the enzyme lactic dehydrogenase frequently shows elevated serum activity in muscular dystrophy, though it is cleared from serum with remarkable speed; in dogs the intravenous injection of purified lactic dehydrogenase immediately raises its serum activity to 10 times the normal value, but after only 1 hour the greater part of it has already been removed from the serum (W18).

In muscular dystrophy, therefore, the maintenance of considerable serum activities of soluble enzymes almost certainly of sarcoplasmic origin, and known to be subject to very rapid and continuous clearance from serum, strongly argues their ceaseless copious discharge from dystrophic muscle. The likelihood at once arises that the dystrophic muscle cell membrane is unduly permeable to these enzymes, that their activities are diminished within the cell, and that because of such lavish discharge even reduced intracellular activities could be preserved only by unusually rapid renewal. These probabilities receive substantial support from the results obtained by other workers.

6.4. Muscular Dystrophy in Mice

In 1955 the appearance of an inherited mammalian muscular dystrophy was reported in mice of the Bar Harbor 129 strain (M9), in which breeding experiments after ovarian transplantation confirmed its transmission by a single mutant autosomal recessive gene (S32). This disease is analogous to, though not necessarily identical with, human muscular

dystrophy not only in its appearances, rapid course, and mode of inheritance, but also in its muscle histology (P3a, R11, W9), electromyographic similarity to human dystrophia myotonica (M4), and, in many respects, its muscle biochemistry (Section 6.6) and serum enzymology (Section 6.5). The ready availability of these animals has allowed thorough investigation of the condition (H0).

6.5. Muscle Cell Membrane Permeability

In 1953 Zierler *et al.* (Z8) observed that, on incubation of isolated diaphragm from normal rats, a glycolytic enzyme system complete from phosphoglucomutase to lactic dehydrogenase migrated out of the muscle cells and into the medium. In particular, he found (Z1, Z2) that the efflux of aldolase in such a system is increased by anoxia in an atmosphere of nitrogen, by lack of glucose or by high potassium ion concentration in the medium, and even by passive transfer to fresh medium. Using the fusiform rat peroneus longus obtained intact by tendon division, this rate of aldolase diffusion could be related to muscle surface area (Z3), and was increased by metabolic inhibitors such as iodoacetate or cyanide thus indicating a cell membrane lability linked to general cell metabolism (Z5); above all, on brief active stimulation in the intact animal before excision and transfer a very great loss of aldolase from the muscle into the circulation was found to have occurred (Z6). It is of interest that, after Zierler had speculated that alterations in aldolase particle size might account for these effects (Z1), it was found by starch gel electrophoresis that rat muscle contained two "aggregations" of aldolase, one protein-bound and the other free, and that the latter was increased in exercised muscle (C3). Dystrophic mice were now found to show hyperaldolasemia (C9, S10), and muscle from them showed a much greater aldolase efflux than normal controls, together with a reduced aldolase content (Z4, Z7) and a higher Na^+ and lower K^+ content (B3, Z7), all of which demonstrated unusual permeability of the dystrophic muscle cell membrane. Meanwhile it was already known that in human muscular dystrophy there was likewise hyperaldolasemia (S16) most probably of muscular origin (Section 6.2), an absolute decrease in muscle aldolase content (D15), and a higher Na^+ and lower K^+ content than in normal muscle (W14).

6.6. Enzyme Content of Dystrophic Muscle

Because of the rapid increase of connective tissue in muscular dystrophy, ultimately far exceeding in quantity any muscle tissue left, determinations of enzyme content of dystrophic muscle are invariably made

against the noncollagen nitrogen of the specimen, which provides a fair estimate of the actual muscle tissue remaining in it. On that basis it has been found that in man the activities of aldolase, phosphorylase, phosphoglucomutase, and of total glycolysis are much lower than normal in dystrophic muscle (A4, H6, R8, S17, V2), and in a recent tabular summary (D12) it is shown how the content of soluble sarcoplasmic glycolytic enzymes is greatly reduced while that of the sarcosomal oxidative enzymes and of GOT and GPT remains normal, or is even slightly increased. Similarly, a substantial decrease in the creatine kinase content of human dystrophic muscle has been reported (H6, O2, R8, V2). It must be noted, however, that the activity of hexokinase remains normal, and that of lactic dehydrogenase may even be increased, especially in the early stages (R8); but though the former statement has been confirmed, the latter is now disputed (H6). Similar diminutions of total glycolysis and of creatine kinase content have been reported in muscle undergoing neurogenic atrophy (V2), but this reduction may be due to feeble metabolism in disuse and not to constant loss by efflux, since here the serum enzymes are not elevated.

The copious muscle enzyme efflux in Duchenne-type muscular dystrophy, giving gross serum elevations despite the rapid serum clearance, may well deplete some muscle enzymes; that so much aldolase still remains may indicate a replacement so rapid that, if applied to the transaminases and to lactic dehydrogenase, the muscle content may be maintained or even increased, since their serum elevations, though considerable, are proportionately much less than that of aldolase.

Less regular results have been obtained for the enzyme content of dystrophic mouse muscle, where reductions in aldolase and in lactic dehydrogenase (W12), phosphoglucomutase (H2), and creatine kinase (N2) have been reported, though other workers have found a normal aldolase content (W8) and normal glycolytic and oxidative cycles (M7). It seems apparent, however, that in dystrophic mouse muscle the lysosomal enzyme activities may be considerably increased (T3), with a cathepsin activity twice normal (W8), and that in human muscular dystrophy a similar increase in cathepsin activity is usual (P9). The close association of these enzymes with active catabolism may be highly significant. It is of interest that a considerable increase in the muscle content of glucose-6-phosphate dehydrogenase has been reported, both in dystrophic mice (M2) and in human muscular dystrophy (H6), implying an excessive production of reduced nicotinamide-adenine dinucleotide phosphate ($NADPH_2$, formerly termed TPNH) and thus of lipids, which may be

related to the extraordinarily complete replacement of skeletal muscle by fat so often seen in Duchenne-type muscular dystrophy.

6.7. Protein and Nucleotide Turnover in Dystrophic Muscle

This has been studied in dystrophic mice by radioactive isotope techniques. The rapidity of incorporation of glycine-C^{14} into the nucleic acids and proteins of the skeletal muscle in dystrophic mice has been found to be higher than in normal mice, and to increase with advancing age, whereas in liver, kidney, and spleen the incorporation rates are similar (C11). By using similar techniques with glycine-C^{14} in normal rats it has been possible to assign a life span of 30 days to the myofibril before its myosin is replaced (D13). By sacrificing normal rats at different intervals after injection of glycine-C^{14} and immediately isolating the muscle aldolase, its exponential decrease of radioactivity has been taken to indicate an intracellular turnover of this soluble enzyme (S19). But after intraperitoneal injection of glycine-C^{14} into both normal and dystrophic mice, and subsequent sacrifice and measurement of the decrease in radioactivity of various tissue fractions, it has been found that while in normal mice there is a turnover of water-soluble muscle proteins as in the rat, with a myosin lifetime of 20 days, in dystrophic mice the turnover of the same proteins is greatly accelerated, together with that of myosin (K13). Further, the results obtained after intravenous injection of DL-leucine-C^{14} into normal and dystrophic mice show that, while no differences exist in liver, the turnover in dystrophic muscle of both myofibrillar and sarcoplasmic proteins is accelerated to such an extent that catabolism exceeds synthesis, so that dystrophic muscle wasting may be due to an increased rate of synthesis being outstripped by still more rapid destruction (S27).

These findings are very strong indications, in the dystrophic mouse at least, of an unusually rapid renewal of soluble sarcoplasmic enzyme proteins; and the analogies with the human form of the disease are sufficiently close to argue great likelihood of similar processes at work in man.

Again, in the dystrophic mouse P^{32} is incorporated twice as fast as in the normal animal into the acid-soluble phosphate of muscle (K7); indeed, by this means it is found that the turnover rate of some acid-soluble nucleotides is greater in dystrophic than in normal mice (Z10). Recently it has been reported that the lives of dystrophic mice may even be prolonged by the administration of certain mixtures of purine and pyrimidine bases (Z9). In man it has been found that the nucleotide fraction

of dystrophic muscle is considerably diminished (R9), and parenteral administration of nucleosides and nucleotides has recently suggested some beneficial effect in human muscular dystrophy (Section 8.3.4).

6.8. Nondystrophic Factors Influencing Muscle Efflux and Serum Enzyme Activity

The enzyme content even of normal muscle is labile, and can be altered by exercise. In contrast to their sedentary controls, healthy male rats made to swim for only 30 minutes daily for 5–8 weeks show a significant increase in the aldolase content of heart and gastrocnemius (H3, H4), though in similar circumstances the lactic dehydrogenase content of the heart alone increases while that of skeletal muscle does not (G6). It is of interest that in man the aldolase content of the skeletal muscle is 50% greater in mountain-dwellers than in others, though the cardiac content remains the same (L4). In addition, the physiological conditions conducive to muscle enzyme efflux (Section 6.5) occur during physical activity since, apart from passive extension of associated muscles, exercise is known to produce relative local hypoglycemia (B1, B2) and hypoxia (K3). Tourniquet ischemia of skeletal muscle has thus been shown to cause a rapid rise of serum aldolase activity on its release (K8), and it is significant that on full voluntary contraction the human quadriceps can develop intramuscular pressures as much as 6 times that of the diastolic blood pressure (S34). Moreover, these factors are likely to have much greater effect in human muscular dystrophy, especially in early cases of the Duchenne type still capable of powerful contraction, since in this condition arteriography (D4) and sphygmography (C16) have disclosed circulatory abnormalities and loss of vascular tone.

Even in normal individuals, however, only moderately strenuous exercise usually causes modest but distinct elevations of the serum activities of aldolase (B6, C5, F3, R3), both transaminases (B6, C5, F3, P12, S21), lactic (B6, C5, F3, T4) and malic (F3) dehydrogenases, and creatine kinase (A1, B6, F2a, R4) which rapidly return to normal after rest, though GPT seems least affected. More sustained military or athletic training programs cause a similar but more persistent rise in these values, but not in that of SGPT (C13, R5). In particular it has been found that serum aldolase activity in untrained subjects rises immediately after 5–10 minutes of exercise, falls to normal shortly afterwards, then rises again briefly from these normal values to further maxima at 30 minutes and again at 90 minutes after termination of the exercise (R3). It is of in-

terest that regular, though far wider and much slower, excursions of serum enzyme elevations are typical during even ordinary ambulation in Duchenne-type muscular dystrophy (Section 8.3.4).

Causes other than physical activity, however, may also elevate these serum enzyme values, sometimes in circumstances where useful information might otherwise be gained (P8). Placental impermeability allows the fetus its own serum values, which in newborn infants usually exceed those of the mother. Cord serum aldolase activity may reach nearly thrice the upper normal adult limit (F4, L3), rising still higher in physiological jaundice (M6) and grossly in icterus gravis (S22), and since these elevations correlate with that of the serum bilirubin it is probable they arise at such times from erythrocyte aldolase discharge on hemolysis. Much lower values by the third week of life (M6) approach adult values after 6 months (B8, C9). Similar, though even more marked, increases of neonatal serum creatine kinase activities have been reported (R4). Cord and neonatal serum activities of both transaminases show wide individual variations from normal values to twice the upper limit of the adult normal range, but no relation has been found between these values and that of serum bilirubin concentration; indeed, even in very severe icterus gravis SGOT may show only a temporary increase while SGPT remains unaffected (K11, K12). Why the cord serum enzyme values should show such elevations long before the appearance of frank physiological hemolysis is a matter for reflection. It is of interest that the neonatal adrenal cortex is proportionately very much larger than in adults (B18), that administration of cortisone or adrenocorticotropin (ACTH) causes hyperaldolasemia in rabbits (S3) while stress increases SGOT in monkeys (C14), and that even a moderate 3-day course of prednisone in 15 normal children has caused a rapid increase in serum aldolase activity, diminishing immediately on prednisone withdrawal though not regaining normal values until 4 days later (C17). Several accounts have also appeared of potent steroids, given for other conditions, causing in patients undoubted proximal myopathic syndromes, sometimes severe, which disappeared on withdrawal of steroid therapy (F1, G4, G5, M5, T5, W15). Again, it is interesting to recall how in the dystrophic muscle fiber the normally subsarcolemmal nuclei tend to assume a central position (Section 3.2), a disposition proper to embryonic muscle (Section 3.1), and to note that starch gel electrophoresis of lactic dehydrogenase from dystrophic muscle biopsy in man discloses an isoenzyme pattern closely resembling that of normal fetal muscle (D16, E2a).

7. Methods of Serum Enzyme Assay

7.1. Aldolase

1,6-Diphosphofructoaldolase is a soluble glycolytic enzyme especially abundant in skeletal muscle, occurring also in the myocardium and to a lesser extent in liver and erythrocytes, so that hemolysis of blood specimens elevates the serum aldolase activity and must therefore be avoided. The molecular weight of muscle aldolase is 147,000–180,000 (D10). Its function is specifically the reversible splitting of D-fructose-1,6-diphosphate (FDP) into equimolecular amounts of the trioses D-glyceraldehyde-3-phosphate (G-3-P) and dihydroxyacetone phosphate (DAP).

Colorimetric assay of serum aldolase activity (B21, E5, F1a, S23) is by incubation at 37°C of serum with buffered FDP substrate in the presence of hydrazine to trap the G-3-P and DAP so formed as their hydrazones, thus preventing back-reaction or the conversion of G-3-P to DAP by serum triosephosphate isomerase. The reaction is terminated by protein precipitation with 10% trichloroacetic acid, the phosphate moiety removed by dilute alkali at room temperature from the triose esters during molecular rearrangement, and, after incubation at 37°C with 2,4-dinitrophenylhydrazine and addition of cold dilute alkali, the optical density of the methylglyoxal osazone read against water at 540 mμ, from which the reading of the corresponding blank, treated in exactly the same way save for addition of substrate only after protein precipitation, is subtracted. Initially, simultaneous determinations of alkali-labile phosphate formed are made (E5) to relate optical density to triose phosphate production and thus to serum aldolase activity. Using an otherwise similar procedure, Friedman and Lapan (F4) avoid this tedious step by using pure dihydroxyacetone as the standard; moreover, aldolase assay may be performed by their method on as little as 0.1 ml of serum, whereas the other colorimetric methods require 1 ml. Since the chromogenic composition of the osazone fades rapidly with time (B7), a smooth and uniform technique is advisable in these colorimetric methods. Where a number of assays are contemplated a modern high-speed instrument is useful, such as the Hilger Spectrochem which reads directly from a moving scale and takes five cells and flow-through attachments.

Spectrophotometric assay (B10, L7, S29) is accomplished by a similar incubation, without hydrazine, where excess added triosephosphate isomerase converts triose phosphate formed to DAP, in turn removed as glycerophosphate by reduced nicotinamide-adenine dinucleotide ($NADH_2$, formerly termed DPNH) and added glycerophosphate dehydrogenase.

The rate of decrease of optical density of the system at 340 mμ measures the rate of conversion of $NADH_2$ to NAD by the DAP produced, and thus the serum aldolase activity. After adding serum to all the reagents at 37°C in the reaction vessel, 5 minutes are allowed for serum lactic dehydrogenase to equilibrate traces of serum pyruvate with $NADH_2$ (K4). Thereafter it is just possible, by very rapid transference, to read optical densities in the instrument cell at zero time and again after a suitable interval, with incubation between these readings in a glass-stoppered tube in a water bath at 37°C. It is far more accurate and convenient to conduct the entire procedure from start to finish in the thermostatically controlled silica cell of a modern, fully automatic, multiple-cell recording spectrophotometer such as the Hilger-Gilford instrument (4 cells) or the split-beam Optica-UK CF4R (5 cells and scanning). Several assays may thus be carried out simultaneously, if need be against serum blanks in isotonic saline instead of water to avoid turbidity. The spectrophotometric method is superior in accuracy to the colorimetric, and only 0.2 ml of serum need be used.

For both methods, serum of very high aldolase activity may be suitably diluted with normal saline just prior to assay, a method proved valid by testing serial dilutions. Even high activities are retained in serum with little change for at least a fortnight at —17°C. Serum aldolase activity may be conventionally expressed by both methods as μl of FDP split per hour by 1 ml of serum at 37°C (B21), and in 50 healthy adults the normal range has been found (R13) to be 2.3–8.8 units per ml (mean 5.7 units). For conversion to International Units, 1 μl/hour/ml = 0.745 μmole/minute/liter (C2).

7.2. Transaminases

These are soluble enzymes operating the reversible exchange of amino groups between α-amino and α-keto acids, and the two considered here are glutamic-oxalacetic transaminase (GOT) which reversibly catalyzes the conversion of aspartic and α-oxoglutaric (α-ketoglutaric) acids to oxalacetic and glutamic acids, and glutamic-pyruvic transaminase (GPT) which similarly converts alanine and α-oxoglutaric acid to pyruvic and glutamic acids. Like aldolase, both are abundant in skeletal muscle which however, displays 20 times more GOT than GPT activity; in the myocardium both activities are proportionally increased by half, but in the liver, though that of GOT remains similar to the myocardial content, GPT activity is 10 times greater than in skeletal muscle (W17). The molecular weights of aldolase and GPT are similar and thrice that of GOT (D10, G10), but recent work assigns to GOT a molecular weight nearly

⅔ that of GPT (J2); and though GPT seems homogeneous, two GOT isoenzymes have been found (B17, W13).

In the widely used colorimetric assay of SGOT and SGPT (R2), serum is incubated at 37°C with phosphate-buffered L-aspartate/α-oxoglutarate or DL-alanine/α-oxoglutarate, respectively, the reaction terminated after a definite interval by addition of 2,4-dinitrophenylhydrazine in dilute HCl, and after a period at room temperature the hydrazones of oxalacetate or pyruvate so formed are treated with dilute alkali and the optical density measured against water at 505 mμ; the results are read from standard curves of optical density against transaminase activity.

Spectrophotometric assay of each enzyme is achieved by a similar incubation at 25°C in the presence of $NADH_2$, where added malic dehydrogenase converts the oxalacetate from GOT to malate (K4, K5, L1) or lactic dehydrogenase the pyruvate from GPT to lactate (W17); in each case the rate of decline of the optical density of the system at 340 mμ measures the rate of conversion of $NADH_2$ to NAD by the oxalacetate or pyruvate produced, and thus the respective transaminase activities. As in the aldolase assay, readings should not begin until 5 minutes after adding serum to all the reagents at 25°C. Rarely lactic or malic dehydrogenase preparations, particularly the latter, possess contaminant transaminase activity leading to erroneously high results; in each batch this may be tested by conducting a complete assay with serum substituted by water, or even dilute pyridoxal phosphate solution lest the contaminant is present as the apo-transaminase (W13).

High-speed colorimetric or automatically recording spectrophotometric instruments are again most useful. Though the spectrophotometric method is more accurate, the colorimetric, requiring only 0.2 ml of serum, is exceedingly rapid and convenient. At high activities colorimetric SGOT assay may be subject to oxalacetate inhibition, unlike the spectrophotometric method (B17), but this may be reduced by saline dilution of serum as before, a method similarly found sufficiently valid for rapid routine work. Serum retains its transaminase activities comparatively unchanged for at least a fortnight at — 17°C. Serum transaminase activity by the colorimetric method specified is arranged for expression in conventional spectrophotometric units (K4), in which one unit of activity under the conditions defined causes a decrease in optical density at 340 mμ of 0.001 per minute per ml of serum; in 50 healthy adults the normal ranges have been found (R13) to be 12–36 units per ml for SGOT (mean 19 units) and 4–24 units per ml for SGPT (mean 12 units). For conversion to International Units, one conventional spectrophotometric unit per ml = 0.48 μmole/minute/liter (W13).

7.3. Lactic and Malic Dehydrogenases

Both are abundant in skeletal muscle, myocardium, liver, and erythrocytes, so that hemolysis must be avoided, and in serum they may be assayed spectrophotometrically by their conversion of phosphate-buffered pyruvate to lactate (R6, W16) or oxalacetate to malate (S25) at the expense of added $NADH_2$, when the rate of decrease of optical density at 340 mμ thus measures the serum activities of the respective enzymes. Recently, however, the reverse reaction has been found best for serum lactic dehydrogenase assay (A2a). In conventional spectrophotometric units the normal ranges are 100–600 units per ml for lactic dehydrogenase (W16) and 42–195 units per ml for malic dehydrogenase (S25); as before, one conventional spectrophotometric unit per ml = 0.48 μmoles/minute/liter (W13).

7.4. Creatine Kinase (Creatine Phosphokinase; ATP-Creatine Phosphotransferase)

This soluble enzyme is specially abundant in skeletal muscle, only one quarter as plentiful in the myocardium and brain, and practically absent from other tissues (C13), so that hemolysis does not affect its activity in serum; in the study of muscle disease this distribution offers great advantages. Its function is specifically the equilibration of creatine phosphate and ADP with creatine and ATP, with equilibrium heavily in favor of the latter compounds. Since its activity in serum is some million times lower than in skeletal muscle, serum assay offers certain difficulties; three reliable methods, however, are available.

Colorimetric assay (H7) is by incubation of only 0.1 ml of serum at 37°C and pH 7.4 with Tris/HCl-buffered ADP and creatine phosphate in the presence of Mg^{++} ions and cysteine to maintain enzyme action; after 30 minutes the reaction is stopped by addition of *p*-chloromercuribenzoate, the mixture deproteinized by $Ba(OH)_2/ZnSO_4$, and the creatine content of the supernatant determined colorimetrically by the α-naphthol/diacetyl method using a 60-minute development at 37°C for creatine chromogen. Controls are prepared simultaneously by the same procedure, without incubation, by replacing ADP with water and adding *p*-chloromercuribenzoate first, and standards in the same way by using a pure creatine solution instead of water.

The constant-pH titrimetric method (C6), especially suitable for low enzyme activities and requiring 1 ml of serum or saline-diluted serum, uses the reverse reaction at 30°C in the presence of Mn^{++} ions and mercaptoacetic acid. It depends on the quantitative liberation of one H^+

ion at pH 9.0 as ATP (4^-) and creatine (1^-) become ADP (3^-) and creatine phosphate (3^-), so that the rate of addition of base to maintain pH 9.0 measures the rate of creatine phosphate formation. A small water-jacketed reaction vessel with a Teflon-covered magnetic stirrer is used, into which dip the pH meter electrodes and the tip of a microburette holding dilute NaOH. The use of a Conway burette is technically difficult but, since the reaction rate is little different between pH 8.5 and 9.5, this may be overcome by flicking the burette stopcock to bring the pH just above 9.0, then reading the stopwatch and burette as the pH meter needle moves downwards over the 9.0 mark, when another flick immediately brings it just above again. The straight line derived by plotting base added against time for about 15 minutes gives μmoles of base required in unit time, thus μmoles of creatine phosphate evolved, as a measure of creatine kinase activity. Though accurate, reproducible, and subject to little interference, the method demands great dexterity and is capable of only one assay at a time. For routine use a modern automatic pH titration apparatus would avoid much tedium.

The spectrophotometric method of Tanzer and Gilvarg (T2) as applied by Aebi *et al.* (A1, C13) measures creatine kinase activity at 37°C in the presence of Mg^{++} ions and Tris buffer at pH 9.0 by the rate of conversion of creatine and ATP to creatine phosphate and ADP; the ADP produced is reconverted to ATP by added phosphoenolpyruvate and pyruvate kinase, and the liberated pyruvate converted to lactate by added lactic dehydrogenase and $NADH_2$. The rate of decrease of optical density at 340 mμ measures the rate of conversion of $NADH_2$ to NAD by pyruvate liberated by the ADP produced, and thus the creatine kinase activity. The reaction is started by adding creatine substrate to all the reagents, including serum, at 37°C in the photometric silica cell, and optical density read serially at 30-second intervals for up to 10 minutes against a similar blank containing Tris buffer instead of creatine, to account for any action of serum alkaline phosphatase on phosphoenolpyruvate or ATPase on ATP. Only 0.3 ml of serum, diluted if need be, is required for both test and blank. For multiple assays by this method automatic spectrophotometry is obligatory.

Serum retains its creatine kinase activity at —25°C for at least a month without change (H7). In the colorimetric procedure (H7), which is especially sensitive, units of creatine kinase activity are expressed as μmoles creatine/hour/ml serum at 37°C, with a normal range of 0.6–4.0 units per ml for men and 0.6–2.6 units per ml for women (H9), thus clearly establishing a sex difference not apparent in the other serum enzymes so far considered.

8. Clinical Applications of Serum Enzymology

Throughout the text, serum aldolase has been determined by the spectrophotometric method (B10, L7, S29) and both transaminases by the colorimetric method (R2). The normal ranges (R13) in conventional units are for serum aldolase 2.3–8.8 units per ml (mean 5.7 units), for SGOT 12–36 units per ml (mean 19 units), and for SGPT 4–24 units per ml (mean 12 units). In each figure the activity of serum aldolase is denoted by an uninterrupted line, of SGOT by long strokes, and of SGPT by short strokes (Figs. 7-15).

8.1. Differential Diagnosis of Myopathic and Neurogenic Weakness

Patients with increasing muscular weakness may offer widely diverse histories, signs and symptoms of their complaint. On examination, the condition may often be related at once to disease of the central or peripheral nervous system or, if clearly defined, to one of the classical myopathies. Doubts may frequently arise, however, and where further

TABLE 1
Neurogenic Muscular Weakness

Condition	Age (years)	Sex	Serum aldolase	SGOT	SGPT
Acute polyneuritis (Guillain-Barré)	13	M	3.7	22	11
Chronic peripheral neuritis	30	F	7.7	20	12
Amyotrophic lateral sclerosis	45	F	7.7	15	6
Motor neuron disease	44	M	7.9	16	8
Werdnig-Hoffman disease	⅔	F	9.9	22	15
Werdnig-Hoffman disease	3½	M	7.6	16	11
Werdnig-Hoffman disease	8	F	5.6	28	18

confirmation seems desirable the patient may have to be admitted for cerebrospinal fluid examination, muscle biopsy histology, electromyography, and other tests, when refined diagnosis becomes possible (W3). Serum enzymology, however, offers both patient and dispensary clinician immediate and convenient assistance, which in the absence of other florid disease can be not only confirmatory but diagnostic. In the hands of the research worker it becomes a valuable source of information.

Serum enzyme activities are normal (A3, A5, C9, C13, D17, E5, G2, H7, K1, M18, O1, O2, P4, R14, S1, S13, S14, S16, S17, S22, S26, T8, W12) in patients with neurogenic muscular weakness (Table 1), but are raised, sometimes grossly, in those with primary disease of muscle. Such elevations in a patient with obscure muscle weakness and without other evident disease are very strong indications of the presence of myopathy.

Elevated serum activities in primary myopathy occur to different degrees in a number of enzymes, including aldolase (A1, A2, A3, A5, B19, C7, C9, E5, G2, H7, K14, O1, O2, P8, R14, S1, S11, S14–S17, S22, S24, S33, T1, T6, T8–T11, W11, W12), both transaminases (A1, A2, A3, B19, C7, D11, K1, K2, K17, M18, P4, P8, R15, S1, S14, S26, T1, T8–T11, W11, W12), creatine kinase (A1, A2, C13, D17, E1, F2a, H7, K16, O1, O2, R4, R12, S14, S33, T11), lactic dehydrogenase (A1, A2, A3, C7, P8, R6, R15, S13, S14, S28, T1, W12), and malic dehydrogenase (C7, P8, S20). Of these, aldolase and the transaminases have been extensively investigated and are widely used, aldolase being very sensitive and the transaminases most easily assayed. Recently creatine kinase has been found most sensitive of all chiefly due to its very low activities in normal serum compared with the others (A2, S14), so much so that modest elevations occasionally occur in neurogenic weakness (F2a, H7) and even in healthy carriers and sibs in Duchenne-type muscular dystrophy (Section 8.3.6). In this form of dystrophy a high degree of statistical correlation exists between the degrees of elevation of all four enzymes (A2, T9).

8.2. Polymyositis

Similar serum enzyme elevations are found in polymyositis (A3, B4a, B20, D2, D17, E5, H7, K10, M15, M18, P1, P4, P5, P7, R15, S15, S26, T8, W12, W19), a nonspecific inflammatory myopathy sometimes associated with neoplastic disease but in general related to the collagen diseases and likewise responsive to corticosteroid therapy. The condition occurs at all ages in both sexes, may be acute or insidious with perhaps a normal erythrocyte sedimentation rate, may or may not be painful, or may be accompanied by an erythematous rash (dermatomyositis). Characteristically the earliest appearance of weakness is in the muscles of the pelvic

TABLE 2
Myositis

Condition	Age (years)	Sex	Serum aldolase	SGOT	SGPT	Days
Acute polymyositis	33	M	85.9	94	56	
Acute polymyositis	27	M	40.8	106	75	
Dermatomyositis	20	F	10.9	59	31	
Chronic polymyositis	35	M	13.4	27	17	
Chronic polymyositis	44	F	9.3	19	19	
Crippled polymyositis	14	M	12.8	25	9	
Wasted and disabled polymyositis	22	M	6.8	19	21	
Successfully treated acute myositis	16	M	33.2	100	60	1
			13.8	60	71	9
			9.7	28	42	22

and shoulder girdles and in the proximal limb musculature; muscle atrophy is usually moderate and occurs late, so that in children or in young adults a confident diagnosis from the Duchenne-type or limb-girdle forms of muscular dystrophy, respectively, may be possible only after extensive investigations, including muscle biopsy with expert histological opinion. The subject has been well reviewed recently (B4a, P7); it is pointed out that, as might be expected, the serum enzyme elevations are greater in the early acute form than in the chronic form, much lower also where considerable muscle loss has occurred due to myositis of long duration, and restored to normal values in the course of successful treatment. Examples are given in Table 2; the last shows such a return towards normal values during 3 weeks of intensive and successful treatment of very acute myositis accompanying acute staphylococcal septicemia (T8).

8.3. Muscular Dystrophy

8.3.1. *Diagnosis at Casual Venepuncture*

As in polymyositis, one might expect that the degree of serum enzyme elevations in muscular dystrophy should likewise depend on the severity and extent of the dystrophic process. From the results obtained in a substantial number of patients (Table 3) it is evident that by far the greatest elevations are found in the Duchenne type, the most severe of all the dystrophies, being much less notable in the other three less rapid and disastrous forms (T10). Serum creatine kinase shows proportionally similar elevations in each type (A2), though in far greater degree (A2, S14) owing to its very great abundance in muscle and its low normal values in serum, and is thus most useful where other enzymes are equivocal, as in dystrophia myotonica (K14, K16, K17).

TABLE 3
Mean Values of Serum Enzyme Activities in the Muscular Dystrophies

	Duchenne	Limb-girdle	Facio-scapulo-humeral	Dystrophia myotonica	Normal mean value
Aldolase	63.61 (22 cases)	6.21 (14 cases)	9.32 (17 cases)	7.89 (12 cases)	5.7
SGOT	90.3 (22 cases)	20.8 (12 cases)	25.5 (13 cases)	24.8 (12 cases)	19
SGPT	78.1 (22 cases)	15.3 (12 cases)	16.3 (13 cases)	18.1 (12 cases)	12

8.3.2. *Effects of Progressive Muscle Loss*

Again, continued loss of dystrophic muscle must diminish its total enzyme efflux. In Duchenne-type muscular dystrophy the highest serum enzyme elevations occur in early childhood, diminish as the disease pro-

gresses, and are least raised terminally (A2, A5, B19, C9, M18, O1, O2, P4, R14, R15, S11, S17, S20, S33, T6, T8–T10, W11). With the exception of the limb-girdle form, where progress may be very variable even in the same individual, the rate of muscle loss in muscular dystrophy is fairly constant, so that the remaining muscle mass is inversely proportional to the duration of the disease, or to age in the Duchenne type with its onset as early as infancy (A1, A2, P7a, P8), and a steady, rapid course (W6). Figures 1, 2, and 3 (pp. 166 and 167) show curves illustrating the relationship between serum aldolase activities and duration of disease, with a diminishing rate of fall towards lower constant values as the muscle disappears with time (T10). Exactly similar findings have been reported for serum creatine kinase in Duchenne-type muscular dystrophy (H9). No such regular relationship is found in the limb-girdle form (C9, T10).

However, it is apparent from Fig. 1 that in Duchenne-type muscular dystrophy the serum aldolase activity declines less smoothly with age in younger than in older patients, and it has been found that the wide individual variations usual in ambulant children (T6) disappear only when they have become bedridden (S11). By casual venepuncture in a larger series of patients, from active little boys to helpless men, where the exact age is taken not in round years but to the nearest day, and where physical status is denoted by circular points for full ambulation and triangular for permanent immobility, it then becomes possible to relate with greater accuracy the serum enzyme elevations to age and physical activity (T8). In Figs. 4, 5, and 6 (pp. 168 and 169) the high values of serum aldolase and both transaminases in young, active patients decline steeply through widely scattered points, then much more slowly in older, immobilized patients through points more closely grouped towards values just exceeding normal. This very sudden alteration in course takes place at the age by which most patients are confined to wheel chairs. Two different processes are evident: the large irregular variations while the muscle mass suffices, and the underlying slow decline with shrinkage once inactivity supervenes. In Duchenne-type dystrophy serum aldolase seems to be a sensitive index, SGOT rather less so, and SGPT least of all though constantly and reliably elevated; perhaps this individuality stems both from its relative dearth in muscle (Section 7.2) and from its slower clearance from serum (Section 6.3). Comparative statistical analyses support this view of aldolase (C9).

8.3.3. *Effects of Physical Activity*

The probable origin of these enzymes from the diseased muscle (Section 6.2) with the known rapidity of their clearance from serum (Section 6.3) implies abnormal muscle efflux due to increased membrane perme-

ability (Section 6.5), and though the resulting diminution in the sarcoplasmic enzyme content (Section 6.6) must be maintained by rapid continuous renewal to sustain this loss (Section 6.7), muscle enzyme efflux

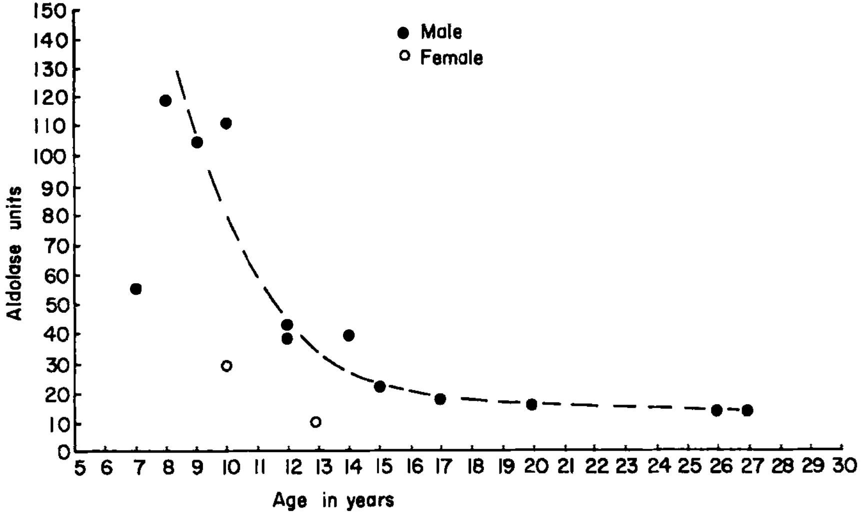

FIG. 1. Relationship between serum aldolase activity and age in Duchenne-type muscular dystrophy.

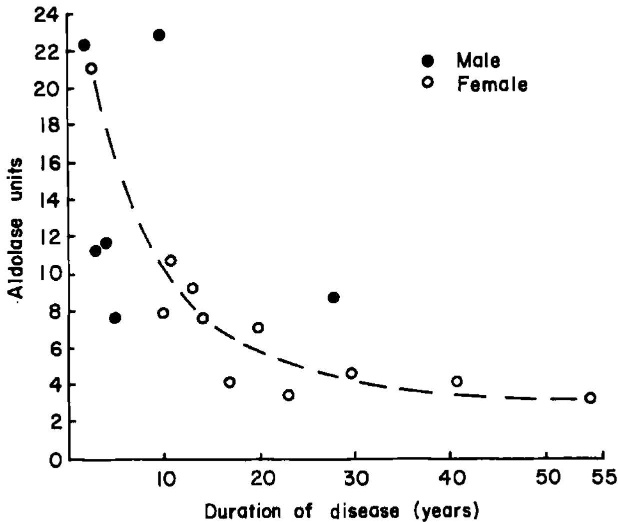

FIG. 2. Relationship between serum aldolase activity and duration of disease in facio-scapulo-humeral muscular dystrophy.

is liable to be increased still more, and particularly in the dystrophic individual, by physical activity (Section 6.8). Further, any influence of rest or of physical activity on serum enzyme values in the dystrophic patient should be proportional, as before, both to the severity and extent of the disease and to the amount of dystrophic muscle remaining.

Figures 7, 8, and 9 (pp. 170 and 171) show the effects after a week in bed, though free to move about in it (W10), of 2 days of ordinary ambulation on the serum enzymes of three little boys at different stages of Duchenne-type muscular dystrophy (T8). Such modified rest has little effect, except on SGPT in the earliest case, but the immediate and extreme

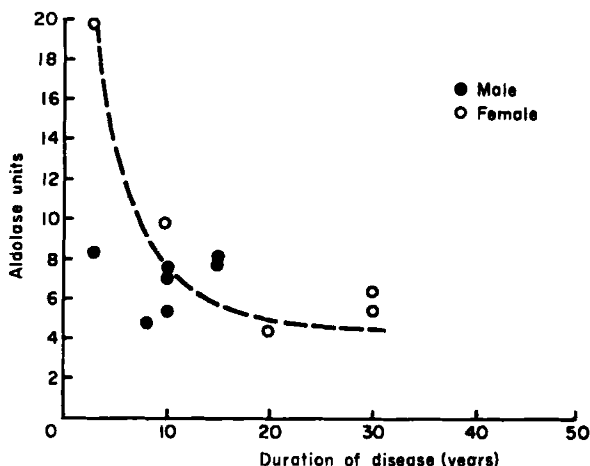

FIG. 3. Relationship between serum aldolase activity and duration of disease in dystrophia myotonica.

elevations of serum aldolase and SGOT values on subsequent ambulation are clearly proportional to the amount of dystrophic muscle left and probably also to the degree of physical activity it permits. They indicate, moreover, both by the small effects of rest, admittedly modified, and by the copious efflux after it, the severe involvement of a large muscle mass with very great cell membrane permeability. Further, this profuse enzyme discharge on ambulation seems to suggest accumulation by rapid intracellular renewal during rest, and possibly also additional rapid generation of enzymes to enable active muscular work, which may itself cause extra efflux.

In less catastrophic forms of muscular dystrophy the same processes are observed, again to a degree commensurate with the severity of the disease and with the amount of muscle left (T8). Figure 10 (p. 173)

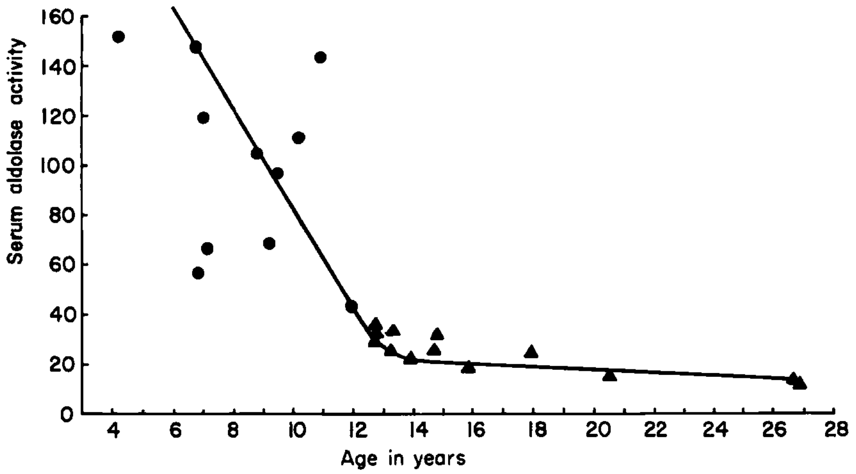

Fig. 4. Relationship between serum aldolase activity and exact age in Duchenne-type muscular dystrophy.

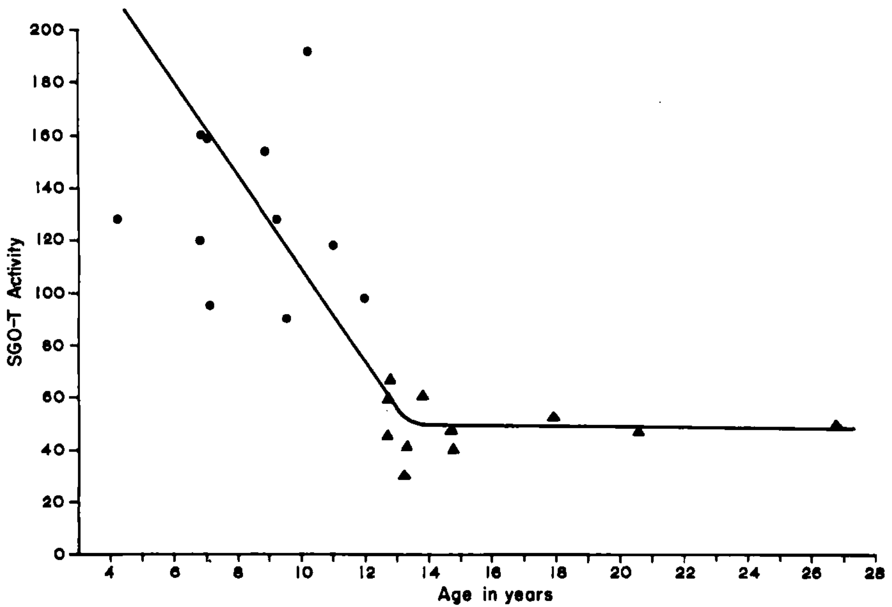

Fig. 5. Relationship between SGOT activity and exact age in Duchenne-type muscular dystrophy.

shows the effects of alternate bed rest and ordinary ambulation in a very wasted young man with advanced rapidly progressing limb-girdle muscular dystrophy. The immediate discharge on ambulation of muscle enzymes accumulated during rest promptly subsides on further rest, and on continued ambulation thereafter the apparent accumulations in his small residual muscle mass are finally discharged and quickly cleared, leaving the serum enzymes as they were.

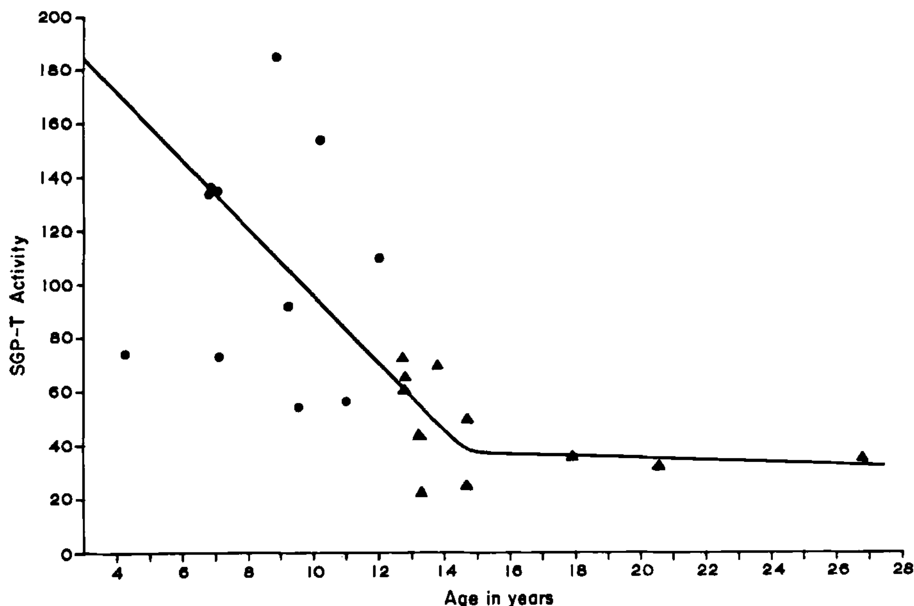

FIG. 6. Relationship between SGPT activity and exact age in Duchenne-type muscular dystrophy.

Again, Figs. 11 and 12 (pp. 174 and 175) show similar effects due this time to early mild limb-girdle muscular dystrophy in a young woman confined to bed during and after normal delivery of healthy twins. After the peak discharge of myometrial enzymes 4 days after delivery, compared in Fig. 13 (p. 175) with similar appearances after normal twin delivery (point A) in a healthy woman, the serum enzyme values slowly ascend for many days, then steadily decline to pre-admission values. Though widespread, the disease in this patient is still so early as to present a large muscle bulk seemingly capable of considerable total enzyme accumulations, but subject to a dystrophy sufficiently mild and slowly progressive as to contain them and to prevent so sudden and complete an efflux as in the young man.

In Fig. 14 (p. 177), the dystrophic process is so slight in a young road laborer with myotonia congenita that only the major environmental change to light employment can reduce his serum enzyme activities to rather lower and remarkably constant values. The elevation of serum aldolase activity, though modest, still supports the disputed inclusion of myotonia congenita as a muscular dystrophy (M1, W6), as do the other elevated findings at casual venepuncture in Table 4 (p. 172).

Thus in muscular dystrophy it is apparent that both the mean elevations of the serum enzyme values and the magnitudes of their variations upon physical activity are proportional to the mass of dystrophic muscle remaining and to the severity of the disease in it. Both are thus greater in early than in evident Duchenne-type dystrophy, less in limb-girdle dystrophy, and least in myotonia congenita. Further, though serum creatine kinase has been found to be an exceedingly delicate index of myopathy (A2, S14), for present purposes serum aldolase is sufficiently

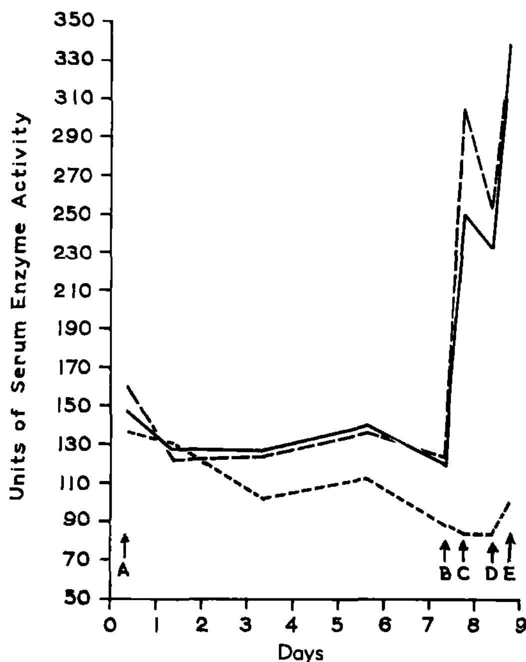

FIG. 7. Early dystrophy.

FIGS. 7, 8, and 9. Effect of physical activity after rest in ambulant Duchenne-type muscular dystrophy. A, on admission before being put to bed; B and C, on morning and evening of first day out of bed; D and E, on morning and evening of second day out of bed.

sensitive, showing elevations even in myotonia congenita, the least dystrophic myopathy, whereas the transaminases tend to show elevations more particularly where rapid muscle destruction and wasting are proceeding, as in Duchenne-type muscular dystrophy (Section 8.3.1).

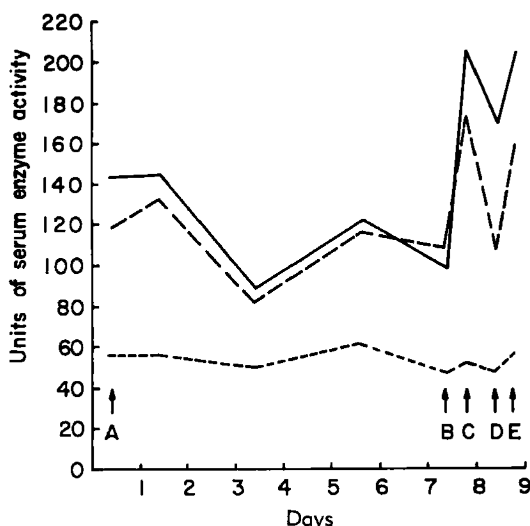

FIG. 8. Moderately advanced dystrophy.

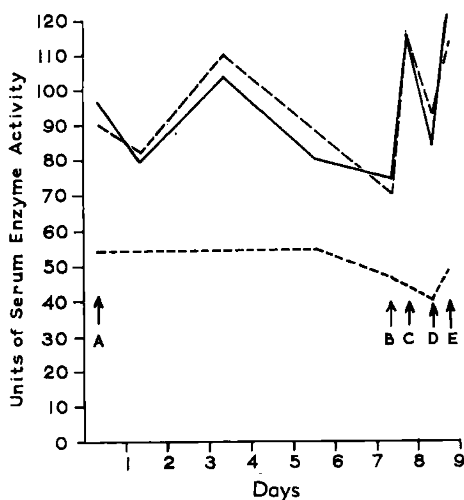

FIG. 9. Advanced dystrophy.

8.3.4. *Measuring the Dystrophic Process for Assessment of Therapy*

By using both the means and variations of serum enzyme elevations it becomes possible to measure objectively the severity and extent of the individual dystrophic process, and thus, in Duchenne-type dystrophy, to observe the effects of prospective therapy (T9).

TABLE 4
MYOTONIA CONGENITA

Condition	Severity	Age (years)	Sex	Serum aldolase	SGOT	SGPT
Myotonia congenita	Severe	20	M	26.5	21	12
Myotonia congenita	Moderate	27	F	19.0	16	13
Myotonia congenita	Mild	33	M	7.9	14	19
Myotonia congenita	Mild	18	F	6.1	15	20
Paramyotonia congenita	Moderate	46	F	10.1	16	18

Figure 15 (p. 177) shows the results of serial serum enzyme assay of morning pre-ambulatory specimens taken not less than thrice weekly for some 3 months from a 7-year-old boy, still ambulant and active, though with plainly manifest Duchenne-type muscular dystrophy confirmed at biopsy. Periods 1, 2, and 3 are each 5-day periods of strict bed rest; during period 1 no treatment whatever is given, but during periods 2 and 3 successive intravenous loading infusions in saline are given of an aqueous solution of certain pure nucleotides and nucleosides (T9). Periods A, B, and C are each periods of free ambulation for about 4 weeks without physiotherapy of any kind; as in period 1, no treatment is given during period A, but during periods B and C daily intramuscular maintenance injections are given of the same nucleotide/nucleoside mixture. The rapid diminution of serum enzyme values in periods 1, 2, and 3 is due to the strict immobility imposed, and is more marked in periods 2 and 3 probably because of therapeutic infusion. During ordinary ambulation in periods A, B, and C, however, the large continuous variations in all three serum enzyme values are used to derive characteristic objective measures of the dystrophic process during each period. The arithmetical means of these values indicate the muscle mass remaining and its total enzyme efflux; their standard deviations measure their scatter about the means and thus the effects of ordinary physical activity on this abnormal efflux. In a series of patients at different stages of evolution, it is found that both measures diminish slowly as the disease progresses and the muscle wastes away (T9). Statistically significant sudden diminutions, however, of either or both measures after treatment, with a corresponding measurable increase in muscle power, may be taken as objective evidence suggesting some

amelioration of the dystrophic process. Though this child after treatment showed an apparently marked and sustained clinical improvement, with better gait and posture and greater facility in ascending stairs, mere clinical observation alone cannot establish objective improvement in a disease like muscular dystrophy. The chief evidence here resides in statistical analysis of the serum enzyme values (Table 5, p. 176), where the means and standard deviations after treatment are significantly much

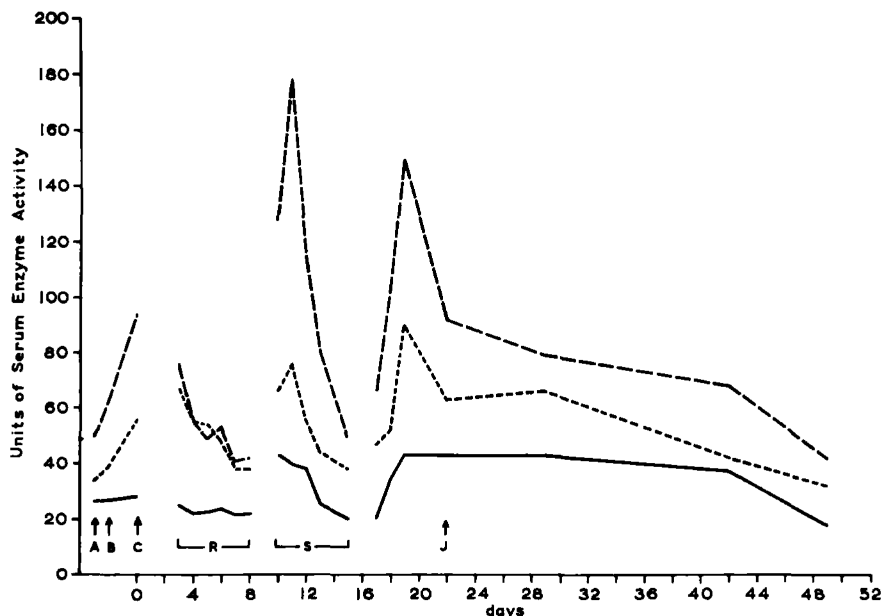

FIG. 10. Effect of alternate rest and physical activity in advanced severe limb-girdle muscular dystrophy. A, first appearance as outpatient; B, admission after 7 weeks of general exercise; C, after 17 days of systematic hospital exercises. Periods R and S, successive intervals of 6 and 7 days of bed rest. J, discharged ambulant from hospital.

lower than those before treatment in period A, supported in Table 6 (p. 176) by the increased aggregates of muscle-group strength testing, on admission and again after treatment, both by the MRC method (M8) and independently by a much more accurate ergometric system (T9).

8.3.5. *Diagnosis of Obscure Myopathies*

Continuous refinement has brought the use of serum enzymology in the diagnosis of myopathy to a high degree of reliability, so that, by careful interpretation of results, important information may quickly be

revealed which might otherwise escape notice. An interesting example of such an occasion is reported (T11) in two patients offered for biochemical opinion. The first was a middle-aged man who all his life had suffered from unusually rapid muscular fatigue with painful cramps provoked by exercise and rapidly relieved by rest, but who showed no signs of weakness or wasting, or relevant abnormality of any system, on ordinary clinical or biochemical examination. The second was an active, 4-year-old boy with slight general muscular weakness, swollen calves with early gastrocnemius shortening, and a myopathic electromyogram, so that a diagnosis of Duchenne-type muscular dystrophy seemed inevitable. Serum enzymology in both, however, gave the information shown in Table 7 (p. 176).

In the absence of other florid disease the presence of primary myopathy was confirmed in both patients by the abnormally high values of

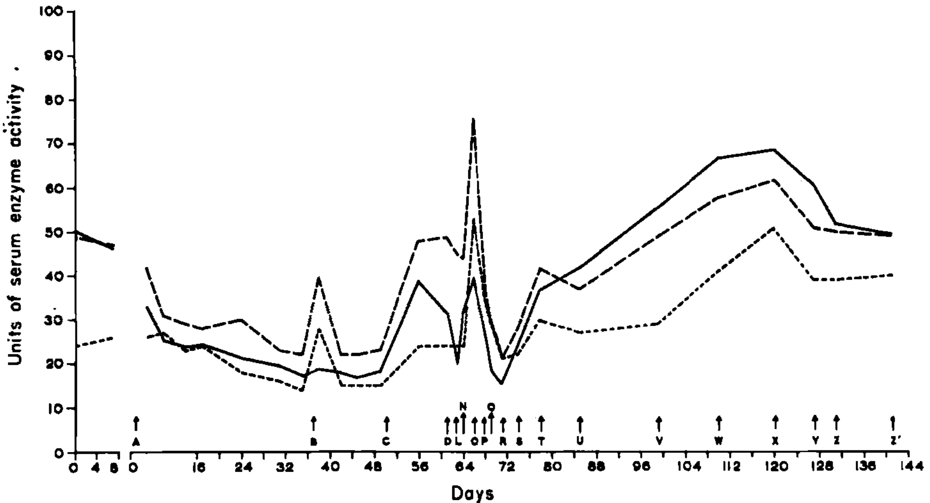

FIG. 11. Late pregnancy, delivery, and the puerperium.

FIGS. 11 and 12. Normal twin pregnancy during early mild limb-girdle muscular dystrophy. A, admission to bed for assessment; B, brief active physiotherapy; C, discharged home under observation; D, admission to bed in early labor; E, 8 hours, and F, 2 hours before first birth in strong labor; G, during uterine relaxation between deliveries.

After placental delivery: H, immediately afterwards, uterus firmly contracted; I, 15 minutes; J, 2 hours; K, 6 hours; L, 12 hours; M, 24 hours; N, 42 hours; O, 91 hours afterwards.

P, Q, R, in bed after delivery before discharge home at R; S, first venepuncture at home; T, 30 minutes after first short walk out of doors; U–Z', remainder of venepunctures at home.

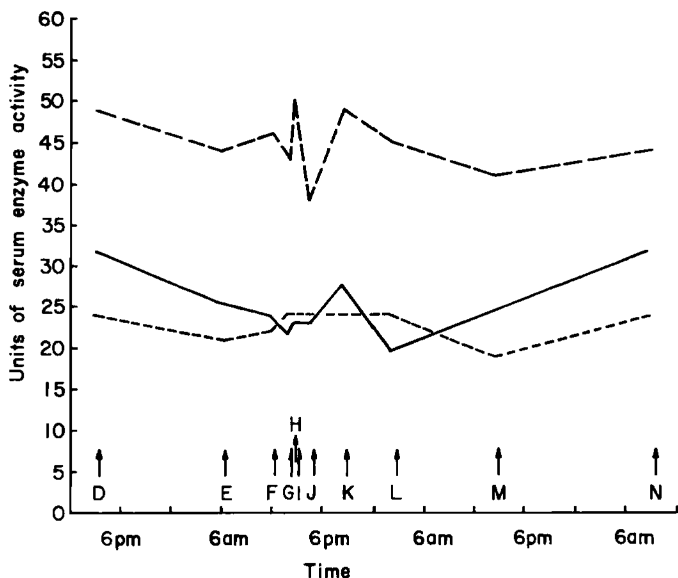

FIG. 12. The course of labor and delivery.

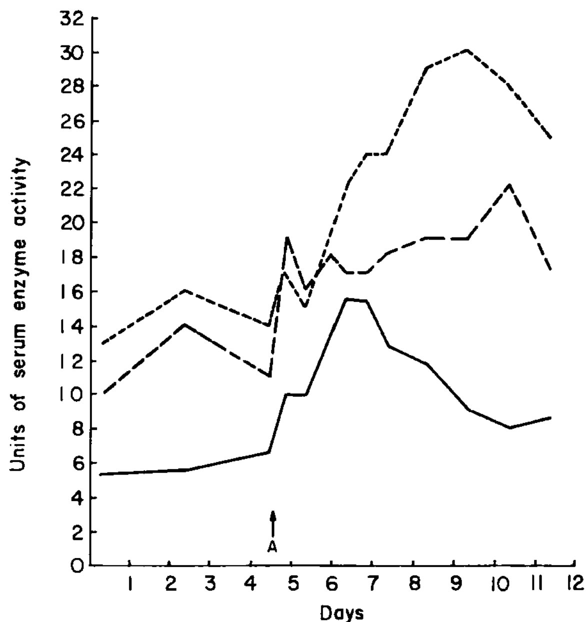

FIG. 13. Delivery (A) of healthy twins from normal mother.

TABLE 5

ANALYSIS OF EFFECTS OF THERAPY ON SERUM ENZYME VALUES[a]

	Mean	S.D.	Difference of means	Significance
Aldolase				
Period A	126.72	27.69	A v B; $t = +2.51$	+
Period B	101.94	28.13	A v C; $t = +4.61$	++
Period C	87.35	19.22	B v C; $t = +1.77$	N.S.
SGOT				
Period A	180.27	41.44	A v B; $t = +2.15$	+
Period B	146.41	42.78	A v C; $t = +4.33$	++
Period C	126.47	25.98	B v C; $t = +1.51$	N.S.
SGPT				
Period A	147.20	38.98	A v B; $t = +2.59$	+
Period B	116.94	24.35	A v C; $t = +5.60$	++
Period C	85.82	17.99	B v C; $t = +4.24$	++

[a] S.D. = standard deviation; N.S. = not significant; v = versus.

Aldolase correlation coefficients:

	v SGOT	v SGPT
Period A	0.89(++)	0.61(+)
Period B	0.95(++)	0.67(++)
Period C	0.81(++)	0.54(+)

TABLE 6

MUSCLE STRENGTH TESTING

	MRC scale		% Increase in strength
	Before	After	on ergometric testing
Arms	43	49½[a]	+32.4
Legs	56½	64½[b]	+57.5

[a] Maximum possible = 50.
[b] Maximum possible = 70.

TABLE 7

SERUM ENZYME ASSAYS IN TWO OBSCURE MYOPATHIES

Enzyme	Adult (G.R.)	Child (J.W.)	Normal ranges
Aldolase	42.6	18.4	2.3–8.8
SGOT	55	107	12–36
SGPT	25	36	4–24
Creatine kinase	0.32	0.38	0.01–0.10[a]

[a] µmoles/min/ml

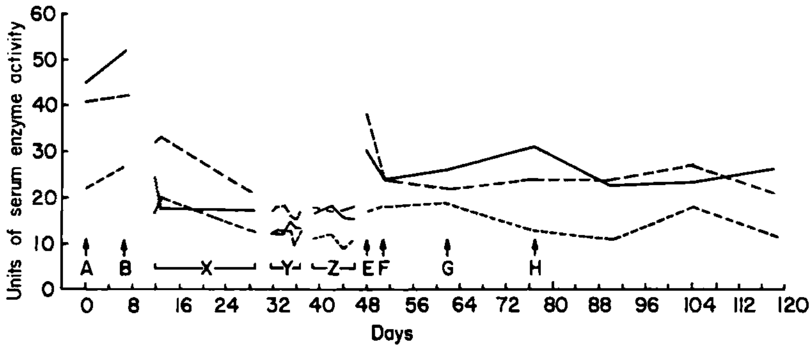

FIG. 14. Effect of alternate rest and physical activity in myotonia congenita. A and B, during heavy employment. Period X, freely ambulant in hospital for 17 days; periods Y and Z, successive intervals of 6 and 7 days of bed rest. E, after 2 days out of bed; F, on discharge; G, at home; H, after 10 days in light employment, so continuing thereafter.

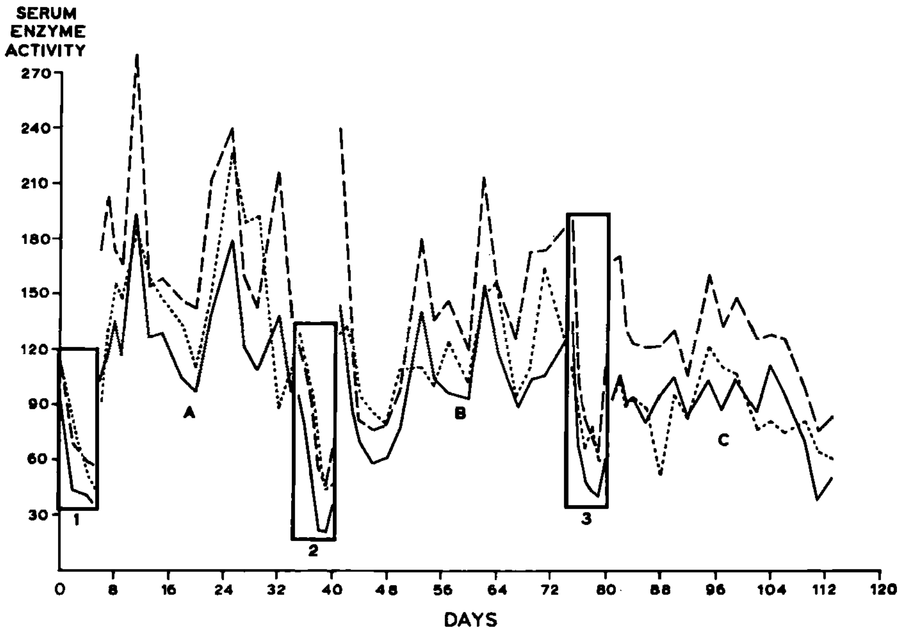

FIG. 15. Measuring therapeutic effect in Duchenne-type muscular dystrophy (see text).

serum aldolase, SGOT, and creatine kinase. In the adult, however, only a severe and rapid myopathy, in marked contrast with his lifelong history and comparative well-being, could give so high a serum aldolase, which, with such modest transaminase activities, did not accord with any known muscle disorder. In the child, Duchenne-type dystrophy was discounted since at this age (from Figs. 4, 5, and 6) serum aldolase (and creatine

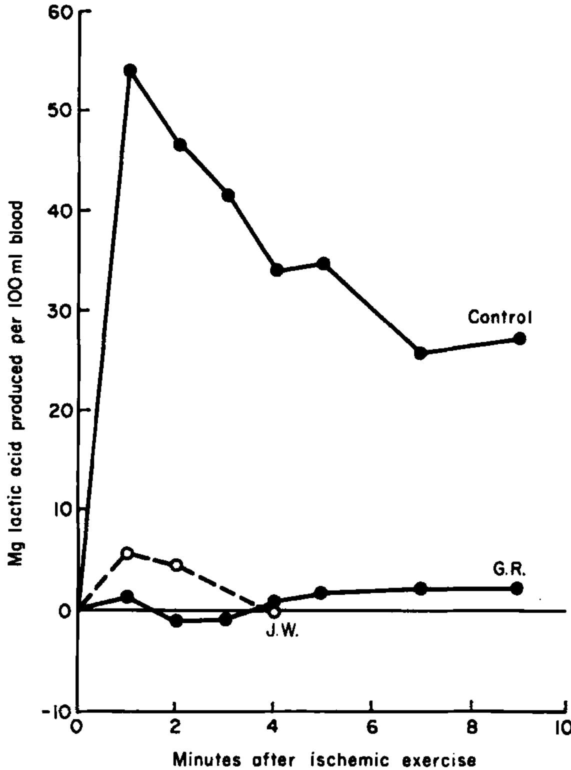

FIG. 16. Changes in blood lactic acid concentration after ischemic exercise.

kinase) would have been some 10 times, SGOT twice, and SGPT 5 times higher than the values found; again, the high SGOT value with only moderate increases of aldolase and SGPT could not be associated with a recognizable myopathy.

However, that both patients had myopathy of some kind was certain, and muscle biopsies were carried out. Formol-$HgCl_2$/paraffin/hematoxylin-eosin and formol/frozen/Sudan IV sections showed neither the pres-

ence of muscular dystrophy nor excessive fat, but instead large numbers of vacuoles in the muscle fibers, which appeared filled with glycogen on unfixed/frozen/periodic acid-Schiff (PAS) staining. Estimation of gastrocnemius glycogen content, normally about 1.5% wet weight, gave values of 4.1% and 11.3%, respectively, in adult and child, and on isolation was proved to have a normal molecular structure in both patients (K8a). Defective muscle glycolysis was therefore evident, and was confirmed by the failure of both the adult (G.R.) and the child (J.W.) to produce a normal amount of lactic acid on ischemic exercise (Fig. 16). Muscle

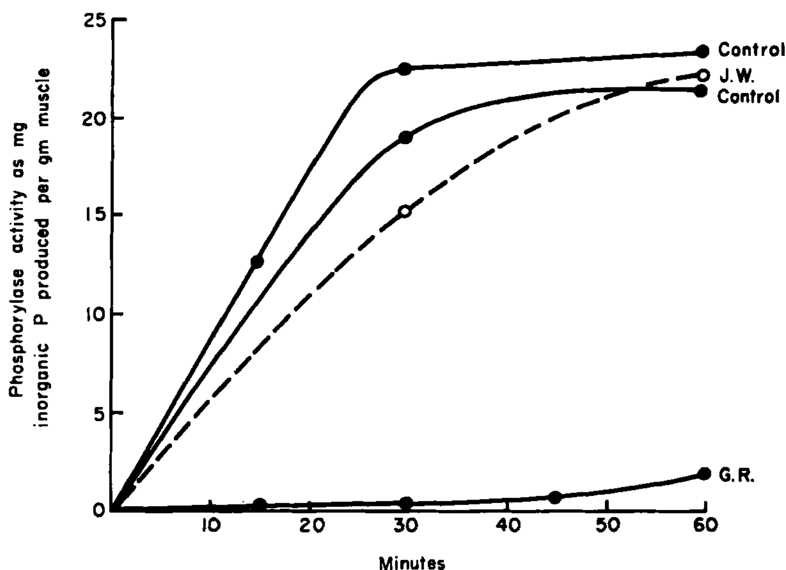

FIG. 17. Phosphorylase activity in homogenates of muscle biopsy specimens.

homogenates from the adult (G.R.) showed a complete lack of phosphorylase activity (Fig. 17), though sufficient was present in those from the child (J.W.); anaerobic glycolysis by muscle homogenates of selected intermediate substrates confirmed that this was the only defect in the adult, while the child showed several partial glycolytic defects, particularly serious but not absolute in respect to phosphoglucomutase. The adult therefore was clearly a case of phosphorylase-defect myopathy (McArdle's disease), while the child suffered from a type of skeletal muscle glycogenosis not hitherto described. It is highly unlikely that either patient would have been investigated so extensively but for the diagnostic indications originally given by serum enzymology.

8.3.6. *Detection of the Carrier State*

In families known to harbor the sex-linked recessive gene of Duchenne-type muscular dystrophy, half the sons of a carrier mother risk having the disease and half of her daughters the carrier state themselves. A small pedigree of this type is shown in Fig. 18. In A a mutation had probably occurred since, with no family history and both brothers unaffected, her son (1) was a sufferer and daughter B a carrier with an affected son (2). The remaining daughter C, however, seems to have escaped the carrier state and has three healthy sons, so that in the fourth generation of this family as it stands the disease will become extinct. It is well to

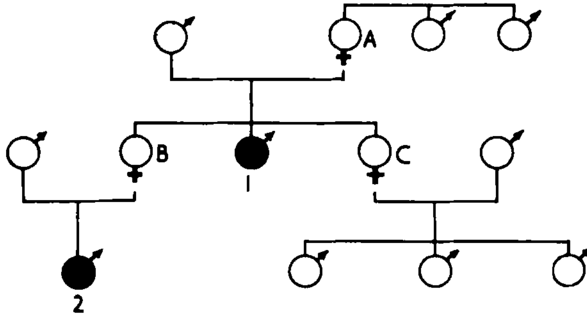

FIG. 18. Typical pedigree of Duchenne-type muscular dystrophy. The black circles represent patients with the disease.

be sure as early as possible how matters are with members of such a family.

Systematic serum enzyme assays in the apparently unaffected male infants disclose gross elevations in those destined to develop the clinical disease, and this as early as 1 year (A1, A2, R12) or even 6 weeks of age (P6, P7a, P7b, P8). Sometimes unaffected brothers of such children show persistent but only modest serum enzyme elevations; Table 8 shows this occurrence in one of two healthy brothers of two patients in the terminal stages, and Table 9 in the healthy nonidentical twin of a disabled dystrophic boy (T8). These findings support recent views (R4) by seeming to indicate that, though Duchenne-type dystrophy almost always shows complete and typical manifestation, in some individuals its expressivity may be so low as to be apparent only as such small serum enzyme elevations; or, much more likely, an unrelated tendency to such small elevations may be inherited separately as a benign variant in the general population (B17a).

On the other hand, occasional female carriers appear to be manifest-

ing heterozygotes, in whose X-chromosomal somatic mosaic (L3a) the carrier X-chromosome is held to be the more active one in more muscle cells than in most carriers (E2, E2b, P7c), and who thus show mild but definite signs and symptoms of undoubted muscular dystrophy, with strongly suggestive muscle histology and marked elevations of serum enzyme values (C9, D19a, D19b, E2, E2b, R4). More often, manifestation in female carriers is quite symptomless and limited to the serum enzymes, so that here assay may have great eugenic value. If the opposite

TABLE 8
DUCHENNE-TYPE DYSTROPHY IN TWO OF FOUR SONS

Condition	Age (years)	Serum aldolase	SGOT	SGPT
Healthy mother	—	7.5	19	14
Healthy father	—	7.1	12	11
Healthy son I	27	12.2	33	22
Dystrophic son II	26	13.2	50	35
Healthy son III	23	7.3	22	16
Dystrophic son IV	20	15.8	47	32

TABLE 9
DUCHENNE-TYPE DYSTROPHY IN ONE NONIDENTICAL TWIN

Condition	Age (years)	Sex	Blood group	Serum aldolase	SGOT	SGPT
Healthy mother	50	F	AB Rh(D)+ve	4.2	13	14
Healthy father	54	M	O Rh(D)+ve	6.4	12	13
Healthy twin I	12¾	M	B Rh(D)+ve	12.0	17	16
Dystrophic twin II	12¾	M	A Rh(D)+ve	29.7	46	73

occurs, however, and the paternal X-chromosome is the more active, occasional female carriers might show no serum enzyme elevations (P7a). Though Chung *et al.* (C9) found that in a minority of heterozygous carriers serum aldolase activity is increased, this assay has found no reliable application in carrier detection (E5, L5, S30).

It was soon noted, however, that serum creatine kinase as well as aldolase may be elevated in carriers (S12, S33), that here creatine kinase is far more sensitive, and that by its assay not only might the carrier state be confirmed in almost every female known to harbor the gene (A1, H8), but the expected proportion of mutant cases could be found by a family absence of both clinical and biochemical evidence of heredity (R12). These results have been confirmed for creatine kinase in a very large series of relatives of Duchenne-type dystrophic patients (R4), although

the test gives no information in relatives of patients with facio-scapulo-humeral or limb-girdle muscular dystrophy; even among the Duchenne-type relatives, however, there is a certain overlap of creatine kinase values not only between patients and carriers, perhaps due to vigorous heterozygote manifestation, but also between carriers and normal individuals, probably for the reasons just discussed (P7a). This latter difficulty may additionally arise because serum creatine kinase values are often markedly elevated by ordinary physical activity in health (Section 6.8), thus requiring special precautions and repeated assays for reliable results (R4); and it must be noted that elevations occur also in myxedema (G9, G11), myocardial infarction (F2a, K6), and even some types of acute abdominal pain (N3). Elevations of serum aldolase activity have likewise been reported in all these circumstances (Section 6.8; R13, S22).

Methods have recently been used for measuring arm-to-arm (total) circulation time separately (D6) or simultaneously with arm-tongue (central) circulation time (D5), and abnormal reductions in the difference (peripheral circulation time) predictably found in dystrophic patients (D3) have been found also in female carriers of Duchenne-type dystrophy (D5a, S18) and may be due to increased metabolism of the diseased muscle. By combining these techniques with simultaneous serum aldolase and creatine kinase assay, successful detection of 85–90% of carriers is said to be possible (D7, D7a, D8). Further, electromyographic studies in known carriers have now disclosed the presence of polyphasic responses intermediate in frequency between those found in normal and in dystrophic individuals, thus providing an additional means of discrimination (V1).

By these methods prospective mothers from afflicted families may be offered what is called "genetic counsel," that is, eugenic advice about the risks their progeny may face. It is computed that, even if only half the carriers could be detected and of these if only half again accept advice, about a thousand cases of Duchenne-type muscular dystrophy might be prevented from appearing in Britain over a period of 20 years (V1). Even so, a high perfection in these tests must certainly be reached to avoid the dismal possibility of "false positives" wrongly barred from procreation or, worse, the disconcerting appearance of afflicted children in suspect families falsely reassured. Recent attempts to confirm the validity of such tests have not always been encouraging (M14a), perhaps owing to the difficulties just discussed, and may disclose some need for more assiduous study both of dystrophic families and of the general population (B17a) before full confidence is justified.

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Acknowledgments

Figures 1–3 and Table 3 are reprinted from the *British Medical Journal*, and Figures 4–17 and all the remaining Tables from the *Journal of Neurology, Neurosurgery and Psychiatry* by permission of the author, the editors, and the publishers, B.M.A. House, Tavistock Square, London, W.C.1, England. Figure 18 is reprinted from the *Annals of Human Genetics*, London, England.

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MUCOPOLYSACCHARIDES IN DISEASE

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1. Introduction

The term mucopolysaccharide was introduced by Meyer (M12) to describe "hexosamine-containing polysaccharides of animal origin occurring either in a pure state or as protein salts." The prefix "muco" was chosen to denote the relationship of this type of substance with mucus, the physiological term for a viscous secretion. Later classifications of materials as mucopolysaccharides involved limitations with regard to protein content (the presence of hexosamine not being necessary) (S16), and the absence or presence of uronic acid and/or sulfate residues (B8, P3). Some confusion has resulted from application of the term mucopolysaccharide to (a) protein-polysaccharide complexes of high protein

content, and (b) lipid-polysaccharide complexes, which are, however, more accurately termed glycoproteins and glycolipids, respectively. Although there is no universally accepted definition of a mucopolysaccharide, the term is nowadays normally applied (J8) to heteroglycans which contain residues of both a uronic acid and a hexosamine. Attention is thereby confined to a group of substances including hyaluronic acid, the isomeric chondroitin sulfates, and heparin which are conveniently termed *acidic mucopolysaccharides*. The definition is, however, usually extended to encompass certain related "amino-polysaccharides" such as keratan sulfate, which accompanies other acidic mucopolysaccharides in animal connective tissues, and the homoglycan chitin.

In most connective tissues of animals, the acidic mucopolysaccharides are complexed with protein or peptide residues. Little is known about the structure of these complexes, and the term mucopolysaccharide is therefore best applied only to the pure polysaccharide. When the latter is complexed with protein, it has been suggested (J5) that a noncommittal term such as hyaluronic acid–protein complex should be used. Many of the names originally assigned to the acidic mucopolysaccharides have since been revised (J5) in an effort to systematize the nomenclature. The more systematic names proposed by Jeanloz (J5) will generally be used throughout this review, but whenever possible synonymic names have been given.

The methods that are available for the isolation of pure mucopolysaccharides have been reviewed (S7, S13) extensively and are therefore not considered further here. Recent evidence suggests that the isolation of mucopolysaccharide-protein complexes is likely to be of increasing importance from a biological and medical viewpoint.

Histochemical tests have often demonstrated changes in the mucopolysaccharides of connective tissues during the onset of certain diseases. However, in view of the unspecific nature of histochemical tests, in most cases a more definite assessment of the changes that have taken place is required. It seems appropriate, therefore, to review the structures of the acid mucopolysaccharides, many of which have been established within the last decade, and to indicate changes that have been observed in these substances in some diseases involving connective tissues.

2. Biological Function of Acid Mucopolysaccharides

One of the principal functions of connective tissues is to support and bind together the organs which in turn form the animal body. Though the connective tissues appear in different forms in various parts of the body, there is a fundamental similarity in the components, and any particular modification of form represents an adaption to function. Three

major components are generally recognized in connective tissues: (a) the cells, (b) the extracellular fibers, (c) the extracellular amorphous ground substance. The acidic mucopolysaccharides form an important part of the amorphous ground substance which lies between the extracellular fibers and the cells. It has become increasingly apparent that connective tissues have physiological functions other than that of a supporting medium. Since all substance going to and from the cells must pass through the ground substance, variations in its state and composition must have a profound influence on the life of individual cells and tissues. In a recent review, Dorfman (D11) has suggested that the acid mucopolysaccharides of connective tissues might participate in a number of physiological and pathological processes, including calcification, control of electrolytes and water in extracellular fluids, wound healing, lubrication of joints, blood coagulation, clearing activity, and maintenance of the stable transport medium of the eye. A possible role of sulfated mucopolysaccharides in hair growth has also been indicated (M22). The participation of the acid mucopolysaccharides in a number of these roles is undoubtedly due to their polyanionic nature resulting from the presence of carboxylic acid and sulfate groups. The physiological functions of a number of the acid mucopolysaccharides are discussed in more detail in the following section.

3. Structural and Related Studies

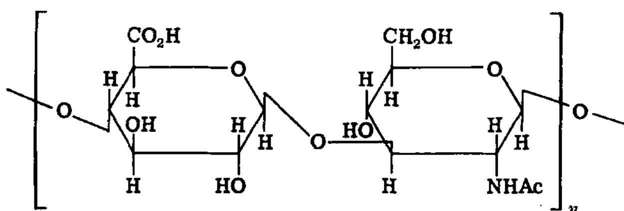
A systematic investigation of the types and quantities of acid mucopolysaccharides in a variety of tissues and fluids was conducted by Meyer *et al.* (M17). The six principal mucopolysaccharides present in connective tissues were identified as hyaluronic acid, chondroitin, chondroitin 4-sulfate (chondroitin sulfate A), chondroitin 6-sulfate (chondroitin sulfate C), dermatan sulfate (chondroitin sulfate B), and keratan sulfate (keratosulfate). The structures of these substances are discussed below together with those of heparin and heparitin sulfate (heparin monosulfate, heparin sulfate). Other reviews (H14, J8) detailing recent progress in the chemistry of the acid mucopolysaccharides have been published so that only salient features of structure need be dealt with here. The acid mucopolysaccharides are usually composed of alternate units of amino sugar and glycuronic acid, the acidic character being enhanced in some by sulfate groups.

3.1. Hyaluronic Acid

Hyaluronic acid, a mucopolysaccharide composed of alternate units of *N*-acetylglucosamine and D-glucuronic acid, is found in the ground substance of many connective tissues. Umbilical cord (M1), pseudo-

mucinous ovarian cyst and follicular fluids (M17), aorta (B7), synovial fluid (M13), and vitreous fluid (M11) are sources of hyaluronic acid. The molecular weight of the purified mucopolysaccharide usually lies within the range 5×10^4 to 8×10^6 depending on the source, the method of isolation, and the method of determination; usually the material is polydisperse (B11).

The structure of hyaluronic acid is now well established. Acidic hydrolysis yields the constituent disaccharide, hyalobiouronic acid, whose structure was shown to be 2-amino-2-deoxy-3-*O*-(β-D-glucopyranosyluronic acid)-D-glucose by degradative studies (W3) and, more recently, by constitutional syntheses (J7, T1). The hexosaminidic linkage has been shown to have a β1→4 configuration by enzyme (W4) and methylation (H11) studies, so that hyaluronic acid may be assigned structure (I).



(I)

Although early chemical investigations were carried out on preparations from which protein had been carefully removed, the existence of a complex, composed of hyaluronic acid and protein, in ox synovial fluid was suggested by Ogston and Stanier (O1, O2) following viscometric measurements. The complex, which contained approximately 25% protein, was readily and irreversibly dissociated so that only the mildest procedures (for example, ultrafiltration) could be used in its isolation. The nature of the linkage between the carbohydrate and protein portions is obscure, but the protein, which appears to be derived entirely from the serum, may be linked through ionic bonds (C8). The original suggestion (O1, O2) that the protein made a significant contribution to the viscosity of hyaluronic acid in solution was later refuted (O5) and it is now certain that the protein contributes little to the physicochemical properties of hyaluronic acid in solution (B2). Hamerman and Sandson (H3) have recently isolated a hyaluronic acid–protein complex from human synovial fluid, using zone electrophoresis. Double gel diffusion in agar showed that the protein formed precipitin lines against anti-γ-globulin, while after injection of the complex into rabbits, antibodies to α_2-globulin were also detected.

Many physiological functions of hyaluronic acid as of a macromolecule appear to depend more on the physicochemical properties of the whole molecule rather than on the properties and types of linkage of the constituent units. These two features of the molecule cannot be considered as completely independent, since the nature and arrangement of the constituent units ultimately determine the properties of the polymer. The equally spaced carboxylic acid groups convey certain properties to hyaluronic acid in solution, and the exceptionally large dimensions of the molecule are attributable to the length of the folded chain and the mutual repulsion between ionized carboxylic acid groups. There now seems to be general agreement in assigning a random-coil configuration to the hyaluronic acid molecule in solution (B11).

The large size of the hyaluronic acid molecules and their random-coil configuration lead to molecular interactions, even in dilute solution. As a result of these interactions, solutions of the polymer exhibit non-Newtonian and elastoviscosity (B11). Synovial fluid shows an increase in viscosity with reduced shearing force and possesses structural rigidity which is reversibly broken down by shearing. Such viscosity behavior makes synovial fluid an ideal lubricant between joint surfaces, which move slowly under considerable pressure for most of the time but which may be required to accelerate violently (O3).

Another important consequence of the interpenetration of hyaluronic acid chains is that, at rest, their solution may be considered as a continuous network of large molecules (O4). Such a structure offers resistance to flow and, while the rate of diffusion of small molecules through the mesh is comparatively little affected, the diffusion of larger molecules, approaching in size that of the mesh, is impeded. Fessler (F1) considers that these effects might also be considered as a possible function of hyaluronic acid in connective tissues.

The large hydrodynamic volume of hyaluronic acid particles results in a high entropy of dilution, and consequently the osmotic pressure of these solutions will be much greater than that predicted on a molecular weight and concentration basis. The presence of the mucopolysaccharide in tissues will therefore result in retention of water and concomitant turgescence; a number of detailed investigations have appeared on this property (H17, H18). Ludwig and his associates (L14, L15) have demonstrated that the development of experimental exophthalmos in guinea pigs is dependent on the accumulation of hyaluronic acid and water in intraorbital tissue. A relationship also exists between the amount of hyaluronic acid present and the amount of water retained in skins of baboons and monkeys during estrus (R3).

Other possible physiological functions of hyaluronic acid may be associated with its macroanionic nature. Aldrich (A4), for example, observed that Ca^{++} was strongly associated with a hyaluronic acid–protein complex, and was not readily displaced by high concentrations of univalent cations. A possible role for hyaluronic acid in regulating the concentrations of Na^{+} and K^{+} in nerve fibers has also been suggested (A1).

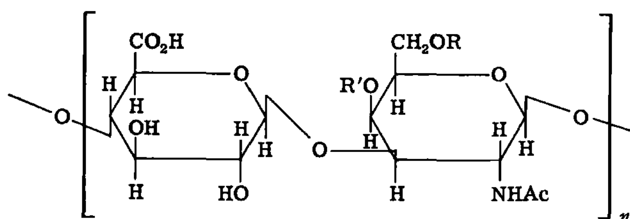
3.2. Chondroitin 4- and 6-Sulfates

Chondroitin 4- and 6-sulfates were originally designated as chondroitin sulfate A and C, respectively, and the close similarity in structure between them is demonstrated by the fact that both mucopolysaccharides yield the same disaccharide (chondrosine) on controlled, acidic hydrolysis together with sulfuric and acetic acids. Chondroitin 4-sulfate or chondroitin 6-sulfate, or both, occur in cartilage and adult bone, and the former mucopolysaccharide is a minor constituent of ligamentum nuchae and cornea (M17). Chondroitin 6-sulfate is a minor constituent of umbilical cord and occurs with dermatan sulfate (chondroitin sulfate B) and hyaluronic acid in heart valves and adult connective tissue (M17). An acid mucopolysaccharide isolated from human plasma resembles chondroitin 4-sulfate in its properties (S4).

Histochemical methods will not differentiate between the isomeric chondroitin sulfates, and identification with any degree of certainty requires isolation of the mucopolysaccharide. A method has been reported (M8) that differentiates mucopolysaccharides sulfated at C-6 from other sulfated mucopolysaccharides, and depends on application of the Morgan-Elson reaction to the oligosaccharides released by the action of testicular hyaluronidase. It is well known that substitution at C-6 does not interfere with the Morgan-Elson determination of *N*-acetylhexosamines, whereas the chromogens are not formed in significant amount if C-4 is substituted.

The structure of the disaccharide chondrosine, which is released in high yield on acid treatment of chondroitin 4- and 6-sulfates, has been established as 2-amino-2-deoxy-3-*O*-(β-D-glucopyranosyluronic acid)-D-galactose (D2, W8). Recently, a constitutional synthesis which confirms this structure has been reported (T1). The D-galactosamine moiety is *N*-acetylated in the parent mucopolysaccharides, and oxidation with periodate indicated (W7) that the sulfate group of chondroitin 4-sulfate is also located on this residue. The assignment of the sulfate group to C-4 and C-6 of the D-galactosamine residue in the respective polysac-

charides was originally based (M7, O6) on differences observed in the infrared spectra in the region 700–1000 cm^{-1}. Chondroitin 6-sulfate (chondroitin sulfate C) has unique absorption bands at 1000 cm^{-1}, 820 cm^{-1}, and 775 cm^{-1}, while with chondroitin 4-sulfate (chondroitin sulfate A), bands in this region occur at 928 cm^{-1}, 852 cm^{-1}, and 725 cm^{-1} (M7). These bands are absent from the spectra of hyaluronic acid and the chemically desulfated chondroitin sulfates, signifying that they are due to the sulfate group; this group is also characterized by a strong absorption band in the region 1230–1255 cm^{-1}. Investigations by Orr (O7) on a synthetic, polysulfated hyaluronic acid had established that the band appearing at ca. 820 cm^{-1} was due to the C-O-S vibration of an equatorially located sulfate group. With the knowledge (W7) that the sulfate ester grouping was probably located on the hexosamine moiety of both isomeric chondroitin sulfates, the mucopolysaccharide (chondroitin sulfate C) with an absorption band at 820 cm^{-1} was assigned structure (II) (sulfate group equatorial, here the C-5—C-6 bond is equatorial), and the other mucopolysaccharide (chondroitin sulfate A) structure (III) (sulfate group axial). The structure of chondroitin 4-sulfate has been confirmed recently by methylation of the mucopolysaccharide and its desulfated analog (J6). Methylation studies (J6) have also provided a chemical proof that C-4 of the D-glucuronic acid moiety is involved in the hexosaminidic linkage. This linkage is assumed to be the same in both mucopolysaccharides, since both are attacked at similar rates by animal or bacterial β-hexosaminidases (L4, M15). With bacterial enzymes the major products of hydrolysis are sulfated disaccharides possessing an unsaturated linkage between C-4 and C-5 of the hexuronic acid residue (L4, S27).



(II) Chondroitin 6-sulfate R = SO_3H R' = H
(III) Chondroitin 4-sulfate R = H R' = SO_3H

The chondroitin sulfate of animal cartilage is largely bound up as a complex with protein which is not derived from collagen (M2). The complex from bovine nasal septa contains 30% protein, 60% chondroitin

sulfate, and 10% moisture; treatment with alkali causes irreversible breakdown of the complex. This complex has been separated by centrifugation into two distinct fractions, each of which is composed of protein and polysaccharide (G3). The nature of the linkage between the protein and polysaccharide is uncertain, but several workers (B5, M26, W2) have suggested that the complex represents aggregates of chondroitin sulfate molecules linked in an end-to-end arrangement by peptide chains. Investigations by Partridge *et al.* (P1) have indicated that each chondroitin sulfate molecule is attached to the protein at one point only, which does not involve a terminal, reducing residue. Sialic acid is also present in a number of electrophoretically homogeneous complexes from cartilage (A6), and Anderson (A7) suggests that the protein moiety may contain a glycoprotein. Sialic acid does not appear to be involved in the linkage between protein and polysaccharide chains, since its removal by neuraminidase did not result in a decrease in viscosity (A7). The association of these polysaccharide-protein complexes with collagen may be partly responsible for the physical characteristics of cartilage.

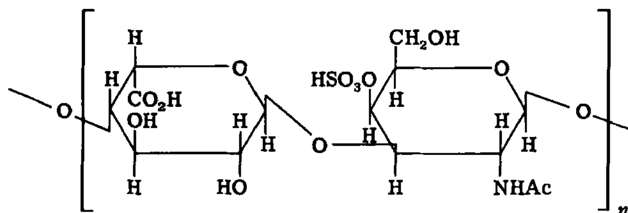
3.3. Dermatan Sulfate (β-Heparin, Chondroitin Sulfate B)

The name dermatan sulfate has been suggested recently (J5) for the sulfated mucopolysaccharide which was originally isolated from pig skin (M14) and named chondroitin sulfate B. The presence of dermatan sulfate has also been established in tendon, heart valves, aorta, sclera, and lung parenchyma (M17). This mucopolysaccharide constitutes 64% of the acid mucopolysaccharides present in adult pig skin compared with a figure of 5–12% for the embryonic skin (L8).

Dermatan sulfate may be distinguished from chondroitin 4- and 6-sulfates in that it is not degraded by testicular hyaluronidase and, furthermore, the desulfated mucopolysaccharide is unattacked by testicular and bacterial hyaluronidases (M17). Further differentiation of dermatan sulfate from hyaluronic acid and the foregoing chondroitin sulfates is readily made on the basis of color reactions given by the different uronic acid components. Dermatan sulfate shows equimolar ratios of uronic acid:hexosamine:sulfate when the uronic acid content is determined by the orcinol (K7) or decarboxylation (T4) methods, whereas significantly lower values are obtained by the carbazole method (D8).

Acidic hydrolysis of dermatan sulfate yielded D-galactosamine, acetic acid, sulfuric acid, and a uronic acid with chromatographic properties indistinguishable from those of L-iduronic acid (H12). The first crystalline derivatives of the uronic acid component of dermatan sulfate were obtained by Jeanloz and Stoffyn (J4, S19) and permit an unequivocal

assignment of the acid as L-iduronic acid. Methylation of the polysaccharide and its desulfated analog has revealed (J3) that the sulfate ester group is located at C-4 of the hexosamine residue. Identical disaccharides, possessing unsaturation at C-4 and C-5 of the uronic acid moieties, are released from dermatan sulfate and chondroitin 4-sulfate by a *Flavobacterium* enzyme (H13), signifying that these mucopolysaccharides are stereoisomers differing only at C-5 of the hexuronic acid residues. Dermatan sulfate can thus be assigned structure (IV), with a repeating unit of (1→4)-*O*-α-L-idopyranosyluronic acid-(1→3)-2-acetamido-2-deoxy-4-*O*-sulfo-β-D-galactopyranose.



(IV)

3.4. Chondroitin

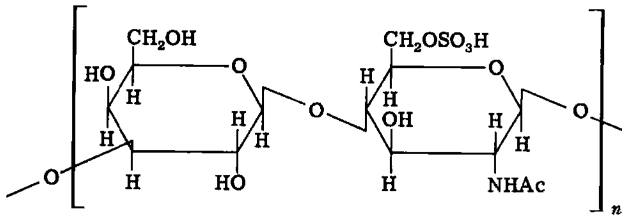
This mucopolysaccharide, possessing a structure similar to those of chondroitin 4- and 6-sulfates but with a small content of sulfate, was isolated from bovine cornea (M16). Chondroitin resembles hyaluronic acid in its rate of hydrolysis by testicular and bacterial hyaluronidases, but was differentiated from hyaluronic acid ($[\alpha]_D$ —65° to —78°) by its optical rotation ($[\alpha]_D$ —21°). Its structural similarity to chondroitin 4- and 6-sulfates was indicated by the fact that chondrosine was released in high yield on controlled, acidic hydrolysis (D3). The isolation of this mucopolysaccharide is of particular interest since it may be a precursor in the biosynthesis of chondroitin 4- and 6-sulfates.

3.5. Keratan Sulfate (Keratosulfate)

Keratan sulfate constitutes approximately half the total mucopolysaccharide fraction of bovine cornea, where it was isolated originally by Meyer *et al.* (M16). It occurs fairly widely in animal connective tissues, and its presence has been reported in nucleus pulposus (G2), aorta (B17), and costal cartilage (M20). Unlike the acid mucopolysaccharides described previously, keratan sulfate contains no uronic acid residue but is comprised of *N*-acetyl-D-glucosamine, D-galactose, and sulfate, in equimolar ratio. The small amount of L-fucose detected in acidic

hydrolyzates of keratan sulfate may be of significance, since a similarity in structure between the desulfated mucopolysaccharide and the blood group substances has been demonstrated on the basis of cross-reactions with blood group antisera (R10). Enzymes which degrade the blood group substances also hydrolyze keratan sulfate, whereas testicular and bacterial hyaluronidases, commercial emulsin, β-galactosidase, and crude liver extracts are without effect. Some workers (J8) are of the opinion that the chemical structure of keratan sulfate resembles more closely that of the glycoproteins isolated from plasma and secretions, with addition of sulfate groups, than that of the acid mucopolysaccharides of connective tissues.

The repeating unit of keratan sulfate has been identified as (1→3)-*O*-β-D-galactopyranosyl-(1 → 4)-2-acetamido-2-deoxy-6-*O*-sulfo-β-D-glucopyranose (V), by comparison of the methylated monosaccharides ob-



(V)

tained on acidic hydrolysis of the methylated polysaccharide and its desulfated methylated analog (H10). Infrared spectral data (J14) are in agreement with the allocation of the sulfate group at C-6 of the D-glucosamine residue, and oxidation of the polysaccharide with periodate (H10) confirms the over-all structure assigned.

The relative amounts of mucopolysaccharides in human rib cartilage at a range of ages from newborn to 74 years was investigated by Kaplan and Meyer (K1). The chondroitin sulfate content appeared to decrease linearly with age, while the keratan sulfate content, which was negligible in the very young, increased to approximately half of the mucopolysaccharide content, usually during 20–30 years, and was thereafter maintained at this value even in senescence. The increase with age in the ratio of D-glucosamine:D-galactosamine in the mucopolysaccharide pattern of human nucleus pulposus was likewise indicative of an increase in the keratan sulfate:chondroitin sulfate ratio (H1). In bovine nucleus pulposus and cornea, the ratio of unbound protein-conjugated keratan sulfate appeared to vary with age and the physiological state of the

tissues (L7). The ratio of chondroitin 6-sulfate to keratan sulfate in the costal cartilage of two cases of Marfan syndrome was 1:1, while in controls a value of 2.5:1 was obtained (M20).

3.6. Heparitin Sulfate (Heparin Monosulfate)

The dextrorotatory ($[\alpha]_D$ +38° to +70°) mucopolysaccharide heparitin sulfate was originally isolated (J12) from beef liver and lung extracts after removal of heparin. Although resembling heparin in general composition, the mucopolysaccharide, unlike heparin, contains acetyl residues and gives a positive response in the periodic acid-Schiff (PAS) reaction (J13). The name heparin monosulfate is sometimes used to describe the mucopolysaccharide, but heparitin sulfate (M18) is usually preferred since its biological relationship to heparin is uncertain. The different values of anticoagulant activity reported for heparitin sulfate are believed to result from contamination of the preparations with higher sulfated homologs (J8). The presence of heparitin sulfate has been demonstrated in human liver during amyloidosis (L3), in human uteri (L10), in bovine and human aorta (D15, K2), and in the oviducts of stilbestrol-treated hens (S5). In addition to these sources, abnormally high amounts of heparitin sulfate have been isolated from the tissues and urine of patients with Hurler's syndrome (see page 219).

Heparitin sulfate is composed of D-glucuronic acid, D-glucosamine, acetyl, and sulfate residues in approximately equimolar ratio (J12). The structure of heparitin sulfate still awaits final elucidation, but it may be distinguished from other sulfated mucopolysaccharides by means of its high positive rotation, electrophoretic behavior, and resistance to testicular, bacterial, and leech hyaluronidases. Final identification, however, usually requires its isolation and analysis.

The ability of heparitin sulfate to dialyze through cellophane membranes suggested that it had a lower molecular weight than other tissue mucopolysaccharides. Molecular weights of 1240–2075 have been recorded (B15) for highly disperse samples isolated from the urine of patients with Hurler's syndrome. Enzymatic studies, with a *Flavobacterium* enzyme adapted to either heparitin sulfate or heparin, indicated the presence of both *N*-acetylated and *N*-sulfated hexosamine residues in the mucopolysaccharide (L5). A tentative structure proposed (L5) for heparitin sulfate is that of a section comprising D-glucuronic acid and *O*-sulfated D-glucosamine-*N*-sulfate residues, to which is attached a relatively sulfate-free portion containing D-glucuronic acid and *N*-acetylglucosamine. Application (C5, C6) of Elson-Morgan and Morgan-Elson reactions to the oligosaccharides released by graded, acidic hydrolysis of

the mucopolysaccharide was indicative of the presence of both 1,4- and 1,6-glycosidic linkages; the possibility of branching in the molecule was also inferred from enzymatic hydrolysis (L5).

3.7. Heparin

Since its isolation from liver in 1916 by McLean (M9) and the recognition of its blood anticoagulant properties, heparin has undoubtedly been the most widely studied of the acid mucopolysaccharides. Heparin has been found in many tissues, for example, liver, lung, heart, kidney, thymus, blood, and spleen. Histochemical methods have demonstrated the presence of heparin in the mast cells, which are located in connective tissues. Molecular weights of 16,000–20,000 have been suggested (M23, W6) for heparin, but many of the physical methods employed are strongly influenced by the polyelectrolyte character of the molecule.

Despite numerous investigations, the chemical structure of heparin is still relatively obscure. The component monosaccharides have been identified as D-glucuronic acid (F3) and D-glucosamine (J11), the latter being *N*-sulfated in the parent mucopolysaccharide. Other sulfate groups present in the molecule are probably located as *O*-esters in the hexosamine moiety, but direct confirmation of this is still lacking. Extensive analysis of different preparations of crystalline barium heparinate shows various proportions of sulfate groups; up to three sulfate groups per disaccharide unit have been reported. This suggests that release of sulfate groups may accompany isolation of the polysaccharide if relatively vigorous methods are used. It is pertinent to record that many apparently conflicting results obtained by oxidation of heparin with periodate may be attributed, in part, to the degree of sulfation of the preparation examined.

In view of the rapid release of *N*-sulfate groups on acid treatment, subsequent hydrolysis of heparin is hindered by protonation of the free amino group with concomitant electrostatic shielding of the adjacent glycosidic bonds (S17). The relative ease of de-*N*-sulfation may be turned to some advantage since *N*-acetylation of the free amino groups affords a product which is more amenable to acidic hydrolysis. The isolation in recent years of disaccharides from preparations which have undergone the aforementioned treatment has contributed much to an understanding of the structure of heparin. One such disaccharide was characterized (D1) as 2-amino-2-deoxy-6-*O*-(D-glucopyranosyluronic acid)-D-glucose, while a second disaccharide, recovered from acidic hydrolyzates of a partially desulfated, acetylated, carboxyl-reduced heparin, was identified (W9) as 2-amino-2-deoxy-4-*O*-(α-D-glucopyrano-

syl)-α-D-glucose hydrochloride. The presence of α-linkages in heparin may also be inferred from its high positive rotation and on the basis of the rate of reaction of de-*N*-sulfated heparin with nitrous acid (F2). The present information is still too inconclusive to allow formulation of a definite chemical structure for heparin.

The metabolism, biosynthesis, and biological activity of heparin are less well understood than its chemical structure and it is appropriate to consider the biological activity at this point. Until relatively recently, the most familiar biological property of heparin was its activity as a blood anticoagulant. More recently its lipemia-clearing activity has attracted considerable attention in view of the growing interest in the role of lipid metabolism in diseases such as atherosclerosis and essential hyperlipemia, which are associated with accumulation of fat in the blood stream. Most of the biological properties of heparin can be attributed (C2, J2) to its ability either to combine with proteins or to displace other radical groups from proteins. The role of heparin in the blood has been the subject of numerous investigations and reviews (A8, B14, S8). The precise mechanism(s) of its action is uncertain but the general pattern is now fairly well established and seems to stem from the formation of protein-heparin complexes. Heparin is believed to inhibit the conversion of prothrombin to thrombin (H16) by thromboplastin (D12). *In vitro* experiments have demonstrated (a) that heparin combines with thromboplastin to form a dissociable protein-heparin complex (C3), and (b) that the formation of thromboplastin is prevented by heparin (D12). The role of heparin in the second stage of the coagulation process is also complicated. Although Howell (H16) could find no antithrombin activity with heparin, he observed that the thrombin-inactivating power of serum or plasma was greatly enhanced by the presence of heparin. It has been demonstrated that heparin requires a cofactor, present in the albumin fraction of plasma, to exert its antithrombin effect (J1). There is evidence (M5) which suggests that fibrinogen has a greater affinity for thrombin than for albumin, but that, on addition of heparin, thrombin reacts readily with the albumin-heparin complex leaving the fibrinogen unchanged. Although a small quantity of heparin is present in normal circulating blood (F4, H9), it is unlikely that the role of heparin as an anticoagulant will be understood until the experimental techniques for investigating this problem are refined.

The lipemia-clearing action of heparin has been studied for the most part in patients with alimentary lipemia, although other lipemic conditions have responded to treatment with heparin (K9). These observations, together with the facts that injection of heparin prevents the ap-

pearance of alimentary lipemia and that antiheparin agents reverse this effect (L1), suggest that native heparin may play an important role in the transport of fat. The clearing of visible fat in the blood by heparin *in vivo* is accompanied by changes in lipoprotein metabolism (E1). Injection of heparin produces a progressive shift of lipoproteins from the larger atherogenic classes (β-lipoproteins) to the smaller nonatherogenic classes (α-lipoproteins). The lipemia-clearing action of heparin does not appear to be associated specifically with any single property or chemical group in the molecule, and certain colloid materials, such as polymetaphosphate, dextran, glycogen, and hyaluronic acid, are known (H6, L2, S9) to cause the disappearance of blood lipids.

The observation that only substances of high molecular weight are active suggests a physical rather than a chemical action. Most antilipemic agents, including heparin, cause the appearance in blood of an antilipemic substance or clearing factor. Since heparin is a more effective antilipemic agent *in vivo* than *in vitro*, the release of some tissue factor may be involved. The mechanism of lipemia clearing by heparin *in vivo* falls naturally into two parts involving (a) the site of action of heparin and its role in the formation and/or liberation of a clearing factor, and (b) the mode of action of the clearing factor on blood lipids and lipoproteins. The observation (F4, H9) that injection of fat into the blood stream causes release of heparin from cell tissue indicates that heparin is intimately involved in the fat-clearing process and does not solely stimulate liberation of a clearing factor. It is apparent, however, that other factors must be involved. Studies with a chemically modified heparin, in which the anticoagulant properties but not the antilipemic properties are destroyed, may throw new light on the clearing action.

4. Biosynthesis of the Acid Mucopolysaccharides

The principle that tissue constituents are in a state of continuous synthesis and degradation is well established. The rate of turnover of the various constituents of connective tissues varies and, whereas the turnover of collagen is very slow, that of the ground substance is relatively rapid. Although the detailed mechanism of synthesis of the acid mucopolysaccharides is as yet unknown, in a number of instances the general pattern of synthesis has emerged.

With hyaluronic acid, much of the informative work on its biosynthesis has accrued from studies with bacteria. Group A hemolytic streptococci have long been known (K6) to produce hyaluronic acid identical with that present in mammalian connective tissues. The participation of uridine nucleotides in the biosynthesis of hyaluronic acid is now well

established, and D-glucose is a direct precursor of both the D-glucosamine and the D-glucuronic acid moieties (R8, R9). Experiments with disrupted and cell-free extracts of Group A *Streptococcus* have demonstrated incorporation of radioactivity from tritiated uridine diphospho-*N*-acetylglucosamine (UDP-G*N*Ac) and uridine diphosphoglucuronic acid (UDP-GA) into the hexosamine and hexuronic acid moiety, respectively, of hyaluronic acid (M4). A possible mechanism of synthesis of hyaluronic acid is shown in Fig. 1, although the exact mechanism of synthesis from

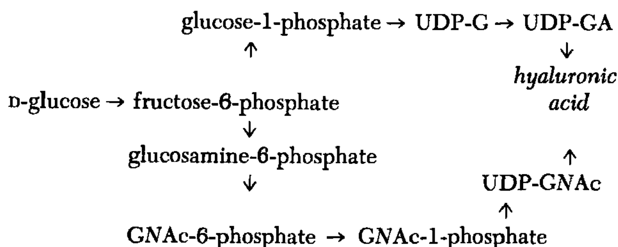
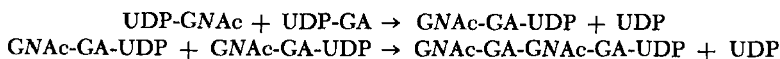


FIG. 1. Possible mechanism of biosynthesis of hyaluronic acid.

the nucleotide intermediates has not been ascertained (M4). One mechanism might involve a stepwise addition of monosaccharide units to the growing polysaccharide chain; a mechanism of this type could presumably operate by increasing preformed polysaccharide chains. Alternatively, the mechanism might involve interaction of two uridine nucleotides in the following manner:



The mechanism of synthesis of hyaluronic acid in mammalian tissues appears to resemble that present in bacteria. The synthesis of hyaluronic acid in tissue culture has been widely studied (G10, H7), and labeled hyaluronic acid is synthesized *in vitro* from uniformly labeled D-glucose by explants of human synovial tissue (Y1). An enzyme system has recently been found in mesodermal tissues which catalyzes the synthesis of hyaluronic acid directly from UDP-G*N*Ac and UDP-GA (A5).

With the sulfated polysaccharides, the mechanism of sulfation has aroused much discussion. The chief point at issue is whether sulfation takes place before or after formation of the polysaccharide chain. The biological sulfate carrier, formed enzymatically in chicken embryo cartilage or liver, has been characterized (R4) as 3′-phosphoadenosine-5′-phosphosulfate (PAPS). Following the isolation of chondroitin, it was suggested that this mucopolysaccharide might act as a sulfate acceptor

in the biosynthesis of the chondroitin sulfates. No incorporation of sulfate-S^{35} into chondroitin could be demonstrated with a particle-free enzyme system from chicken embryo condyles in the presence of $Na_2S^{35}O_4$, adenosine triphosphate, and magnesium chloride (A2, A3); subsequent work showed (A2) that chondroitin did not influence incorporation of D-glucose-C^{14} into chondroitin sulfate. Greiling and Bauditz (G8), on the other hand, reported the presence of an enzyme in rat liver extracts which transfers sulfate to chondroitin. In this connection, Whitehouse and Lash (W5) concluded from a study of the biogenesis of cartilage in tissue culture that cortisone, hydrocortisone, and their chemical analogs inhibited the formation of acid mucopolysaccharides in connective tissues by preventing sulfation of preformed polysaccharide chains.

An extensive investigation of the sulfation mechanism has been conducted (S24–S26) recently with a sulfotransferase derived from the isthmus of hen oviduct. This system catalyzed a 64% transfer of sulfate from $PAPS^{35}$ to added acceptors, including chondroitin, chondroitin 4- and 6-sulfates, dermatan sulfate, and various sulfated oligosaccharides (S24). At least two types of sulfate transfer were catalyzed by the enzyme preparation: (a) sulfation of an unsubstituted *N*-acetylglucosamine residue, and (b) sulfation of an *O*-sulfated *N*-acetylglucosamine residue (S25). The first of these mechanisms appeared to be more rapid. Although transfer of sulfate to chondroitin occurred, the observation that a number of model monosaccharides also underwent sulfation readily leaves the mechanism of biological sulfation uncertain. The recent isolation (S21) of uridine diphospho-*N*-acetylgalactosamine sulfate (UDP-*N*AcGal-S) from hen oviducts has again highlighted the mechanism of sulfation since this nucleotide may be a precursor of sulfated polysaccharides.

Following the demonstration (L13) that glutamine stimulated synthesis of hyaluronic acid by bacteria, it was found that glutamine also accelerated incorporation of sulfate-S^{35}, acetate-C^{14}, and D-glucose-C^{14} into chondroitin sulfate in cartilage (R5). Glutamine is believed to participate in the formation of D-glucosamine during the biosynthesis of these mucopolysaccharides. Support for this contention is derived from the fact that D-glucosamine stimulates incorporation of sulfate-S^{35} into cartilage to approximately the same maximal level as glutamine, whereas D-galactosamine functions in this capacity only at much higher concentrations (R6). The presence of an epimerase in cell-free extracts of *Bacillus subtilis* (G6) and rat liver extracts (G6, M3), which catalyzes formation of uridine diphospho-*N*-acetylgalactosamine from uridine diphospho-*N*-

acetylglucosamine, is therefore of particular significance. In accord with the foregoing evidence, it seems likely that an epimerase of this type is involved in the biosynthesis of the D-galactosamine moiety of chondroitin 4- and 6-sulfates.

With dermatan sulfate, biosynthesis of the L-iduronic acid residue poses another problem. Incorporation of D-glucose-6-C^{14} into rat skin indicated that L-iduronic acid is derived from D-glucose without scission of the carbon chain (R7). A sulfotransferase has been isolated from rabbit skin (D4) which is specific for sulfation of a chemically desulfated dermatan sulfate. However, the degree of sulfation achieved by this enzyme amounted to only one sulfate group per five or six disaccharide units.

The chondroitin sulfate–protein complex in rat costal cartilage is metabolized as a single unit (G9). Subcutaneous injection of a mixture of DL-lysine-C^{14} and $Na_2S^{35}O_4$ resulted in labeling of both mucopolysaccharide and protein moieties. Since no differences in the rates of turnover of these moieties were apparent, it seems that the entire complex is synthesized as a unit and then extruded into the matrix. The suggestion (G9) that chondroitin sulfate may be released from cartilage *in vivo* by the action of proteolytic enzymes is not without support (T3).

5. Mucopolysaccharides in Pathological Conditions

Connective tissues can be affected locally by inflammation, or the cells, stimulated to excessive growth, may form local benign or malignant neoplasms. Because of their localized character, neoplastic diseases are not included in the concept of connective tissues or collagen diseases, the term being restricted to diseases in which the tissue is implicated diffusely. Changes in the mucopolysaccharide pattern have been demonstrated in a number of diseases involving connective tissues and these will chiefly be considered in the ensuing pages. A number of syndromes in which there appears to be a heritable disorder of acid mucopolysaccharide metabolism (a so-called mucopolysaccharidosis) will also be discussed.

5.1. Rheumatoid Arthritis

The maintenance of the normal, anomalous viscosity index of synovial fluid is essential for its proper function as a lubricant between joint surfaces. The concentration and the degree of polymerization of hyaluronic acid in synovial fluids of patients with rheumatoid arthritis and other degenerative joint diseases have been the subjects of widespread investigation. Ragan and Meyer (R1) ascribed the changes in synovial fluid in

rheumatoid arthritis to an overproduction of incompletely polymerized hyaluronic acid. This suggestion lacked much substantial evidence since it was based on measurement of the viscosity of whole fluids. Sundblad (S22) also observed a large increase in hyaluronic acid production by synovial tissues under pathological conditions; these changes were most marked in rheumatoid and "infectious" arthritis. Compared with normal synovial fluid ($[\eta] = 39.3$), the average intrinsic viscosities ($[\eta]$) determined for pathological fluids were: traumatic arthritis $[\eta] = 36$–39; degenerative joint disease $[\eta] = 30$; rheumatoid arthritis $[\eta] = 26$; arthritis of infectious or unknown etiology $[\eta] = 24$. Both the ultracentrifugal and viscosity behavior was affected in rheumatoid fluids which had not lost the property of mucin clot formation (J10).

The lower degree of polymerization of hyaluronic acid present in rheumatoid fluids (B3, B10, B13, B18) undoubtedly impairs the effectiveness of the fluid as a lubricant between joints. Another factor contributing to this may be the lower concentration of hyaluronic acid found in certain rheumatoid effusions. Special viscosity and elastic properties are exhibited by hyaluronic acid in synovial fluid in concentrations in excess of 0.2 g/100 ml (B11); the concentration of hyaluronic acid in inflamed joint fluids of rheumatoid arthritis is generally less than this value. In a recent investigation, Hamerman and Sandson (H4) found that, apart from a reduced anomalous viscosity, the hyaluronate-protein complexes of synovial fluid differed from normal and showed increases in both protein content and amounts of basic amino acids.

The lower mean degree of polymerization of hyaluronate generally observed (B3, B10, B13, B18) in the synovial fluid of rheumatoid arthritis might arise either from depolymerization of the normal substance or from disturbance of its synthesis. The recent demonstration (H8) that a β-*N*-acetylglucosaminidase is present in abnormal amount in the synovial membrane in rheumatoid arthritis and allied joint diseases, suggests that depolymerization of hyaluronic acid is brought about enzymatically. The work of Pigman and his associates (P4) has demonstrated that irreversible depolymerization of hyaluronic acid in solution is also accomplished by a number of reducing agents (L-cysteine, L-ascorbic acid, thiols, metal ions, and hydroquinones) in the presence of molecular oxygen. This reaction is referred to as an ORD reaction (oxidative-reductive depolymerization) and resembles a mild hyaluronidase action. The common occurrence of reducing agents in tissues and cells suggests that the ORD reaction may have biological significance. It is pertinent to record that Chazov (C4) observed an accumulation of nonspecific substances, such as L-ascorbic acid and thiolactic acid, in the serum of

rheumatic patients. Synovial fluids from patients with rheumatoid arthritis were also found to contain increased concentrations of copper, iron, and aluminum ions (N3).

Local treatment of rheumatoid arthritis with hydrocortisone usually results in restoration to normal of the qualitative hyaluronic acid changes occurring in joint fluids (J9, S23). The increase in viscosity and hyaluronate concentration of synovial fluid after treatment with hydrocortisone is believed to result either from the influence of the hormone on synthesis of hyaluronic acid, or, indirectly from its anti-inflammatory action (J9, S23). The molecular sizes of hyaluronate in rheumatoid fluids usually show a continuous but skewed distribution; treatment with hydrocortisone was found to reduce the less polymerized fractions, which were marked in severe cases (N1).

5.2. Arteriosclerosis and Atherosclerosis

The role of heparin in fat-clearing processes has already been discussed (see page 211). Other data indicate (B16, G4, G7) that in the morphogenesis of both human and experimental atherosclerosis, the deposition of lipid substances in the aorta is preceded by an increase of metachromatically stainable mucoid substances. The total concentration of acid mucopolysaccharides in aorta is high compared to most other tissues, and histochemical observations suggest that they are located principally in the intima. Buddecke (B17) has reported that chondroitin 4-sulfate and dermatan sulfate together constitute half of the acid mucopolysaccharides normally present in aortic tissue, together with hyaluronic acid (one seventh), chondroitin (one seventh), keratan sulfate, and a heparin fraction. A somewhat different distribution of acidic mucopolysaccharides in aorta is claimed by Kaplan and Meyer (K2), who found that heparitin sulfate is present in greatest abundance. There appears (K2) to be no significant changes with age of the total mucopolysaccharide concentration in aorta, although an increase in the chondroitin sulfate:hyaluronic acid ratio has been reported (B6).

The anticoagulant activity of the aortic mucopolysaccharides and the associated ability to stimulate the production of lipid-clearing activity are considered (G7) to be of significance in the development and progression of atherosclerosis. Immunoelectrophoresis has been employed to demonstrate (G4) that one part of the lipids in sclerotic aortic intima is identical with plasma β-lipoprotein. The deposition of plasma β-lipoproteins may be associated with the ability of some specific mucopolysaccharide of sclerotic aortic intima to bind β-lipoprotein.

In arteriosclerotic processes, the mucopolysaccharide spectrum changes; Buddecke (B17) has demonstrated that the amounts of sulfated mucopolysaccharides are increased by 50%, whereas the amounts of hyaluronic acid and chondroitin fall to 40% and 15% of their original values, respectively. The increase of sulfated mucopolysaccharides in arteriosclerotic aortae is related to the increased deposition of calcium.

5.3. Pretibial Myxedema

One of the manifestations of generalized or localized pretibial myxedema is a pronounced edema of the corium, with accumulation of mucinous material in the edema fluid. Both hyaluronic acid and a sulfated mucopolysaccharide fraction were isolated in increased quantity from the affected area of the skin of a patient with localized pretibial myxedema (W1). Following this observation, the relationship between the functional status of the thyroid gland and the mucopolysaccharides of skin has been discussed in numerous publications. Analysis of the acid mucopolysaccharides of human skin has revealed the presence of dermatan sulfate, hyaluronic acid, and a small amount of chondroitin 4- or 6-sulfate (L9).

Alterations in the metabolism of acid mucopolysaccharides in skins of hypothyroid animals have been studied by determining the extent of sulfate-S^{35} incorporation by skin. Kowalewski (K10) demonstrated an increased uptake of sulfate-S^{35} in hypothyroid rats whereas, with thyroidectomized rats and guinea pigs, uptake of sulfate-S^{35} was less than in normal animals (D16, M25). Gabrilove and Ludwig (G1), on the basis of histochemical tests, attributed the myxedematous process to a deficiency of thyroid hormone(s). Support for this contention was obtained recently by Schiller *et al.* (S6), who demonstrated a differential effect on the metabolism of hyaluronic acid and sulfated mucopolysaccharides (principally dermatan sulfate and chondroitin 4-sulfate) in the skins of rats when synthesis of thyroid hormones is blocked with propylthiouracil. An increase in concentration of hyaluronic acid with a decrease in concentration of sulfated mucopolysaccharides (cf. reference K10) was found in hypothyroid animals; these changes were restored to normal on administration of thyroxine. The slow rate of disappearance of labeled carbon from the hyaluronic acid fraction in the hypothyroid rat suggested that, although the rate of synthesis is normal, the rate of disappearance is decreased, resulting in accumulation of hyaluronic acid in the skin. On the other hand, the rate of synthesis of the sulfated mucopolysaccharide fraction was apparently decreased.

5.4. Hurler's Syndrome (Gargoylism)

Gargoylism is a syndrome which comprises a large number of abnormalities. In particular, the skeleton is severely affected with dwarfism, a large and deformed skull, a shortened neck, and characteristic abnormalities of the thoracic and lumbar vertebrae. The underlying pathological process in gargoylism appears to be an inherited disorder of the metabolism of acid mucopolysaccharides. Histochemical studies of the skins of patients with gargoylism have shown (H2) the participation of the epidermal and the dermal cells, and support the view that a generalized disturbance of mucopolysaccharide metabolism is involved.

The urine of patients with gargoylism, of both the autosomal recessive and sex-linked types, contains abnormally high amounts of acid mucopolysaccharides. Determination (T2) of the acid mucopolysaccharides in urine in relationship to preformed creatinine is useful in distinguishing certain cases of gargoylism from other conditions with which they are sometimes clinically confused (e.g., cretinism and chondro-osteodystrophies of the Morquio-Brailsford type). Another simple diagnostic test for gargoylism is based on the presence in the lymphocytes of abnormal inclusions of acid mucopolysaccharides which are revealed by staining (M24).

Numerous investigations (B9, B15, C1, D10, L6, M19, M21, T2) have revealed the presence of large amounts of dermatan sulfate and heparitin sulfate in the urine and the tissues in cases of gargoylism. In addition, small amounts of chondroitin 4- and/or 6-sulfate are present in the urine of these cases (L6). The nature of the acid mucopolysaccharides normally present in urine is by no means settled, but chondroitin 4- or 6-sulfate, or both, are usually found in largest amount (D5), while recent studies (K8, L6) have shown the presence also of heparitin sulfate and dermatan sulfate. The knowledge that dermatan sulfate and heparitin sulfate are normal constituents of certain connective tissues led Meyer and his associates (G11, M19, M21) to suggest that the hereditary defect in gargoylism lies in faulty differentiation of the fibroblasts, resulting in abnormally high production of these mucopolysaccharides, which are partly stored in the tissues and partly excreted. The finding (L6) that chondroitin 4- or 6-sulfate, dermatan sulfate, and heparatin sulfate are present in "normal" urine, as well as in the urine of cases of gargoylism, has prompted the suggestion that the disturbance is of a quantitative rather than of a qualitative nature. No correlation has been found between the supposed genotype (autosomal recessive and sex-linked types) and the type of mucopolysaccharide excreted, nor, has any relationship

been observed between the amounts of acid mucopolysaccharides excreted and the severity of the disease (M19). Kaplan and Meyer (K3), in seeking the possible genetic defect in gargoylism, demonstrated that long-term injection of dermatan sulfate in dogs was accompanied by the appearance in the urine of an acid mucopolysaccharide resembling heparitin sulfate. Although unequivocal identification of this glucosamine-containing mucopolysaccharide was not accomplished, this observation suggests that the basic defect in gargoylism might involve a single mucopolysaccharide.

Although the therapy of gargoylism is not well advanced, the discovery (L11) of an apparently related heritable disorder of acid mucopolysaccharide metabolism in cattle may facilitate study of the pathogenesis and the therapy of this condition.

5.5. Marfan's Syndrome

The considerably increased (20–40-fold) excretion of acid mucopolysaccharides suggests that Marfan's syndrome also represents a defect in the metabolism of connective tissues (B4). The major mucopolysaccharides excreted were indistinguishable in their electrophoretic mobilities from hyaluronic acid and chondroitin 4- or 6-sulfate. Examination of the levels of mucoproteins and acid mucopolysaccharides in the serum of cases of Marfan's syndrome revealed an increase in the latter, while the former is decreased. Bacchus (B1) considers that this pattern might be of clinical and pathogenetic significance.

5.6. Morquio-Ullrich's Disease and Morquio's Disease

The metabolic disorder in Morquio-Ullrich's disease differs from that in Hurler's syndrome since substantial quantities of keratan sulfate have been identified (P2) in the urine of patients with this disease. The identification of the mucopolysaccharide was based on chemical, chromatographic, and infrared spectral data. In view of this rigorous characterization, it seems likely that the glucosamine-containing mucopolysaccharide reported by other workers (M6) in the urine of cases of this disease is also keratan sulfate.

A definite difference in the urinary excretion of acid mucopolysaccharides was also observed (S2) in Morquio's disease. Although the nature of the mucopolysaccharide excreted in Morquio's disease awaits definite characterization, it does not appear to be keratan sulfate.

5.7. Hereditary Deforming Chondrodysplasia (Diaphysial Aclasis)

The amounts of acid mucopolysaccharides excreted in the urine of two families with this disease were comparable to those excreted in

Hurler's syndrome and suggested (L12) that this condition represents another heritable disorder of connective tissue and mucopolysaccharide metabolism. Although identification of the excreted mucopolysaccharide(s) was not made with certainty, preliminary chemical analyses indicated its similarity to chondroitin 4- or 6-sulfate.

5.8. Eye Diseases

The nature of the acid mucopolysaccharides in bovine cornea and sclera was investigated by Polatnick *et al.* (P5). Although these tissues occupy adjoining positions in the developed eye, their gross properties are different. Bovine cornea was found to contain 52% keratan sulfate, 22% chondroitin 4-sulfate, and 20% chondroitin, in agreement with values reported by other workers (M17). Keratan sulfate, the major mucopolysaccharide of cornea, was absent from sclera which contained dermatan sulfate, in greatest amount, together with hyaluronic acid and chondroitin 4- and 6-sulfates. Autoradiographic techniques have demonstrated (D9) that half of the sulfated mucopolysaccharides of sclera are replaced every 12 days whereas, in cornea, a similar replacement occurs in 32 days. Smelser and Ozanics (S11) have attributed the over-all slow metabolism of sulfated mucopolysaccharides in cornea to an unusually low rate of metabolism of keratan sulfate.

Acidic mucopolysaccharides play an important role in keeping the cornea transparent, and one of their functions is to maintain an orderly arrangement of the fibrils of the corneal stroma. Swelling of the ground substance alters the spatial arrangement of the fibrils with resultant clouding of the cornea. Clouding of the cornea usually occurs in diseases such as Hurler's syndrome (see page 219) in which there is a generalized disturbance of mucopolysaccharide metabolism. Harris (H5) has found that acid mucopolysaccharides can alter markedly the hydration properties of cornea. Several studies (D9, E2) have been conducted on the healing of corneal grafts. Incorporation of sulfate-S^{35} into corneal keratan sulfate indicated that the mucopolysaccharides of corneal grafts have a faster turnover than normal. Vascularizing corneas also incorporated sulfate at a rate exceeding normal during the active process (S11). Sulfated compounds synthesized in a vascular cornea have a shorter life than similar material in the normal avascular structure.

An acid mucopolysaccharide, which was degraded by hyaluronidase, was present in abnormal amount in spaces of the trabecular meshwork in eyes containing melanomas, probably due to an alteration of the mucopolysaccharides in the vitreous (D13). In contrast to normal conjunctivitis, smears from eyes with vernal conjunctivitis are stained with

toluidine blue (N2); this has been attributed to the presence of hyaluronic acid.

5.9. Other Diseases

The concentration of acid mucopolysaccharides in serum, and their excretion in urine, are increased in patients with rheumatoid arthritis (D7), lupus erythematosus (D6), diabetes (C7), and leukemia (R2, S10) and other malignant diseases (R2). The daily urinary excretion of acid mucopolysaccharides was within the normal range in cases of acute hepatitis, but was usually increased in chronic hepatitis and in florid cirrhosis (K5). A decrease in the amount of acid mucopolysaccharides excreted was found in primary hepatoma, whereas in most cases of obstructive jaundice the amount was markedly increased (K5).

Numerous reports, which rely chiefly on histochemical tests, have indicated that in pathological states of skin, especially in inflammation and malignancies, there is an increase in the level of acid mucopolysaccharides. High mucopolysaccharide contents of biopsy skin sections were demonstrated in chronic lupus (M10) and in some cases of venous edema (Z1); in scleroderma, however, the mucopolysaccharide content of the affected skin was low (M10). Skin lesions from patients with lupus erythematosus, dermatomyositis, lichen sclerosus et atrophicus, and poikiloderma showed significant increases in material which was stained histochemically (S20). In two cases of urticaria pigmentosa, hyaluronic acid was found in the histologically abundant mast cells (I1); injection of hyaluronidase into the involved skin areas apparently cured the patients. An increased concentration of acid mucopolysaccharides has been observed in sun-damaged skin (G5, S1, S12, S18).

The possibility that insulin regulates utilization of D-glucose for the synthesis of mucopolysaccharides in the ground substance of connective tissues was explored by Schiller and Dorfman (S3). Incorporation of sodium acetate-C^{14} into hyaluronic acid and dermatan sulfate in the skins of alloxan-diabetic rats was found to be approximately one third of that in the skins of either normal or partially fasted animals. These data suggest that synthesis of connective tissue mucopolysaccharides is inhibited in insulin-deficient animals.

Biopsies of gastrocnemius muscles in cases of poliomyelitis revealed that mucopolysaccharides were present in increased amount in the thickened connective tissue between atrophied muscle fibers (B12). Even though the vascular system of atrophic muscles was relatively unaffected, these sclerotic changes probably impair the nutrition of muscle fibers.

Numerous recent publications (D14, H15, S14, S15) testify to the great

interest in mucopolysaccharides (particularly the chondroitin sulfates), which are considered of importance as necessary components of both normal and pathological ossification processes. Kasavina and Zenkevich (K4) have studied the mucopolysaccharides of bone fragments and bone callus in fractures in rabbits at various stages of regeneration. In bone callus, the acid mucopolysaccharide concentration reached a maximum on the 7th day after fracture, and by the 55th day, when the process of formation of bone tissue was essentially complete, the level approached that typical of mature bone tissue. These workers (K4) consider that the morphological stages of regeneration of bone tissue in rabbits correlate with the mucopolysaccharide content.

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PROTEINS, MUCOSUBSTANCES, AND BIOLOGICALLY ACTIVE COMPONENTS OF GASTRIC SECRETION[1]

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[1] The investigative work upon which this paper is based has been supported over a period of years by research grants-in-aid: AM-00068, AM-04391, and AM-03778 from the National Institute of Arthritis and Metabolic Disease, P.H.S. and C-05601 from the National Cancer Institute, N.I.H., P.H.S., and grant #P-305F from the American Cancer Society which is gratefully acknowledged.

[1a] The author acknowledges the helpful cooperation of those investigators and publishers who gave their kind permission to reproduce their figures.

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Introduction

For many years, the facility of the hydrochloric acid assay in gastric juice and the unavailability of good quantitative techniques for fractionation of proteins and mucosubstances have directed the interest of researchers toward the study of hydrochloric acid in the stomach. The only other material in gastric juice, studied and quantitated for many years and representative of the nondialyzable gastric secretory products, was pepsin. Study of other large molecular components has been hampered by the complexity of gastric juice, technical difficulties encountered in its fractionation, and lack of adequate quantitative methods for the assay of these materials.

Gastric secretion represents a very complex mixture of electrolytes, water, carbohydrates, proteins, peptides, and amino acids, which are partly in solution and partly in more or less stable suspension. The large molecular materials of gastric secretion include: enzymes, mucosubstances, serum proteins, peptides and products of proteolytic degradation of gastric proteins and mucoproteins, and blood group substances,

as well as various biologically active materials (gastric intrinsic factor, vitamin B_{12} binders, inhibitors of gastric secretion, antigens and antibodies, and other little-explored metabolically active substances).

The complex nature of gastric secretion is further due to: (1) its continually changing composition, which varies with the stimuli applied to its secretion and the particular phase of the stimulation, (2) derivation of gastric materials from many cellular sources, which include not only the gastric glands and surface epithelium, but the blood serum as well, (3) enzymatic degradation of proteins and mucous substances in the gastric lumen, (4) formation of aggregates or complexes between polysaccharides, peptides, and proteins in the native juice, (5) contamination of gastric secretory products by extraneous materials such as food, saliva, blood, and duodenal content.

The interest in large molecular components of gastric secretion is growing, especially because of their relation to several important biologically active materials of the human body.

This review deals only with the large molecular materials from the stomach of man in health and disease. Gastric materials from other species will be discussed only to the extent necessary to corroborate some inferences, to replace missing data on the human stomach, or to approach some results from the point of view of comparative physiology. Low molecular materials contained in gastric juice, i.e., hydrochloric acid and other electrolytes, will obviously be omitted from the discussion.

Each class of macromolecule present in the gastric secretion will be discussed here, separately, with emphasis on selected sections of special interest to the reviewer.

1. Enzymes

The activity of the proteolytic enzymes in gastric juice is of great biological significance. New concepts and findings have been recently developed regarding their nature and mutual relation. Other enzymes have played a far less important role in gastric function, or their role in the stomach has not as yet been adequately understood.

1.1. Proteolytic Enzymes

1.1.1. *Pepsin and Pepsinogen*

The name "pepsin" was coined by Schwann 125 years ago (S11). About 35 years ago, Northrop (N9, N10) obtained a crystalline material from the bovine gastric mucosa exhibiting proteolytic activity below pH 2, which represented highly purified pepsin.

The chemistry and activity kinetics of proteolytic enzymes have been

studied principally on bovine and hog sources. Only recently, the work of Taylor (T14–T24), Tang *et al.* (T2–T6), Seijffers *et al.* (S17–S19b) and Hirsch-Marie, Rapp *et al.* (H20a, H20b, R1) has contributed important physiological and chemical information as to the nature and activity of human pepsin and pepsinogen. Van Vunakis and Levine (V3b) and Arnon and Perlman (A10, P5) have shown that human, bovine, and hog pepsins are chemically not identical. Immunological differences between these materials point to the same conclusion (V3b). It is incorrect, therefore, to directly apply the chemical, physicochemical, and physiological data from hog and bovine pepsinogens and pepsins to human proteases.

The work of Herriott *et al.* (H16–H18) has demonstrated that bovine and hog pepsinogen contain 14% nitrogen and 0.84% phosphorus. It is resistant to inactivation and still reversibly denatured at pH 9. It is not destroyed by boiling in a salt-free solution, but is partly denatured at 70°C and irreversibly denatured by raising the pH above 12. This distinguishes pepsinogen from pepsin, which is practically destroyed at 70°C or at pH above 8.0 and certainly at pH 9.0. The isoelectric point of pepsinogen is at pH 3.7 and its molecular weight is 42,500 (H21). Following activation to pepsin, it splits off several peptides, of which one, the so-called pepsin inhibitor, has a molecular weight of about 1500–3100 (H16, H17). At pH above 5.4, it is combined with the pepsinogen molecule. However, as soon as pepsin is formed, the inhibitor undergoes gradual destruction, which proceeds at the highest rate at pH 3.5–4.0. During activation of pepsinogen, other peptides are also split off, as shown by Van Vunakis and Herriot (V3, V3a), which have different molecular weights ranging from 1000 to 3000 and different charges.

The possible relationship between formation of intrinsic factor and the formation and secretion of pepsinogen and other proteolytic proenzymes or enzymes (G20) is discussed in Section 6.1. As a result of splitting off peptides, the nitrogen content of pepsin is less than that of pepsinogen, by about 15%. Furthermore, the molecular weight of pepsinogen, after activation to pepsin, decreases from 42,500 to 34,500, its specific optical rotation changes from —61° to —71° (P5) and its isoelectric point from 3.7 to below 2.0 (H16–H18, V3–V3b). The amino acid composition of pepsinogen and pepsin is also different: while pepsinogen has 18 basic amino acid residues (11 lysines, 3 histidines, and 4 arginines), pepsin has only 4 (one lysine, one histidine, and two arginines) (P5). Thus, a large part of the basic amino acids is lost by pepsinogen during the activation into pepsin. Whereas pepsinogen forms needles on crystallization, pepsin forms hexagonal pyramids. The molecular weight of pepsin is

34,500, but its apparent molecular volume is 80,000, suggesting that the pepsin molecule is either hydrated or nonspherical. This is in line with the concept that both pepsin and pepsinogen form straight peptide chains. Transformation of pepsinogen into pepsin occurs spontaneously in solution below pH 6.0 by autocatalytic reaction (H16, H17). This transformation is slow at a relatively high pH, but almost instantaneous at pH of 2 or below. In strong acid solution (pH 2 or below) there is a gradual loss of pepsin activity, which is faster the lower the pH.

Hunt (H54) reported the possible existence of a synergistic enzyme, which potentiated activity of crystalline hog pepsin at pH 2. He considered the potentiating effect of dilution on gastric juice peptic activity to be due not to dilution of the inhibitor, as assumed by Bucher *et al.* (B41, B42), but to the additional effect of the synergist.

The chemical methods for assay of pepsinogen and pepsin will not be described here. The companion review deals only with the physicochemical methods of separating the large molecular components of the stomach (G21). It should only be mentioned that proteolytic activity may be determined by using various proteins as substrates (hemoglobin [A9, A9a], serum albumin [H53], ovalbumin [Mett], gelatin), and measuring the changes in (a) viscosity, (b) optical rotation, (c) clot formation, (d) loss of solid substance, (e) turbidity, and (f) appearance of peptides (see H21, O3). The latter is by far the most exact method and is best measured by change in optical density at 280 mμ (N12) or by the Folin-Ciocalteu phenol reaction (A9, B41, G51). Other valuable methods are based on breaking of protein, peptide, or amide bonds and measuring the degree of proteolysis by formol titration (N11, N12) or colorimetry using nihydrin reagent.

The effect of various pharmacological stimuli on pepsin secretion will not be discussed here; the reader is referred to the review by Hirschowitz (H21). This applies to the effects on pepsin and pepsinogen of cholinergic and anticholinergic drugs, alcohol, histamine, vagal stimulation (G52), gastrin (B21), caffeine extracts, secretin, Diamox, other secretory inhibitors, and various hormones (adrenal cortex [G63, H23], medulla, thyroid and parathyroid) not to be discussed here. Nor will this review discuss the very extensive literature on the abnormalities of pepsin secretion in various diseases of the gastrointestinal tract (see H12a). The reader is referred to extensive reviews by Northrop *et al.* (N12), Hirschowitz (H21), and Taylor (T24).

It is well known that the proteolytic activity at pH 2 (peptic activity) is low or absent in pernicious anemia, though occasional patients secrete small quantities of pepsin in the stomach (G21). Neither histamine stimu-

lation nor intravenous injection of insulin causes here an appreciable rise in pepsin activity. In hypochromic iron-deficiency anemia (T24), in atrophic gastritis (G57, S33), and after subtotal gastrectomy, pepsin concentration and output are frequently decreased and even abolished. In a large number of sprue patients, peptic activity is also reduced or absent. However, in peptic ulcer and especially duodenal ulcer, pepsin output in the gastric juice is at a high level (H12a). Although the concentration of pepsin in gastric juice in this disease does not differ from that in young normal controls, the volume of secretion is increased, which results in a higher output of pepsin. This is similar to the situation observed by Gray and his associates (G63) after stimulation of gastric secretion by adrenocorticotropin (ACTH) and corticosteroids. Following administration of steroids, output and concentration of pepsin in the basal secretion were about double in the work of Hitzelberger from our laboratory (H22, H23).

1.1.2. *Cathepsin and Gastricsin*

In 1940, Freudenberg and Buchs demonstrated that human gastric juice exhibits two maxima at pH 2 and 4 (F8, F9). They considered it

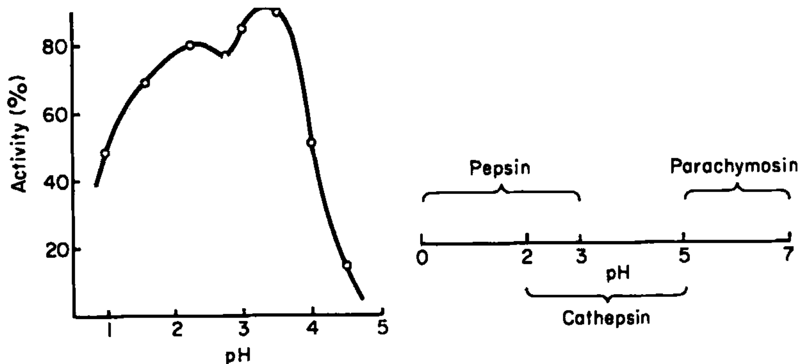

FIG. 1. pH Activity curve of a human gastric juice and structure of gastric protease. From Buchs (B48).

possible that the second maximum might represent an enzyme related to the intracellular "cathepsin." These observations (Fig. 1) were confirmed by Buchs (B45–B48), who found not only two different activity peaks for the gastric proteolytic enzymes, but also reported differences in responsiveness to activating and inhibitory agents and to temperature. "Gastric cathepsin" was noted to be more resistant to heat.

Merten and Ratzer (M32), using the hemoglobin digestion technique,

also found two maxima of proteolytic activity in human gastric juice; that at pH 1.5 was referred to as pepsin, and that at pH 3.0 as cathepsin. However, Christensen noted that the more denatured a substrate is and the more expanded its peptide chain, the more readily it will be hydrolyzed at higher pH's (C17).

For some time, attempts were made to separate peptic from catheptic activity. Buchs tried unsuccessfully to separate them by charcoal adsorption and other precipitation methods (B45–B47).

Taylor was unable to separate catheptic from peptic activity, either by ammonium sulfate and sodium chloride precipitation, or by ion-exchange chromatography, free boundary electrophoresis (Fig. 2), or differential ultracentrifugation (T24, T26).

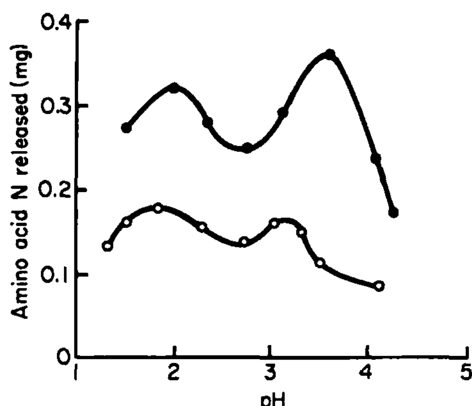

FIG. 2. pH Activity curves of two anodal-moving components separated from concentrated human gastric juice in the Tiselius apparatus. •, Major, faster component, mobility 2.99×10^{-6} cm²/volt/sec; o, minor, slower component. Electrophoresis was carried out in 33 m*M* acetate buffer, pH 2.5. Protein concentration, 0.51 g/100 ml. Human serum albumin as substrate; temp. 37°; time 3 hr. From Taylor (T15).

Salt fractionation yielded two different proteolytic enzymes from gastric juice and from extracts of gastric mucosa. Using salt fractionation, Philpot and Small (see T24) found that pepsin could be split into fractions of two different solubilities. This was corroborated by Herriott and and his associates (H18). The more soluble fraction acted near pH 4, the other near pH 2.0.

Richmond, Tang, Caputto, and Trucco, in Wolf's laboratory, using column chromatography, reported the successful separation of the two proteolytic enzymes from human gastric juice (R5, R6, T2–T6). Of these, one had optimum proteolytic activity at pH 2 (pepsin), while the other

showed activity at pH 1.0–3.2 with a plateau at pH 1.0–2.0 and gradual (though slight) increase of activity up to its peak at pH 3.2. They gave the name "gastricsin" to this second protease, optimally active at pH 3.2 (R6), which was preferred over the old term "gastric cathepsin" (B45–B47, F8, F9) (Fig. 3).

These materials also differed in electrophoretic mobility at pH 5.0 in acetate buffer. The first peak (pepsin) in buffer of pH 5.0 moved 9 cm

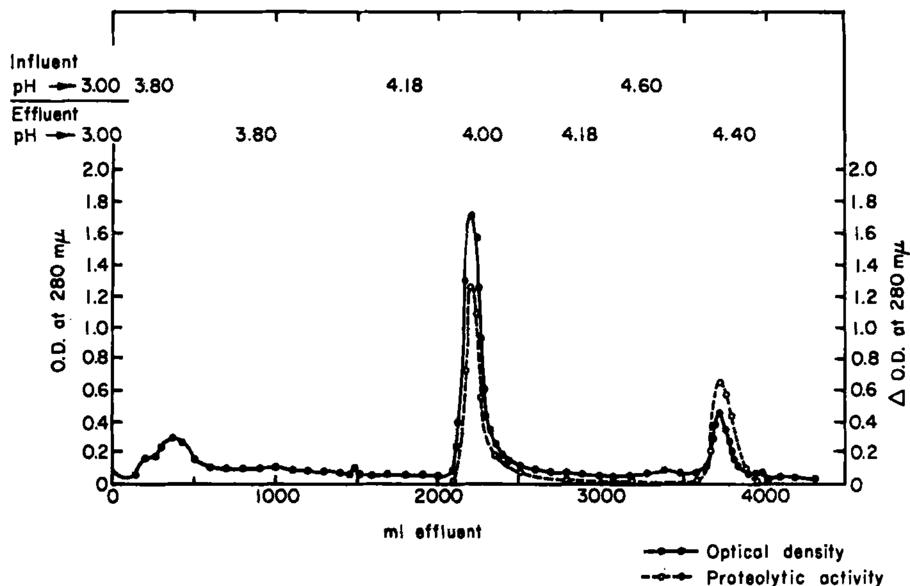

Fig. 3. Separation of the two proteolytic enzymes in the gastric content. From R. Caputto *et al., Gastroenterology* 37: 439-444 (1959).

toward the anode from the application point. Peptic activity could be eluted from this band in acetate of pH 5. Gastricsin did not show significant migration from the origin on paper (crystalline hog pepsin migrated 9.7 cm).

On starch gel electrophoresis, gastricsin migrated 6.5 cm toward the anode after 22 hours as one single narrow band in acetate buffer of pH 5.0. These two materials also differed in heat sensitivity: while pepsin at pH 2.0 and 65° C lost 69% of its activity, gastricsin under these conditions lost 44.8%. Conversely, at pH 3.2 pepsin was inactivated only 11.2%, while gastricsin was inactivated 22.3% (Fig. 4). Both enzymes hydrolyzed synthetic carbobenzoxy-glutamyl-L-tyrosine, which is a specific substrate for pepsin. Gastricsin was crystallized as it came from

the column by repeated ammonium sulfate precipitation at pH 5.0 and cooling. This material crystallized in the form of needle-shaped crystals of high specific activity and was homogeneous on ultracentrifugation, where it showed a single symmetrical sedimentation boundary. Cysteine slightly activated gastricsin, but ascorbic acid did not (T6). Thus, crystalline gastricsin apparently differed from both human and hog pepsin in having (1) a higher pH optimum, (2) slower electrophoretic migration

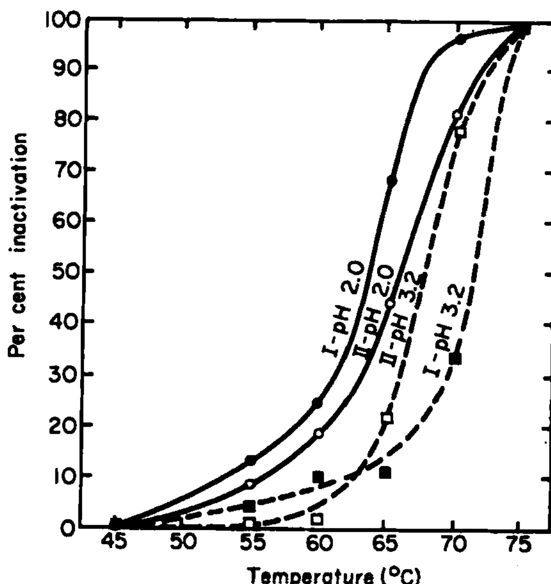

FIG. 4. Heat inactivation of human pepsin (I) and gastricsin (II) at pH 2.0 and 3.2. The enzyme solutions were incubated for 10 minutes at the indicated temperature and the proteolytic activity measured. The losses of activity were expressed as percent of the inactivation relative to that of the solution incubated at 45°. From Tang *et al.* (T6).

on paper and starch gel, and (3) less sensitivity to heat inactivation. The less-soluble material of Philpot and Small (see T24) is probably identical with the slower-migrating protease, which is eluted on Amberlite IRC-50 columns as a gastricsin at pH 4.4.

Taylor (T24) repeated some of Tang's experiments. Materials eluted from an Amberlite IRC-50 column at pH 4.4, by the latter's method and corresponding to his gastricsin, exerted proteolytic activity with two maxima: one near pH 2 and the other near pH 3.3.

Fisher also repeated Tang's work, using human fundic mucosal extracts (cit. T24). He found two and sometimes three proteinases,

but each digested proteins with two maxima near pH 2.0 and 3.5. This suggests a similarity of gastricsin to the parapepsin 2 from hog stomach of Ryle and Porter (R13, R14), which also digests proteins with two pH maxima, although it is homogeneous by several criteria. Thus, the identification of gastricsin as a separate protease, as claimed by Tang and Wolf (T4), has met the objection of other authors whose materials, though homogeneous, showed several pH optima for proteolytic activity (C17,

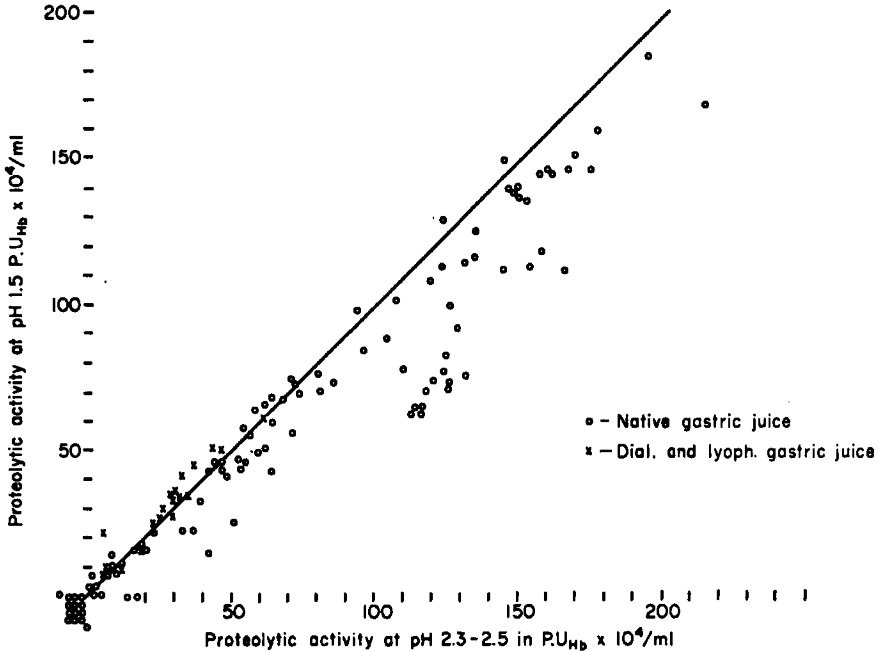

FIG. 5. Proteolytic activity of gastric juices at pH 1.5 and pH 2.3–2.5. From Glass *et al.*, unpublished data.

C25, H10, M23, T24, T30). On the other hand, crystalline pepsin was heterogeneous on electrophoresis (H24).

Miller *et al.* (M40) studied proteolytic activity at pH 1.5 and 3.5 in 93 samples of gastric juice collected from 30 patients. They found that gastric juice activity at pH 3.5 was usually less than at pH 1.5. However, the ratio of activity varied from one individual to another, and also in the same gastric juice during a single collection following injection of histamine. They concluded that gastric pepsin (active at pH 1.5) and gastric cathepsin (active at pH 3.5) are two distinct enzymes secreted at different rates. According to Tang and Wolf, the amount of gastricsin

in normal human gastric juice is about one half to one third that of human pepsin (T4).

In collaborative work (not yet published) from our laboratory, with Ishimori and Rich, we studied 436 samples of gastric juice from 280 individuals. The assays for proteolytic activity were performed at several pH's simultaneously, ranging from 1.5 to 3.5, by a modification of the Anson-Mirsky hemoglobin method (G51). A comparison of the values obtained at pH 1.5 and 2.3 is shown in Fig. 5. Differences of the mean activity at these pH's were highly significant (P below 0.05) in the native gastric juices. In spite of statistically significant differences in the proteolytic activity assayed at the two pH's 1.5 and 2.3, these two proteolytic activities formed parallel regression curves and the coefficient of correlation between these proteolytic activities was 0.92–0.96 in native and lyophilized gastric juice. This suggests that pH 1.5 and 2.3 most probably represent two activity areas of the same enzyme.

We also compared the proteolytic activity at pH 1.5 and at 3.5 in 87 native gastric juices of which 25 were anacid. The difference of the means between these two activities was also highly significant statistically but the coefficient of correlation was only 0.76. Furthermore, the regression curves of the two were not strictly parallel. At low levels of proteolytic activity, that at pH 3.5 was most often higher than that at pH 1.5. Conversely, at high levels of proteolytic activity, 43 of 48 samples showed higher activity at pH 1.5 than at 3.5. Individual differences between various gastric juices in regard to activity at pH 1.5 and at 3.5 were very great. In only about 60% of gastric juices was the absence of proteolytic activity at pH 1.5 associated with absence of activity at pH 3.5. In the remaining 40%, however, there were obvious discrepancies: in one half, some proteolytic activity was detected at pH 3.5, while at 1.5 it was not detectable; in the other half, proteolytic activity was detected at pH 1.5 and no activity at 3.5. This speaks in favor of two separate enzymes rather than of two activity maxima (Fig. 6) at pH 1.5 and 3.5.

Extending their work, Tang and Tang studied the zymogen of pepsin and gastricsin in human gastric mucosa (T3). They obtained a fraction from the alkaline extract of human gastric mucosa by stepwise precipitation with ammonium sulfate of 25–80% saturation. The precipitate was dissolved in buffer of pH 7.5 and dialyzed and chromatographed on a DEAE-cellulose column. After the breakthrough peak and two subsequent small peaks appeared, a large peak emerged at 0.07 molar concentration of phosphate buffer, which contained proteolytic activity. It overlapped with another minor peak, which also exhibited activity. The material in these peaks was homogeneous on ultracentrifugation and

starch gel electrophoresis, where it migrated anodically 5.8 cm from the origin. When it was activated at pH 2.6, it yielded proteolytic activity maxima at both pH 1.8 and 3.0. Activation of this proenzyme with 0.5 *N* HCl (pH below 1.0) for 40 minutes yielded pepsin and gastricsin in a concentration ratio of 2.30:1. However, activation of the proenzyme for

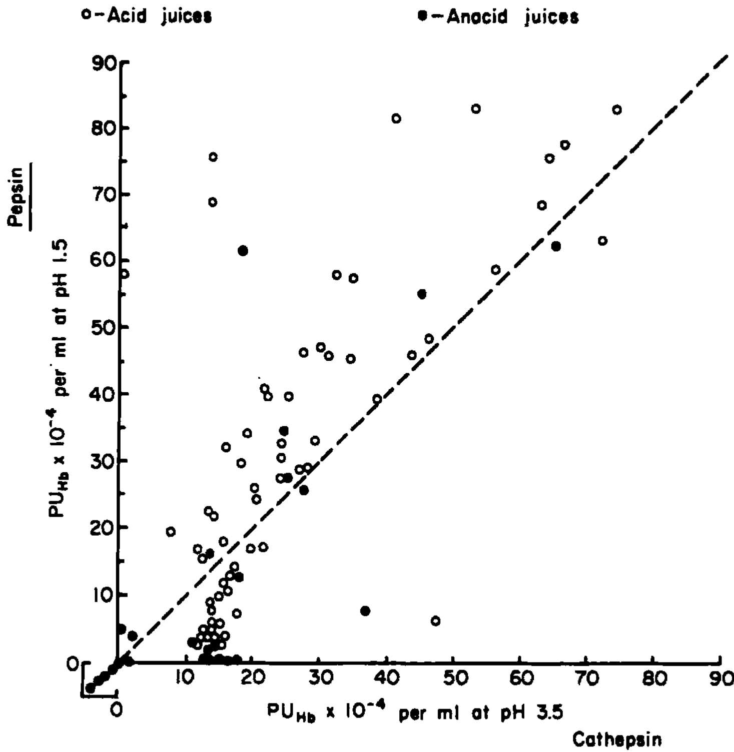

FIG. 6. Pepsin and cathepsin in 87 native gastric juices from 43 individuals, normals and with gastric pathology. From Glass (G16).

the same length of time at pH 2 and 3.5 yielded these two enzymes in a concentration ratio of 1.33–1.36:1. The two enzymes were identified as pepsin and gastricsin by activity curves, starch gel electrophoresis, and chromatography. The authors did not completely rule out the alternative that two separate zymogens for the two enzymes may be present in human gastric mucosa. They tended to conclude, however, that this zymogen in man is possibly a common precursor of both pepsin and gastricsin (T3). This concept substantiates Buchs' (B45) general idea of the uniformity of gastric protease which, depending on external con-

ditions or possibly on local acidity (B46), appears as pepsin, cathepsin, or parachymosin, the latter being active at the pH range 5–7. The inability of various authors (see T24) to separate these enzymes is Buchs' evidence for this concept.

The activation of one zymogen to two proteolytic enzymes, covering a vast range of activity at a wide pH range, may be of important physio-

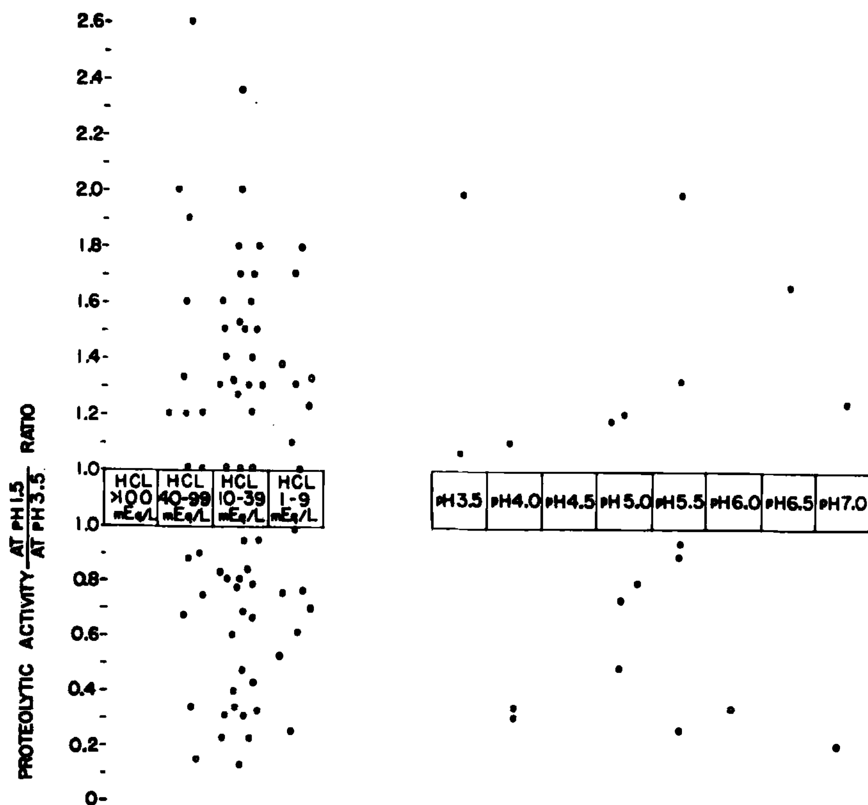

FIG. 7. Ratio of proteolytic activity at pH 1.5 to that at pH 3.5 in 94 native gastric juices. From Glass *et al.*, unpublished data.

logical significance. In this way, the stomach could maintain a high proteolytic activity over a wide pH range by forming either more pepsin or more gastricsin, depending on the actual needs of the pH prevalent in the gastric lumen.

In order to add more information to this subject (in our unpublished work with Ishimori), the ratios of the proteolytic activity at pH 3.5 and 1.5 of gastric juices from 94 individuals were correlated with acidity

and pH's of the same gastric juices in which activities were determined (Fig. 7). However, no correlation whatsoever was found between these ratios and pH's of the gastric juice. At pH 1.5–2.0 the ratio of these two proteolytic enzymes ranged from 0.2 to 2.0. The same was true for gastric juice at pH 1.0 as well as for pH's above 3.0 where a very similar range of ratios was found. We also studied the relationship of the ratio of proteolytic activity at pH 1.5 to that at 3.5 during histamine stimulation in the same gastric juices to their actual pH's. Again, no correlation was obtained between this ratio and the pH changes in the gastric juice. Thus, no support was obtained for the concept of Buchs (B45–B49) and Tang and Tang (T3) that the ratio of pepsin and gastricsin formed depended on gastric juice pH.

The recent fractionation work of Seijffers *et al.* (S17–S19b) on DEAE-cellulose and that of Grabar and Burtin's laboratory by Rapp *et al.* (R1), Kushner *et al.* (K32), and Hirsch-Marie *et al.* (H20b) utilizing agar gel electrophoresis and immunoelectrophoresis, resulted in differentiation of 3 to 4 proteases in human gastric juice, each having its own proenzyme. Detailed discussion of this work is presented in the companion review (G21) of the author, in this volume.

1.1.3. *Fundic and Pyloric Pepsin*

The basic controversy now revolves around the problem of the existence of a separate pepsin active at pH 1.5–2.0 and a separate gastric cathepsin (gastricsin) active at pH 3.2–3.5. The alternative concept is that the activity at pH 1.5–2.0 and at 3.2–3.5 belongs to a similar protease (pepsin), with the difference that one is derived from the fundic glands (fundic pepsin) and the other from the pyloric glands and perhaps chief neck cells (pyloric pepsin) (T22, T24).

The fundic and pyloric pepsin concept dates back to the past (H9, K19, K29) and early part of this century, when it was advocated by Glässner, Ivy (I6), Linderstrøm-Lang and Holter (L11), Lim and Dott (L10), and Meulengracht (M33a). Ivy and Oyama (I7) considered pepsin in pyloric juice as a contaminant or technical error; however the concept of two pepsins was upheld by Taylor (T16, T22, T24) and Grossman and Marks (G72). It appears from Taylor's work (T14, T16) that the proteolytic activity of pyloric pepsin has two maxima: one at 1.6–2.0, and the other at 2.6–3.2. On the other hand, the proteolytic activity of pepsin from the fundic glands has two maxima at somewhat higher pH, at pH 1.8–2.4 and at 3.4–3.9 (Fig. 8). Accordingly, in patients with pernicious and hypochromic anemia associated with atrophic lesions in the fundus and body of the stomach, in whom the pyloric glands

are preserved, the pH maxima of the proteolytic activity would be at lower pH values than in normal stomach.

Taylor studied the activity curves of all the proteinases at different pH's in various diseases of the human stomach (T18–T20). He found that in pernicious anemia, cancer of the stomach, and duodenal ulcer the activity curves of proteolytic enzymes differed from those of normals. "Only one patient exhibited a curve with two normal maxima, and the

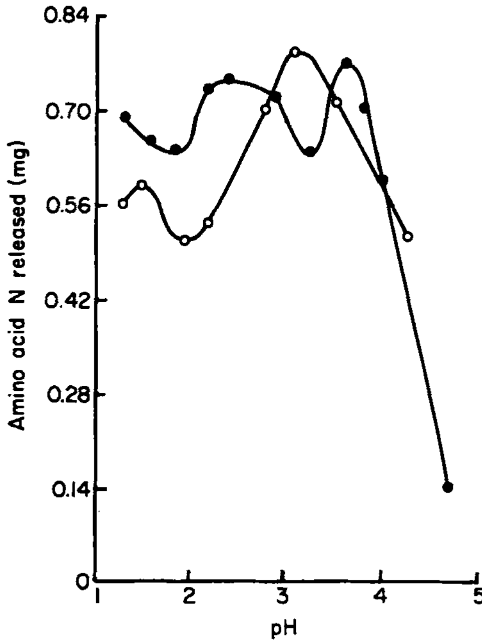

FIG. 8. pH Activity curves for the digestion of plasma protein by normal human fundic (•) and pyloric (o) mucosal extracts. Temp. 37°; time 3 hr. From Taylor (T15).

actual activity was low. Three secreted inactive juices, and four secreted juices with moderate activity which gave the two pH maxima that are characteristic of pyloric mucosal extracts. Thirteen secreted juices exhibiting low activity with only single maxima: in five of them the single maximum occurred above pH 4 and thus at a point where normal gastric proteinase activity should have declined rapidly. This might indicate the secretion of some abnormal enzymes in this disease" (T24). Also, extracts of the gastric cancer tissue showed an abnormal activity curve with only one maximum. This may corroborate the concept that, in gastric cancer, some abnormal proteolytic enzymes form as compared

to those from the normal mucous membrane. Abnormal activity curves were also found in hypochromic iron-deficiency anemia with gastric atrophy. This has been interpreted by Taylor as being a replacement of normal fundic glands by pseudopyloric glands, due to intestinalization of gastric mucosa and proliferation of the neck chief cells, which are morphologically similar to the pyloric glands. A decreased number of fundic glands in pernicious anemia would result in decreased amount of pepsin of fundal origin in gastric juice. Also, the majority of cases of idiopathic steatorrhea (non-tropical sprue) exhibited proteolytic activity curves of a pyloric type (T24). In duodenal ulcer, Taylor found activity curves with three instead of two maxima, due either to higher proportion of pyloric than fundic pepsin, or to the presence of some abnormal pepsin (T24).

Extracts of the uninvaded gastric mucosa from patients with gastric adenocarcinomata show two maxima, at pH 1.6–2.4 and 3.3–3.8, thus resembling normal gastric juice and mucosal extracts (T16). Extracts of the adenocarcinomata from the same patients differed in that only one pH maximum occurred, at pH 3.2–3.4. With egg albumin as substrate, carcinomatous extracts failed entirely to digest this substrate. Extracts of metastatic deposits behaved like the primary growth in failing to digest egg albumin and in digesting other proteins, with a single maximum at pH 3.2–3.4 (T21). The proteinase activity was shown to arise from the carcinomatous cells themselves. Thus, these results could be explained if gastric adenocarcinomatous cells synthesized either a proteinase quite different from those of normal gastric mucosa, or a proteinase in which the center or centers responsible for maximal activity below pH 3 were missing (T21).

Buchs (B49) reported that extracts from the pyloric antrum and upper duodenum of man show proteolytic activity with two pH optima between pH 1 and 4, of which the second peak at a higher pH is larger than that at a lower one. He considered this, however, as evidence for the presence of pepsin and cathepsin in pyloroduodenal secretion.

1.1.4. *Proteinase Active at pH 7.0*

A third proteinase appears to exist in the gastric juice, which exerts proteolytic activity at a neutral pH of about 7.0, i.e., in the range where pepsin and cathepsin no longer are active. The existence of a protease called parachymosin, active at pH 5.0–7.0, has been described in the past (Fig. 1) (see B46). Buchs (B46) considered it to be an integral part of the gastric protease, the two other components of which were pepsin and cathepsin. In the early thirties, Castle's group found some paral-

lelism between the absence of this proteolytic enzyme from gastric juice and lack of intrinsic factor in patients with pernicious anemia (T7, T8). There were some initial speculations that intrinsic factor might be related to this proteolytic enzyme active at pH 7.4 (T7, T8). It became evident later, however, that this proteolytic enzyme was not identical with intrinsic factor (B27, G3a).

Taylor detected some proteolytic activity at a neutral pH (T17) in pernicious anemia and hypochromic iron-deficiency anemia. He concluded (T22, T24) that there are three proteinases in man: (1) fundic pepsin, which is derived from the chief peptic cells of the fundic glands, (2) pyloric pepsin, which is formed in the pyloric glands and probably also in the chief cells of the neck of the fundic glands, (3) proteinase, acting at a neutral pH, of rather weak activity, which is formed only in traces in normal individuals but is found in appreciable amounts under some pathological conditions. In the pig, there are also three pepsins: (1) pepsin, which corresponds to human pepsin of fundic origin, (2) parapepsin II, which is similar in action to pyloric gland proteinase of man, (3) parapepsin I, which is probably identical with rennin, the milk-clotting enzyme of calves. According to Taylor (T24), perhaps all these enzymes, including hog stomach parapepsins and cathepsin, are derived from a single primitive intestinal proteinase, which becomes differentiated during the embryonic stage of development. This concept is supported by the presence of two active centers in the pepsin molecule. There is indication that, in some gastric diseases, abnormal proteinases may be found which show abnormal curves of proteolytic activity.

1.1.5. *Rennin*

Rennin is absent from the human stomach, but is present in that of newborn calves (see D2). Since pepsin may perform rennin's function, i.e., milk coagulation, rennin is superfluous in the stomach of man.

1.1.6. *Gelatinase*

Gelatinase is a separate proteolytic enzyme, since it liquefies gelatin about 400 times faster than pepsin does. It is probably derived from chief cells and has been thoroughly studied by Northrop (N10a).

1.1.7. *Peptidases*

Peptidases of the human stomach were studied by Taylor (T23). Normal gastric mucosal extracts from the fundic part of some human stomachs hydrolyze di- and tripeptides, namely, alanylglycine at pH 6.8 and glycylglycylglycine at pH 7.2. Pyloric mucosal extracts also hydrolyze

alanylglycine and, with some difficulty, glycylglycylglycine. These enzymes are not present in the normal human gastric juice, but are contained inside the mucous membrane (T23).

The fundic mucosal extracts from peptic ulcer patients hydrolyze both these peptides more readily than do extracts from normal mucous membrane, although the activity curves vary from case to case (T23). Extracts from pyloric mucosa in peptic ulcer patients also have more peptidase activity than those of normals, especially in regard to glycylglycylglycine substrate. The pH of the maximal activity is higher here than in normals. Gastric juice of ulcer patients does not contain peptidase activity.

In patients with carcinoma of the stomach, the fundic mucosa not involved in the cancer digests these two peptides more readily than extracts from the normal stomach (T21). This peptidase shows activity with two maxima: one at pH 7.0–7.6, and another at pH 8.2–8.5. Similar to extracts from pyloric mucosa, extracts from mucosa of gastric cancer patients attack alanylglycine more readily than glycylglycylglycine (T23).

1.1.8. *Effect of Proteolysis on Large Molecular Materials in Gastric Juice*

It is well known that proteolysis *in vitro* results in degradation of various large molecular materials of gastric secretion. This effect *in vitro* has been demonstrated in regard to pepsin itself (N12), serum albumin (G16, G76, H45, K4), some mucoproteins (H52), mucus (H49), gastrone (K31, S29), intrinsic factor (C8, C11, C13), and vitamin B_{12} binders (G14, G62, U2). The same effect is exerted by proteolytic enzymes within the gastric lumen upon serum albumin (G16, G76, H45), intrinsic factor, and vitamin B_{12} binders (G14, G62, U2). Since the proteolytic degradation is very rapid, and develops after 5–10 minutes at 37°C at a proper pH (C11, G16, G62), it is obvious that these effects cannot be prevented completely by accelerating the gastric collection and blocking further degradation of the gastric juice outside the stomach by immediately cooling and neutralizing it (G62).

Attempts have therefore been made for intragastric neutralization of gastric secretion by placing various buffers in the stomach and keeping their *in situ* content at an adequate level during the entire gastric collection. This procedure, first suggested by Gullberg and Olhagen (G76) and consisting of intragastric instillation of phosphate buffer at pH 7.2–7.4, was then modified by Piper *et al.* (P12), who introduced 5–10% bicarbonate, by Hirsch-Marie and Burtin (H20), who instilled borate

buffer and Cornet *et al.* (C24a) who introduced TRIS or THAM buffers, with satisfactory results.

The *in situ* neutralization of gastric juice certainly prevents intragastric proteolysis and permits studying the macromolecular components of gastric secretion without demonstrable peptic degradation, as they are secreted into the gastric lumen. However, the basic shortcomings of this method are as follows:

(1) It is unphysiological. The intragastric proteolysis is the normal process to which all the macromolecular materials in the stomach are subjected and following which they perform their normal various biological functions, such as lubrication, digestion of food, reabsorption in the intestine, effect on lipid metabolism, binding of vitamin B_{12}, intrinsic factor activity, etc. Thus, the biological activity of materials collected from the stomach, under conditions of intragastric neutralization, does not reflect their real activity in the body.

(2) It prevents the study of physiological mechanisms of gastric secretion of macromolecular materials, since the dilution of gastric secretion and its neutralization by buffers markedly modify gastric secretory activity and motor function, and render it impossible to quantitate gastric components.

(3) Use of highly alkaline solution, such as bicarbonates, causes excessive liquefaction of mucus, due to depolymerization of mucosubstances and to potentiation of the activity of mucolysin at this pH. This would result, unavoidably, in changing the composition of gastric mucosubstances.

(4) The alkaline buffers in the stomach may extract some preformed materials from gastric mucosa, such as pepsinogen, which normally would not enter the gastric lumen.

Thus, the technique of intragastric neutralization has some merit for special research problems, but cannot be considered a solution for overcoming the complexity of problems faced in studying macromolecular components of gastric secretion.

In order to counteract the continuation of proteolysis of gastric materials after their collection, Gräsbeck (G62, G62a) advocated immediate and irreversible inactivation of all proteases. This he achieved by raising the pH of gastric juice to 10.0 for 10 minutes and adjusting it back to pH 7.0, at which pH the gastric juice may then be stored under refrigeration for a length of time without further degradation. Some depolymerization of the mucosubstances by this procedure seems to be unavoidable, however, despite its merits.

The effect of proteolytic degradation on the mucosubstances of the

gastric juice was studied by Ohara and Ishimori in our laboratory (G16), using paper electrophoresis. As a result of proteolysis, the peptide moiety in some of the mucosubstances decreased, and some additional amido black-stainable bands appeared close to the application point. In patients with gastric hypersecretion and duodenal ulcer, the peak of the carbohydrate moiety of mucosubstances was often found more centrally on paper electrophoresis than in patients with anacidity where it was localized more anodically. It was suggested (unpublished work from our laboratory done in association with Ishimori) that this may be due to removal of the sialic acid from the carbohydrate moiety of gastric mucosubstances at low pH, resulting in their less negative electrical charges. Similar findings were reported from our laboratory in regard to gastric juices of patients treated with corticosteroids (H23).

The effect of peptic digestion is also very manifest on vitamin B_{12} binders. This is discussed in Section 6.2, where the formation of the peptic degradation products of the intrinsic factor-related and nonrelated binders is outlined.

The effect of peptic degradation on intrinsic factor has been long known, ever since the outset of the studies on intrinsic factor (C5–C10). Gastric juice containing acid and pepsin gradually deteriorates as a source of intrinsic factor (G20). This deterioration, however, is not complete. Some intrinsic factor activity in normal human gastric juice kept under refrigeration at an acid pH could still be demonstrated several months later on patients with pernicious anemia (see G20).

Castro-Curel, in our laboratory, using an *in vitro* assay of intrinsic factor activity on guinea pig intestinal mucosal homogenates, demonstrated almost complete loss of intrinsic factor, however, by acid human gastric juices if stored for over 2 weeks, even in a frozen state (C12, C13).

Another striking demonstration of the effect of peptic digestion on intrinsic factor activity has been provided by Castro-Curel in regard to ammonium sulfate precipitates from human gastric mucosa (C11). As a result of the incubation at pH 1.5, of this material, containing pepsinogen, the intrinsic factor activity decreased 60–80% within 1 hour. The exposure of the material to proteolytic activity at pH 3.5 for the same length of time resulted in the deterioration of the intrinsic factor activity to 30 or 40%, while incubation at pH 7 for the same length of time did not affect the intrinsic factor activity of the material at all.

The effects of gastric proteolysis on serum albumin *in vivo* and *in vitro* are discussed in detail in Section 3. These effects are characterized by the appearance of albumin digestion products, peptides, in the electro-

phoretic partition of gastric juice. They are seen *in vivo* in gastric juice of patients with duodenal ulcer and gastric hypersecretion (G16, G42, G58), as well as in patients treated with corticosteroids (G16, H23). The effects of proteolysis on gastrone activity are discussed in Section 7.

1.2. NONPROTEOLYTIC ENZYMES

1.2.1. *Lysozyme*

The role of lysozyme in the gastric secretion evoked interest when one group of investigators reported abnormally high concentration of this

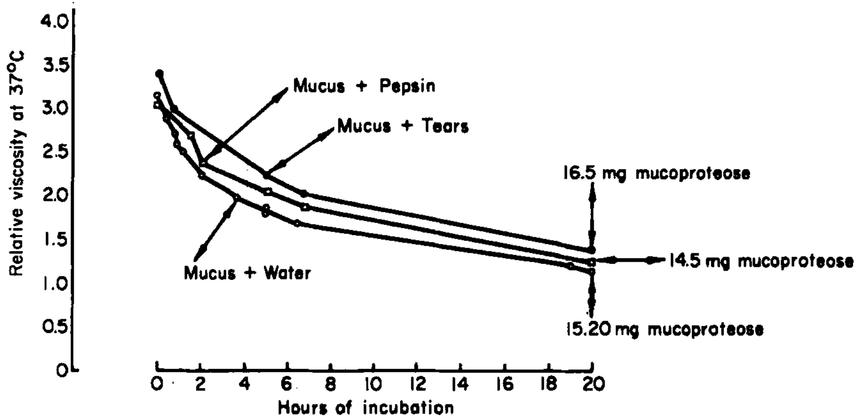

FIG. 9. Treatment of gastric and colonic mucus with lysozyme. Effect of lysozyme in human tears on viscosity of gastric mucus. To three samples of this preparation, 2 ml each, were added, respectively: 0.5 ml distilled water, 0.5 ml solution containing 5 mg crystallized pepsin, and 0.5 ml 50% solution of human tears in water. Incubation at pH 6.5 and 37° C. The mucoproteose content was determined after 24 hours of incubation. From Glass *et al.* (G50).

enzyme in gastric juice of subjects with peptic ulcer (M36). Since lysozyme is known to dissolve mucus of a microorganism, *Micrococcus lysodeicticus*, it was speculated that this may hold true for the gastric mucosa and that peptic ulcer might be due to the excessive lysozyme concentration in gastric secretion, resulting in increased mucolysis (M36). This concept was also extended to the formation of ulcerations in the colon, since a high lysozyme titer was observed in feces of patients with ulcerative colitis.

In collaboration with Pugh, Grace and Wolf, we were unable to demonstrate a mucolytic action of lysozyme *in vitro* on human gastric mucus or its fractions (G50). We treated with lysozyme human gastric mucus obtained directly from the exposed mucosa of a subject with

gastric fistula, or neutral gastric secretions, collected from patients with gastric anacidity. We used viscosimetry and chemical quantitation of the mucin degradation product (mucoproteose) to measure the effect of lysozyme upon gastric mucosubstances. However, gastric mucus was not measurably liquefied or digested by lysozyme from egg white or human tears. This suggested that lysozyme had nothing to do with the splitting of gastric mucus (Fig. 9). Therefore, it could not attack the protective

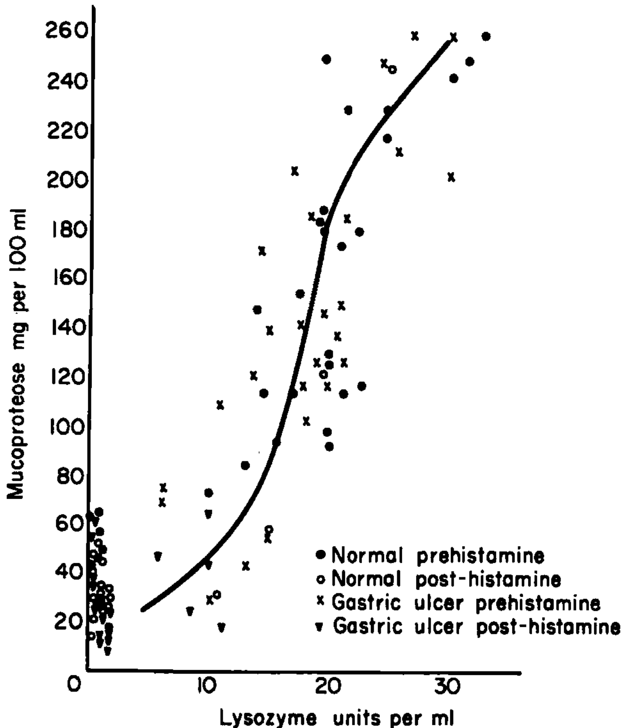

FIG. 10. Relationship of lysozyme to mucoproteose. From Gray *et al.* (G64).

mucous coating of the stomach, nor contribute to the formation of gastric ulceration by virtue of its dissolving or splitting action on the gastric mucus (G50). The purpose of its presence in the gastrointestinal lumen is probably related to microbiological aspects of its activity. The lysozyme concentration and output in gastric juice fall (R3) after histamine stimulation and parallel the concentration of surface epithelial mucosubstance (mucoproteose) (G64) (Fig. 10). Furthermore, gastric juice of 7 patients with pernicious anemia contained normal concentrations of lysozyme. Gray *et al.* (G64) concluded, therefore, that surface epithelial

cells are the source of gastric lysozyme. Wada (W1) found increased amounts of lysozyme in gastric juice of patients with gastric atrophy and gastric cancer, which fits this concept.

1.2.2. *Urease*

This enzyme splits urea and forms ammonia. It was found by Luck and Seth (L14, L15) in gastric mucosa of dogs. Glick (G59) demonstrated its presence histochemically in the mucosa of the body of the stomach, with highest concentration in the parietal cell zone. The antral mucosa had little, if any, urease (L11a). No urease activity could be detected in cancerous stomach (G59). Its concentration showed a positive correlation with the secretory acid response to histamine. Martin (M10) found a quantitative relationship between urease activity and his "gastroglobulin" fraction, precipitated by acetic acid–acetone from human gastric juice. Urease, according to Hollan (H26), is rapidly destroyed by HCl–pepsin digestion and, therefore, is found only in anacid or hypoacid gastric juice. In the most extensive review of this subject, Kornberg and Davies (K27) concluded that urease was of bacterial origin and did not play an essential role in HCl formation and in the gastric physiology.

Rappoport and Kern (R1a) recently found lower concentrations of gastric urea nitrogen in cirrhotics, which was interpreted as indicating greater urease activity in the stomach of these patients. In this connection, the possibility was raised of increased ammonia formation in the cirrhotic stomach.

1.2.3. *Carbonic Anhydrase*

This enzyme was previously considered to be of primary importance in the formation of hydrochloric acid in the stomach, but later *in memoriam* was written for this theory by its originator (see D2). This enzyme has recently become the subject of renewed interest, since it possibly participates to a limited extent in the removal of CO_2 from the gastric mucosa during the formation of hydrochloric acid (see D2).

1.2.4. *Mucolysin*

For 50 years, it was assumed that pepsin and HCl were instrumental in dissolving gastric mucus (see B1) and thus contributing to the formation of gastric ulcer (A3–A5). However, results in our laboratory indicated that the autolytic digestion of mucus occurs, rather, when mucus is incubated at a neutral pH and at body temperature (see Fig. 9) (G26,

G33, G34). Mucus undergoes liquefaction under conditions which preclude the participation of HCl and pepsin in this process.

This mucolysis results in the formation of a tyrosine-containing degradation product in the gastric content which has features of a proteose, and which we named "dissolved mucoproteose" (G26). We have therefore assumed that a specific enzyme may cause the physiological liquefaction and removal of mucus. We have given the name "mucolysin" to this hypothetical enzyme, although nothing is known about its nature or kinetics as well as its relationship to proteinase active at pH 7.0.

Other workers (J2) confirmed the following: (a) loss of viscosity of mucus, when incubated at the pH range 6–7, (b) independence of this process from acid-pepsin digestion, and (c) formation of tyrosine-containing split products as a result of the degradation. Hollander and Janowitz (H36, J2) worked on canine gastric mucus secreted by gastric pouches. They found that it loses its initial high viscosity upon incubation at 37°C, and that the pH optimum for this mucolysis is 6.0–6.5. This process was accelerated by the addition of cysteine, but was retarded by oxalate or citrate ions, chelating salts which remove calcium, or organic mercury preparations. The addition of trypsin, papain, or pectinase to gastric canine mucus also caused liquefaction, but lysozyme, salivary amylase, deoxyribonuclease, snail digestive juice, hyaluronidase, soybean trypsin inhibitor, and chymotrypsin were unable to liquefy the mucus. They referred to this enzyme as "mucinase" although this name was used by Roger 60 years ago (R9) for a coagulase coagulating intestinal mucus, which confuses the semantics (G33).

Thus, along with the secretion of surface epithelium mucus and its mechanical shedding, there must be a constant digestion of mucus by a mucolytic enzyme present in the mucus itself (G26, G33, G34). However, the mechanism of removal of mucus from gastric mucosa with formation of its degradation product, detectable in the gastric juice as "dissolved mucoproteose" (G27) needs clarification.

1.2.5. *Lipase*

Gastric juice contains a lipase which is a tributyrase (see D2). It splits tributyrin at a rate 2.5 times higher than tricaprin, and at a rate 50 times higher than trilaurin. Its activity against tristearin is nil, and its activity optimum is at pH 5.5. The cellular origin of gastric lipase is unknown, but as the lipase of plasma is also tributyrase, possibly gastric lipase is secreted by an endocrine-exocrine partition mechanism, similar to that of pepsinogen (see H21).

Matsumoto confirmed that the gastric lipase was inactive at a low pH, its optimum being pH 5.0–5.8 (M26). Human lipase was twice as powerful as that of canine gastric juice and activity of the latter could be shown in the absence of pancreatic juice reflux (M26). Since the gastric juice pH, when buffered by food, falls into the activity range of gastric lipase, it is probable that the latter may exert its lipolytic activity in the human and canine stomach.

1.2.6. *Other Nonproteolytic Enzymes*

Schenker noted increased lactic dehydrogenase activity in gastric juice from gastric cancer patients (S4). Smyrniotis *et al.* (S32), in an extension of these studies, found that the mean value of this activity was 55 units/ml in 27 controls; in 22 gastric ulcers the mean was 227, in 20 pernicious anemia cases 474, but in 26 gastric malignancies 897. It was abnormally elevated in 24 of 26 gastric malignancies and in only 2 of 27 gastric ulcers. However, this enzymatic activity of gastric juice was found to be dependent on blood contamination (L9a) and duodenal reflux (S32). Also Macoun *et al.* (M3) and Piper *et al.* (P10) found increased lactic dehydrogenase activity in gastric juice of patients with adenocancer of the stomach, but normal activity in patients with anaplastic gastric carcinoma.

Piper *et al.* (P10) found other nonproteolytic enzymes in human gastric juice, such as: (a) phosphohexoisomerase, (b) glutamic-oxalacetic transaminase, (c) alkaline phosphatase, (d) isocitric dehydrogenase, (e) glutamic-pyruvic transaminase, (f) leucine aminopeptidase, (g) α-ketoglutaric dehydrogenase, (h) ribonuclease, and (i) β-glucuronidase. The concentration of phosphohexoisomerase and glutamic-oxalacetic transaminase was similar in cancer patients and controls.

Of these enzymes, β-glucuronidase was of great significance. It was elevated in those with gastric carcinoma and its level clearly demarcated benign from malignant lesions. The anaplastic cancer group showed lower activity, however, than the adenocancer. The salivary contamination could be a major source of error. Increased values of β-glucuronidase in gastric juice in cancer of the stomach were also found by other investigators [Kim and Plant (K17a)].

No significant differences have been found in the concentration of enzymes (c)–(g) in gastric juice of normals and those with diseased stomach, including cancer. Therefore, they have no diagnostic significance.

Ribonuclease, according to Piper *et al.* (P11), has two pH optima, one

at pH 4.5–5.0, another at pH 7.0–7.5. It is heat-labile and inhibited by copper ions. Whether it originates in the gastric juice is unknown, but its concentration in the extracts of the fundic and pyloric mucosa was similar. The source of ribonuclease in gastric juice may be due to cellular

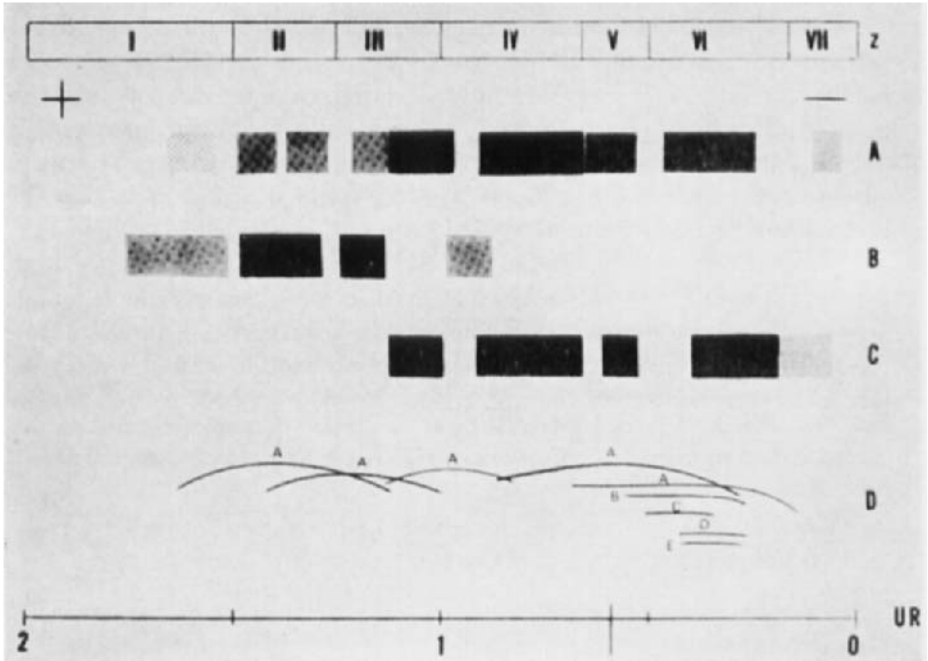

FIG. 11. Electrophoretic distribution in agar of gastric mucosal extract: protein (A); protease activity at pH 2.2 (B); carboxylic esterase activity (C); and immunoelectrophoretic pattern (D). The relative mobility is shown at the bottom (UR) with 0 representing the location of the uncharged dextran, levan and 1 the migration of human serum albumin. The zones of mobility (Z), arbitrarily defined on the basis of protein distribution, are indicated at the top. Each antigen and enzyme is designated by the zone in which it is found. The antigens are alsc designated by a letter. From Kushner *et al.* (K32).

breakdown, diffusion of the enzyme from the blood, or direct gastric secretion.

Rapp with Burtin (R1) found 5 carboxylesterases in the gastric human mucosa on agar gel electrophoresis (see Fig. 11) using special substrates. These carboxyl esterases were decreased and differently distributed in the gastric mucosa of patients with gastric cancer.

2. Mucosubstances

Mucous substances secreted by the stomach are derived from several cellular sources: (1) columnar cells of the surface epithelium, (2) mucous neck cells of the glands of the fundus and body, (3) mucous cells of the cardiac glands, and (4) mucous cells of the pyloric glands (B1). The total of these materials can be referred to as "gastric mucin" (G8, G11, G27), in line with the terminology of Meyer (M34, M35) for whom this term has only physiological meaning denoting a viscous secretion, and pertaining to the sum total of materials secreted by the mucous cells of the gastric mucosa. Chemically, the materials secreted by the above

TABLE 1
MUCOSUBSTANCES OF THE GASTRIC SECRETIONS

I. Aminopolysaccharides (Kent), Mucopolyuronides (Blix)
   1. Chondroitinsulfates: *N*-acetyl-D-galactosamine, D-glucuronic or L-iduronic acids, sulfates
   2. Heparin (alpha): glucosamine, glucuronic acid, sulfates
   3. Hyaluronic Acid(?): *N*-acetylglucosamine, uronic acids

II. Mucopolysaccharides (Kent), Mucoids (Meyer), Mucoids and Glycoids (Winzler)
   1. Fucomucins (Blix, Werner): protein, L-fucose, *N*-acetylglucosamine, *N*-acetylgalactosamine, D-galactose
   2. Sialomucins (Odin): protein, sialic acid, *N*-acetylgalactosamine

III. Mucoproteins (Kent)
   1. Serum proteins: α-globulins, γ-globulin
   2. DL-Hexose-hexosamine type: serum mucoprotein (protein, mannose, galactose, hexosamine)

mentioned mucous cellular structures may be classified, according to Levene (L8–L8c) and Meyer (M34, M35), as mucopolysaccharides, mucoids, and mucoproteins, depending on their chemical composition, especially the content of protein moiety and that of hexosamine (below or above 4%, respectively). On the other hand, the term "mucosubstances," as used by Kent (K15, K16), does not necessarily refer to viscous or mucous materials, but pertains to all those substances which contain hexosamine in their molecule. They are classified according to Kent (K15) into aminopolysaccharides, mucopolysaccharides, and mucoproteins. The characteristic features of these various mucosubstances, as understood by various authors, are listed in Table 1 and discussed in various excellent reviews (B15, B22, B24, L8, L8a, P9, S34, W9) and textbooks (K16, S39) (also see Brimacombe and Stacey, this volume, p. 199).

All mucosubstances form part of the nonparietal gastric secretions (G31, G32) in which they have been grouped, together with other nonparietal materials, by Hollander under the name "alkaline component" (H27, H33). Until recently, the gastric mucin was grossly separated into two main fractions characterized physically or physiologically, rather than chemically (B1): one was the "visible mucus," which forms a mucous lining of the surface of the gastric mucosa. The other, which is dissolved in the gastric juice and cannot be separated by simple filtration or centrifugation, has been called by Babkin and his associates "dissolved mucin" (B1). It confers the feature of viscosity on gastric juice and is intimately related to many of the biologically active materials of the stomach (see Section 9).

2.1. Visible Mucus and Mucous Barrier

Visible mucus forms the external layer of Hollander's so-called protective gastric mucous barrier (H28–H30). According to him, the internal layer of this barrier consists of the preformed mucosubstances contained within the juxtaluminal portion of the surface epithelial cells themselves.

About 100 years ago Harley, followed by Pavlov, stated that mucus protects the stomach from the chemical action of its own gastric juice (see H28). The nature of the protective effect of this mucous layer has then been analyzed, studied, and discussed by many authors, pointing out the possible mechanisms of this protective action. The fact that over 13,000,000 surface epithelial cells secrete mucus, according to Fontaine (F7), and more than 3,000,000 glands are present in the pyloric area of the stomach, according to Policard, led to the acceptance of the protective role of mucus.

This protective role is related to several physical properties of the mucus, which are as follows.

2.1.1. *Physical Properties*

Adhesiveness prevents detachment from the surface of the epithelium, and causes firm attachment to the underlying cellular layer.

Cohesiveness lends to mucus its protective property preventing direct contact and mixing of intraluminal materials with the surface of the cells.

Viscosity determines its lubricating effects and also prevents its rapid washing off the mucosal surface. However, viscosity is a function of the pH of the milieu and is highest at pH 4.6, but decreases with rising or falling pH (J2, K3, M39) (Fig. 12). Below pH 4.6 mucus breaks and precipitates. *N*-Acetyl-L-cysteine decreases mucus viscosity (S20).

Gel formation facilitates hydration of mucus and its imbibition of

water and electrolytes and permeability to passage of electrolytes in both directions, and provides it with adsorptive capacity.

2.1.2. *Adsorptive Capacity*

The adsorptive capacity of gastric mucus has been considered by many authors to be of utmost importance in protecting the stomach against digestion.

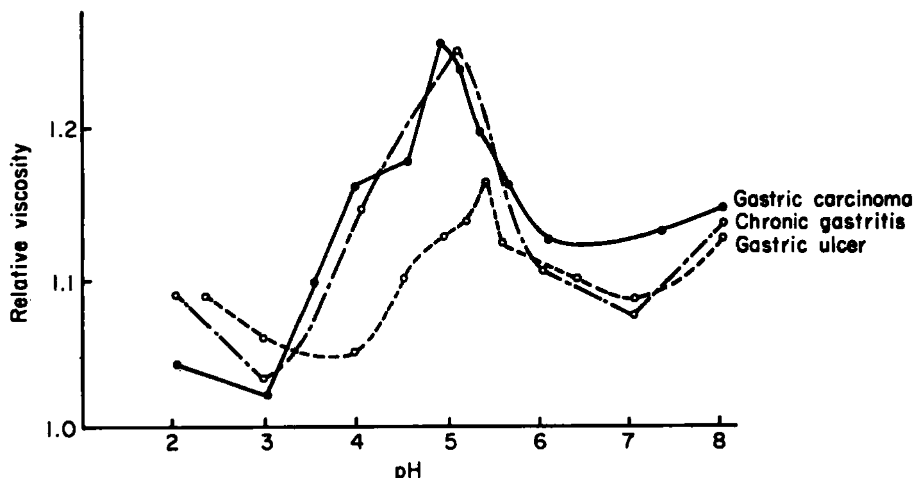

FIG. 12. Change of visible mucus in several gastric diseases. Changes of viscosities by pH correction. From Wada (W1).

The work of Bucher (B43, B44), Bradley and Hodges (B30, B31), Zaus and Fosdick (Z2), and Komarov (K24) supports the concept that the most significant mechanism in the antipeptic activity of mucus consists in its ability to adsorb pepsin. This adsorption may prevent pepsin from acting on the surface epithelium directly, at high concentration. Boldyreff (B23) drew similar conclusions from *in vitro* measurements of peptic activity before and after addition of canine gastric mucus. Schmid (S7) found adsorption of pepsin to the mucoproteose fraction of gastric mucin *in vitro.*

Heatley (H4–H5) demonstrated that mucus and pepsin can be separated by filtration through a sinter filter, which would raise doubts as to whether pepsin really becomes adsorbed to mucus. It appears more probable that the layer of visible mucus only makes a barrier between the surface epithelium and the acid-pepsin solution in the gastric lumen, which slows down the gross mixing of the intraluminal materials with the gastric mucosa. According to Heatley (H4–H5), it is unneces-

sary to postulate an interference of mucus with diffusion, binding, or inactivation of pepsin (Fig. 13). He assumes that within the thickness of the mucous barrier, there is a continuous concentration gradient of pepsin and electrolytes which diffuse through it in both directions.

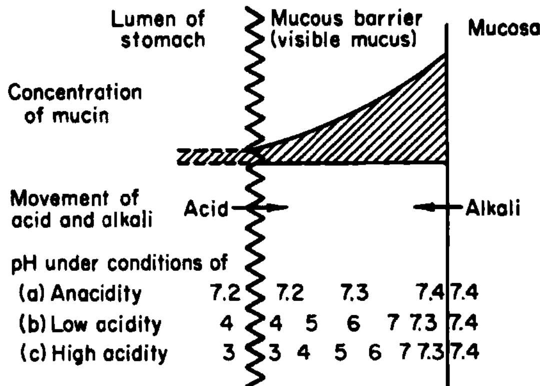

FIG. 13. Diagram of imaginary concentration and pH gradients within the "visible mucus" layer on the gastric mucosa when contents of stomach are: (a) anacid; (b) weakly acid; and (c) strongly acid. The direction of movement of acid and alkali are also shown. From Heatley (H5).

Therefore, one can conceive of the protective mucous layer as a dynamically maintained barrier, and not a static one.

2.1.3. *Antipeptic Activity*

The early investigations of Weinland, Schwartz, and Blum led to the concept that the antipeptic activity of mucus was due to the presence of a special antipepsin (see B1). Komarov (K24) and Zaus and Fosdick (Z2) then considered the possibility that the content of mucoitin sulfuric acid in mucin causes its antipeptic activity. Levey and Sheinfeld (L9) demonstrated the inhibition of peptic activity by other sulfate-containing polysaccharides, such as heparin and chondroitin sulfate, both of which are present in the gastric mucin. Carrageenin from seaweed, another sulfated polysaccharide, also has potent antipeptic activity (H51, V4). Its effect decreases as the pH is raised above 2.0 (V4), as well as when the substrate level is high. Heatley, however, was unable to show significant peptic activity inhibition by pig pyloric mucosubstance at the concentration which normally occurs in the stomach (H4).

In unpublished work from our laboratory on inhibition of peptic activity by hog gastric mucin and commercially available mucoitin sulfuric acid, we also were unable to find significant inhibition of the peptic

activity by these materials at physiological concentrations. This creates doubt as to the physiological significance of this inhibitory activity.

2.1.4. *Acid-Binding Capacity*

One of the much discussed properties of mucus, which at one time was considered to be the main factor in its protective role against acid-peptic digestion, was its alleged buffering ability and acid-neutralizing capacity (B1, J10). This concept was introduced by Pavlov over 70 years ago (see B1) and elaborated further by other investigators in this century (B1, B2, B23, D4, M4–M6). Also Bolton and Goodhard (B25), Helmer (H12), and Leriche (L6, L7) and his associates, Fontaine and Monceaux (F7, M46–M47), assigned a significant buffering effect to mucus.

Other workers in the past, however, Baltzer (B7, B8), Bonis and Kalk (B26, K3), Bucher (B43, B44), Kapp (K7), Mitchell (M41), and Udaondo and Zunino (U3), considered that mucin plays no significant role in the buffering of HCl in the stomach, and that the frequently described decrease in mucus content in peptic ulcer may be instrumental in ulcer formation by a mechanism other than decrease of its buffering capacity.

More recently, Hollander *et al.* (H27–H30, H32) measured buffer capacity of viscous mucus collected from dog Heidenhain pouches following application of topical mucigogues, and found it to be significant and in the range 9–84 (mean 40) millinormal, when measured by electrometric titration against HCl. This would indicate that on the average 100 ml mucus may buffer about 40 ml 0.1 normal HCl.

Electrometrical measurements of the binding capacity of the dialyzed visible gastric mucus and dialyzed dissolved mucin fractions, made in association with Pugh and Wolf (G51a), showed us, however, that the binding capacity was by far smaller than that reported for the undialyzed human or animal mucus. In this we concurred with the early work of Bonis (B26). The total buffering capacity of mucosubstances dissolved in gastric juice was only 1.2–10.3% of the free acid present under fasting conditions (G51a). This amounts to a very insignificant factor in the buffering of acid of stimulated gastric secretion. The neutralizing capacity of mucus may thus have a slight effect when the rate of acid secretion is low, but at moderate or high acid secretory output, its significance dwindles. The buffering effect upon HCl of gastric mucus is obviously due to the dialyzable materials contained therein (G51a), including mineral bases (bicarbonates and phosphates) which are chiefly responsible for its alkalinity. Though the mucin has little buffering power

and does not significantly inhibit peptic digestion, the bicarbonate and phosphates secreted with it help to neutralize the HCl and, by raising the pH, make the action of pepsin less effective.

2.1.5. *Protective Role*

This has been well demonstrated in an experimental subject with gastric fistula by Wolf and Wolff (W20, W21): strong irritants and corrosive agents failed to cause more than slight to moderate engorgement of the gastric mucosa, but caused marked inflammatory reaction with tissue destruction when applied in similar concentration on the skin. This protection is provided by the layer of tenacious mucus adherent to the gastric mucosa which protects it from physical and chemical injury. According to Florey (F5, F6), mucus is secreted in response to local nervous and hormonal stimuli, partly in liquid form; this jellies upon contact with acid. Some of it then adheres to the surface of the mucosa and, by virtue of its physical properties, impedes mechanical mixing which would bring the gastric juice in contact with the surface cells. Through its lubricating properties, it also protects the mucosa from mechanical trauma. Beneath this layer lies the undischarged mucin in the continuous palisade of gastric surface cells (H28–H30), so that any protection afforded by secreted mucus is probably reinforced by the mucin contained in the underlying cells.

2.1.6. *Deficiency of Gastric Mucin in the Pathogenesis of Peptic Ulcer*

This concept has been repeatedly advanced by Leriche and his school (F7, L6, L7, M46–M47) ever since Kaufman introduced it in 1908 (K10). It found some support in the early work of Anderson, Farmer and Fogelson, who reported decreased mucin content in patients with peptic ulcer (A3–A5). However, with application of more exact iodometric methods (G6, G7) for the quantitation of mucin in the gastric juice, neither the reviewer (G6, G7) nor others who worked with this technique abroad (K6, K20, M52) and in this country (T33) could demonstrate mucin deficiency in patients with peptic ulcer. This was true for gastric juice collected both under fasting conditions and after alcohol or histamine stimulation. Similar negative findings were recorded by authors who worked with other methods (B35, I3).

Some time ago we determined total dissolved mucin and its fractions during fasting and after alcohol test meals, histamine injection, and i.v. insulin in gastric juices of 60 patients with peptic ulcer and 106 controls with various gastric disorders (G30). In each of the 841 gastric specimens, the total dissolved mucin and its two fractions (mucoprotein and

mucoproteose) were determined by the tyrosine method (G27) and correlated with the volume of gastric secretion and free and total gastric acidity. The results, which were evaluated statistically, did not corroborate deficiency of gastric mucin in peptic ulcer. Neither fasting basal secretion nor gastric juice collected after humoral or central vagal stimulation showed significant decrease of dissolved mucin in patients with gastric or duodenal ulcer, as compared to controls (G30). Output of gastric mucoproteose in duodenal or gastric ulcer decreased only insignificantly, as compared to controls. Decrease in concentration of this mucin fraction, which was manifest in most cases, was due only to dilution of the surface epithelial secretion by the increased volume of parietal secretion in these patients. Since gastric mucoproteose represents the split and dissolved product of surface epithelial mucus, lack of significant decrease in its output does not corroborate the concept of the defect in the protective layer of the surface epithelial mucus in ulcer disease, as well as increased degradation of the surface epithelial mucus in peptic ulcer.

Nevertheless, recent literature again revives the old concept of mucin deficiency in peptic ulcer. Zaidi and Mukerji (Z1) produced acute peptic ulcer in guinea pigs with massive doses of histamine. To prevent histamine-induced ulceration, capsicum was applied topically as a mucosal irritant. Gastric mucin was quantitated by determining the glucuronic acid content of the gastric juice, which is obviously subject to criticism, as not all gastric mucoproteins contain uronic acid (G54–G56). Results indicated that the higher the mucus production, the lower the severity of the ulcerative process (Z1). It was therefore concluded that mucin has a neutralizing effect on the acid gastric juice and, by adhering to the mucosal surface, forms a barrier against peptic ulceration.

In addition, steroid ulcer has been related recently to mucin deficiency caused by this therapy. Menguy and Masters injected steroids into rats with denervated antral pouches (M28). They found substantially decreased mucus secretion, as well as compositional change of antral mucus characterized by decrease in its sialic acid concentration (Fig. 14). They concluded that the steroids interfered with the rate of the mucous barrier renewal, caused decreased mucus production, and lowered the threshold of gastric mucosal susceptibility to peptic digestion. Robert and his associates studied the relationship of the mucus secretion to the development of ulcers in fasting rats (R7), as well as in those given large doses of steroids (R8). They determined mucus content by quantitation of hexosamine and found decrease in concentration and output in rats who developed ulcers. The latter appeared only in that portion

of the stomach containing the least mucus, namely the body, while the antrum, with twice the amount of mucus, remained intact.

Although these findings may apply to an experimental cortisone ulcer in animals, Hitzelberg in our laboratory obtained less definite results in man (H22, H23). Fifteen arthritic patients received 1 g prednisolone in 6 weeks. In the course of treatment, there was never an indication of peptic ulcer formation, although the output and concentration of pepsin in the basal secretion increased on the average about 2-fold. Paper electrophoresis of the dialyzed and lyophilized gastric juice, following steroid administration, frequently showed decrease of total carbohydrate

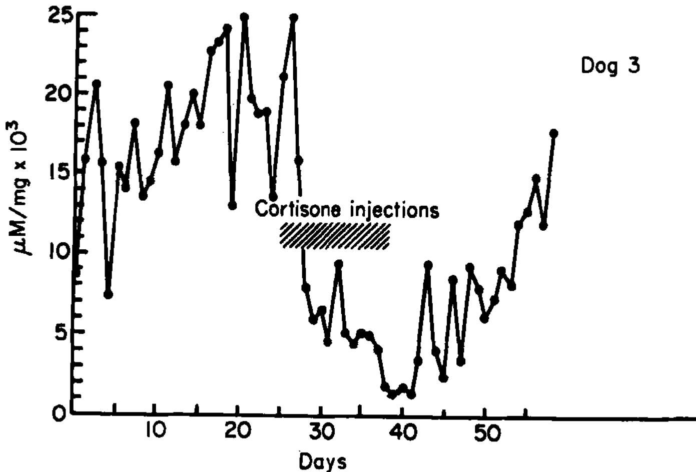

Fig. 14. Effect of cortisone on sialic acid concentration in antral mucus. From Menguy and Masters (M28).

content in gastric juice, as quantitated by periodic acid-Schiff staining of gastric electropherograms, followed by tracing and integration of strips in Analytrol. Total carbohydrates calculated in the integration units showed on the average a decrease from 134 to 113 after 21 days of steroid treatment, and a decrease at the conclusion of administration to 100 units per 2 mg of dialyzed and lyophilized gastric juice (Table 2). This corroborates, to some extent, the decrease in the amount of mucosubstances in the gastric secretion in some steroid-treated patients. Of greater significance in these cases was the increase in pepsin concentration and output. In some of our steroid-treated cases, changes were found in electrophoretic mobility of the carbohydrate moiety of gastric mucosubstances, which became less negatively charged (H23) (Fig. 15). This may corroborate Menguy's findings (M28) of decreased sialic acid con-

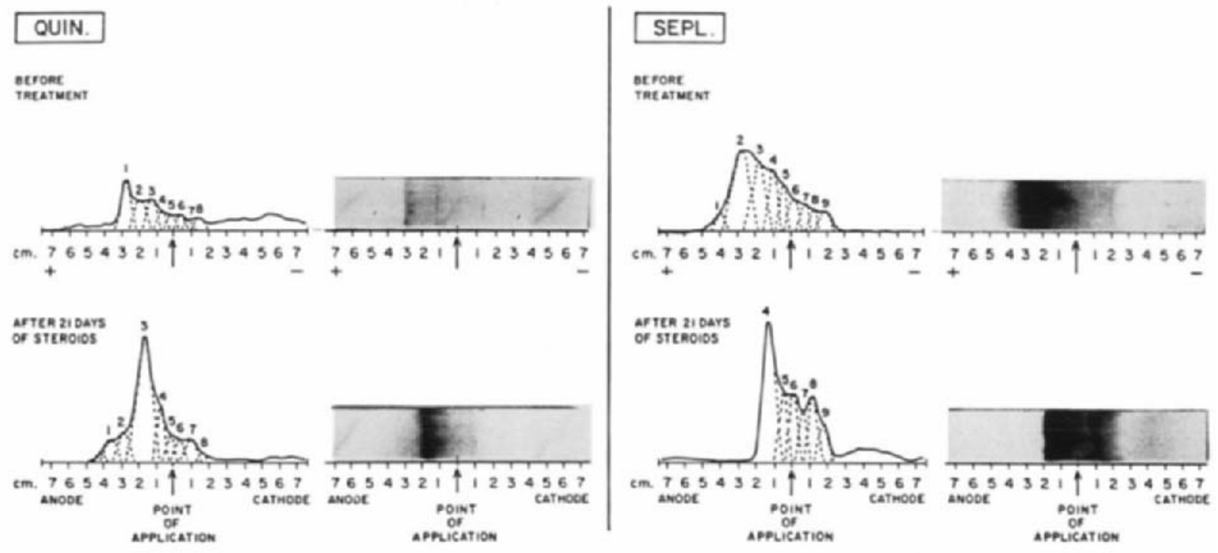

FIG. 15. Effect of corticosteroids on PAS-stained electrophoretic patterns of carbohydrates in fasting gastric juice of 2 patients with rheumatoid arthritis. From Hitzelberger and Glass (H23).

tent in mucus of steroid-treated dogs, since sialic acid supplies negative charges to some of the mucosubstances (sialomucins).

Upon histochemical examination of gastric mucosal biopsy specimens in some of our cases treated with steroids, evidence was also found for possible changes in the mucous lining itself (G49). This was character-

TABLE 2
CARBOHYDRATE-CONTAINING MATERIALS IN PAPER ELECTROPHEROGRAMS OF THE GASTRIC JUICE OF 10 INDIVIDUALS BEFORE, DURING, AND AFTER STEROID TREATMENT

	Arbitrary integration units, calculated from electrophoretic strip[a]							
	Before steroid treatment		Steroid treatment (after 21 days)		At conclusion of steroid treatment (day 42)		After 21 days of steroid treatment (2 course)	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range
Total carbohydrates	134	42–254	113	48–187	100	53–163	102	37–158

[a] Of 2% solution (2 mg.) of dialyzed and lyophilized gastric juice in borate buffer 0.1 ml. was applied to the center of a Whatman No. 1 strip, run against borate buffer of pH 9.0 and 0.24 ionic strength at 120 volts and 0.4 mA per centimeter for 5½ hours and after oven-drying was stained with PAS stain.

ized by changed staining properties of the mucous barrier and, at times, its dissolution. The information available on these lesions is too scanty to assume that they facilitate the proteolytic enzyme penetration through the mucous barrier into the deeper layers of the gastric mucosa, resulting in steroid ulcer. Further work is needed in this direction.

2.1.7. *Control of Mucus Secretion*

There is no evidence for humoral control of the cells secreting mucous material (B1, G11, K26). Histamine rather decreases total gastric mucus output in man (G27, G52). It is probable that surface epithelial cells secrete mucus in response to topical mechanical and chemical stimuli (H27a, H29), while the deeper mucous cells, such as mucoid neck cells, are activated rather by nervous (vagal) stimuli (J9, M53). Squeezing out preformed mucus from the mucous cells by contracting the gastric wall following vagal stimulation may also be involved (B1). Electrical stimulation of the vagus (Ushakoff, cited in B23) increases gastric mucus secretion in dogs and discharge of the mucous material from the pyloric glands of the cat, but not from superficial epithelial cells (J9, M53). Boldyreff (B23) noted increased flow of gastric mucus following insulin in dogs. Sham feeding in dogs also increases secretion of mucin (K26).

Topical application of acetylcholine in dog stomach stimulates formation of cell-free mucus (J3).

Mersheimer *et al.* (M31) found that gastric mucosa of dogs sacrificed at varying time intervals after bilateral vagotomy shows normally preserved surface epithelial cells and mucoid neck cells of gastric glands. They were filled with secretory granules when the animals were sacrificed during the resting phase, but were partly discharged of their content when the animals were sacrificed during the digestive period. This indicated that the secretory mucoid neck cell response to food stimuli was retained in the vagotomized stomach, and that the mucous material was produced normally in animal stomachs with completely severed vagal nerves. This suggests that the preserved vagal innervation in the dog stomach is unnecessary for mucus formation and mucus discharge from the surface epithelium and mucoid neck cells (M31).

2.2. Dissolved Mucin

Another mucous fraction, which does not form the mucous barrier of the stomach but is dissolved in the gastric juice, has been called "dissolved mucin" by Babkin and his associates (B1). As recognized 55 years ago by Kaufman (K10), dissolved mucin does not exert a protective effect upon the gastric mucous membrane. This fraction cannot be separated from gastric juice by either filtration or centrifugation, but only by precipitation with suitable agents, such as acetic acid–acetone mixture (M9, W6, W6a), sulfosalicylic acid–acetone (B7), trichloroacetic acid–acetone mixture (G6, G7, G23, G24), tungstic acid (M9), sulfosalicylic acid–alcohol (B35), ethanol or methanol at low pH (K2, K3, N5, N6), or tungstic acid from perchloric acid filtrates (E6). It confers the feature of viscosity upon gastric juice. Its composition certainly varies widely, depending on method used for its precipitation and gastric juice from which it was recovered. Webster (W6) and Webster and Komarov (W6a) precipitated dissolved mucin from neutralized filtered gastric juice by adding acetic acid at 1% concentration and 1–1.5 volumes of acetone. Its elementary analysis demonstrated 13.8% nitrogen, 52–75% carbon, 6.97% hydrogen, and 0.8–1.29% organically bound sulfur, of which sulfur of organic sulfates was 0.25%. It showed positive reactions for proteins, but also gave strong reduction after 3-hour hydrolysis with 2 *N* sulfuric acid, equivalent to 12.6–12.8% glucose. However, orcinol (Bial) and resorcinol (Selivanoff) tests were negative. Its composition differed markedly from the "surface epithelial mucin" ("visible mucus") which, after alcohol precipitation, contained 12.31% N, and after hydrolysis with sulfuric acid yielded as much at 31.3–35.0% sugars. Com-

parison of the composition of these two mucous fractions as well as other materials processed from the stomach, is given in Table 3.

TABLE 3
COMPOSITION OF VARIOUS FRACTIONS FROM HUMAN GASTRIC JUICE (IN % W/W)

Materials	N	Proteins	Tyrosine	Sulfate	ASH	Ref.[a]
Surface epithelial	12.3				2.0	(1)
mucus	6.7		4.2			(2)
"Floating" mucus	10.1					(3)
"Precipitating" mucus	8.9					(3)
"Mucoprotein"	13.8–14.0			0.8	3.9	(4)
"Gastroglobulin"	11.5–12.8			1.4–1.8		(5)
"Dissolved mucin"			5.2			(6)
	14.7	61.8	6.6			(7)
"Glandular mucoprotein"	12.6 ± 0.4		7.5 ± 0.6			(8)
	11.2					(3)
"Mucoproteose"	5.7–7.3		3.9 ± 4.2			(8)
	8.2					(3)
"Trichlorac. ac. ppt."	12.1					(3)
"Winzler's mucoprotein-like ppt."	6.7	56.5	4.1			(7)

Materials	Total carbohydrates	Hexoses	Hexosamine	Fucose	Sialic acid	Uronic acid	Ref.[a]
Surface epithelial							(1)
mucus	*						(2)
"Floating" mucus			7.1		3.1	0.8	(3)
"Precipitating" mucus			17.0		4.3	0.7	(3)
"Mucoprotein"							(4)
"Gastroglobulin"	**						(5)
							(6)
"Dissolved mucin"		23.5					(7)
"Glandular mucoprotein"	13.5	5.4	4.7	2.2	6.6	1.2	(8)
			8.8		2.0	4.8	(3)
"Mucoproteose"	61.2	32.1	18.9	8.5	1.7	1.7	(8)
			15.1		3.1	2.1	(3)
"Trichlorac. ac. ppt."			5.8		1.9	1.2	(3)
"Winzler's mucoprotein-like ppt."		6.9					(7)

* Reducing substances content: 31.3–35.0.
** Reducing substances content: 12.6–12.8.

[a] Authors, years, and reference numbers:
(1) Webster (1931); (W5)
(2) Glass and Boyd (1949); (G26)
(3) Werner (1953); (W9)
(4) Webster and Komarov (1932); (W6)
(5) Martin (1933); (M9)
(6) Glass and Boyd (1948); (G23)
(7) Espada *et al.* (E6)
(8) Glass *et al.* (1948); (G36)

Martin prepared material (M9) from human gastric juice related to dissolved mucin which, according to him, contained three protein-like compounds, in addition to the visible mucus. Of these, the first was precipitated by 50% saturation with magnesium sulfate, by heating, or by 0.5 volume acetone, after prior addition of acetic acid at 2% concentration. He named it "gastroglobulin." Its isoelectric point was about pH 3.5 and it contained 11.48–12.80% N and 1.37–1.76% S. It did not reduce copper sulfate without hydrolysis nor form osazones with phenylhydrazine. This material crystallized from brucine-pyridine-ammonia and its relation to pepsin was assumed to be "so close, in fact, that one wonders if the enzymes do not form most, if not all, of the proteins" (M9). Martin obtained a second protein material from the gastric juice filtrate after gastroglobulin precipitation, by adjusting the pH to 3.5 and adding tungstic acid. The precipitate gave all protein tests, except the Millon test; it strongly reduced copper sulfate after hydrolysis with 2% sulfuric acid, and produced osazone crystals. In addition, a third protein body was obtained (M9) by adjusting the pH 6.8–7.0. The slight precipitate yielded a positive biuret test and reduced copper sulfate after hydrolysis. It was found in only very small amounts.

The existence of more than one mucoprotein in the dissolved mucin fraction of the gastric juice was further substantiated in our laboratory (G26, G27, G36). We found (G27) that the composition of dissolved mucin varied markedly, depending upon the stimulus applied to gastric secretion. These variations included degree of hydration, extractability with 60% alcohol, and content of tyrosine, nitrogen, and reducing substances. We therefore postulated that, in man, at least two but probably three different mucous substances were present within the mixture of mucosubstances called dissolved gastric mucin (G11, G27) (Fig. 16).

Similar inferences were drawn regarding dissolved mucin from dog stomach by Grossberg *et al.* (G70), who determined hexosamine and uronic acid contents of canine gastric juice and mucus before and after histamine and sham feeding. Determinations were performed after acid hydrolysis and showed marked variations in the mutual ratio of these two components, depending on the nature of the stimuli applied. This suggested the existence of at least two different kinds of mucoprotein in these secretions—one containing uronic acid, and the other hexosamine with or without small amounts of the former (Fig. 17).

Further support for the heterogeneity of dissolved mucin was added by the electrophoretic studies of these authors (G71), demonstrating in canine gastric juice the presence of several components endowed with various electrophoretic mobilities and differing in hexosamine and uronic

acid contents. These data have been extended further by the fractionation studies of gastric juice in our laboratory performed with the use of combined electrophoretic and chemical methods (G55, G56) (also see G21). They all point to a heterogeneity of dissolved mucin in gastric juice.

Over 10 years ago, we referred to dissolved mucin as a mixture of at least three mucosubstances (G11). They were designated as (1) soluble

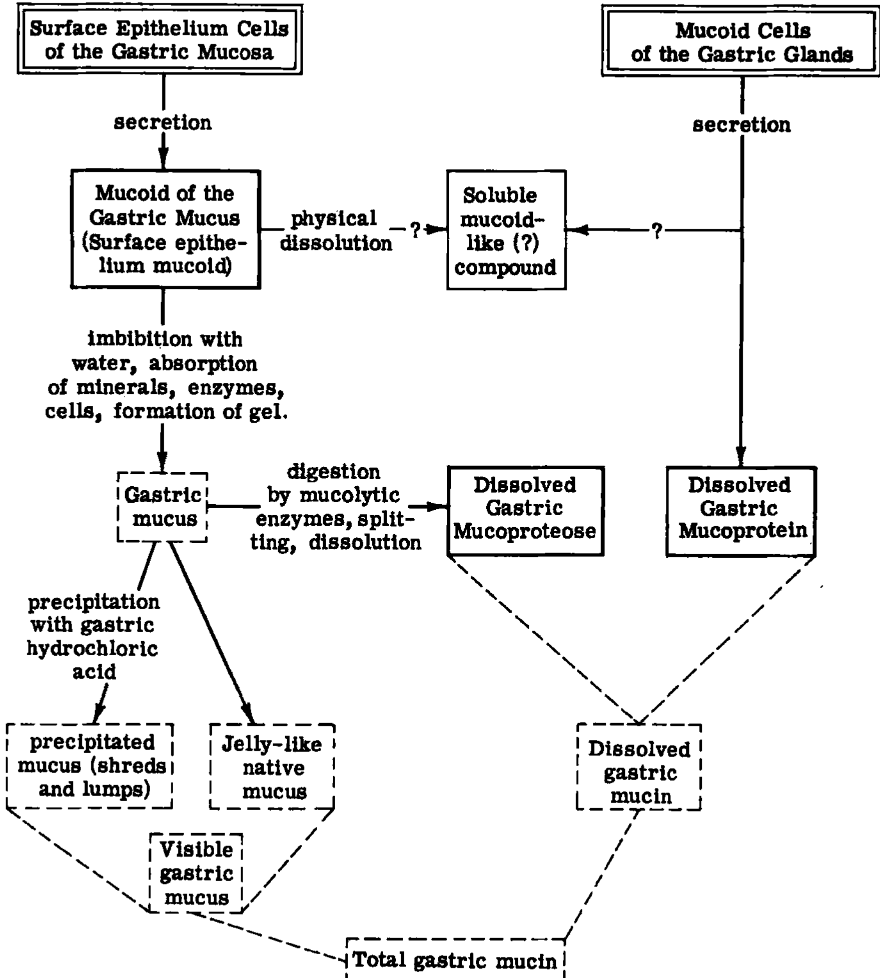

FIG. 16. Classification and mechanism of origin of mucous substances of the stomach. From Glass and Boyd (G27).

mucus, (2) dissolved (glandular) mucoprotein, and (3) dissolved mucoproteose.

Now these component fractions of dissolved mucin, separated by simple chemical precipitation, may be considered as only prototypes of more elaborate and purified materials which can be obtained at

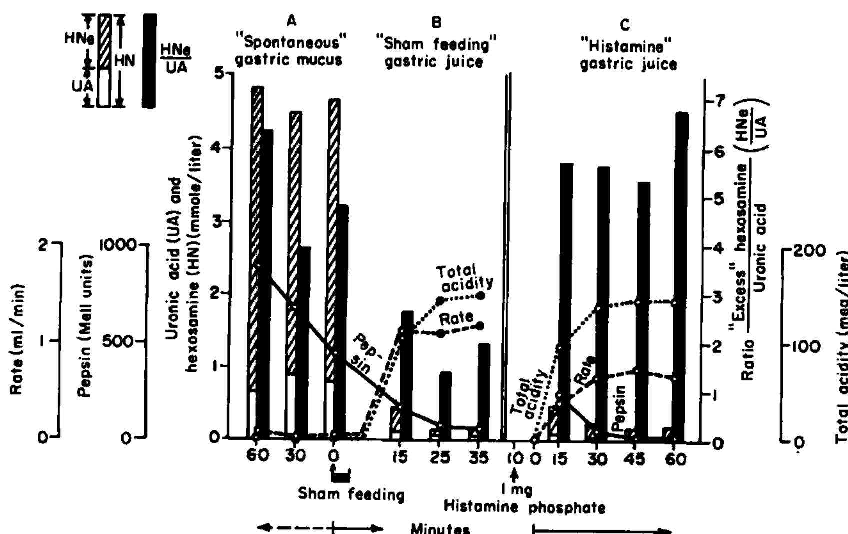

FIG. 17. Relative and absolute concentrations of uronic acid, total hexosamine and excess hexosamine in spontaneously secreted mucus and in gastric juice secreted in response to sham feeding and to histamine.

Dog A, female, 13 kg, gastric fistula and esophagotomy, operated March 1945. Sections A and B represent an experiment of November 22, 1948, and section C an experiment of January 4, 1949. In the latter 2 mg atropine sulfate were injected 42 minutes before the injection of histamine phosphate. Rate of secretion and concentration of total acid and pepsin are shown by line graphs in order to more completely characterize the samples. The spontaneous mucus specimens represent 30-minute samples. UA = uronic acid; HN = total hexosamine; HN_e = excess hexosamine; mmoles/liter. From Grossberg *et al.* (G70).

present by means of more advanced physicochemical methods. This progress will become evident from the information discussed in the companion review of the author in this volume (p. 373).

2.2.1. *Soluble Mucus*

Soluble mucus was considered to be the transient physical dissolution product of the visible surface mucin before it undergoes further enzymatic degradation in the stomach (G11). It precipitates from filtered

acid gastric juice with 0.5 volume 10% trichloroacetic acid, similarly to salivary mucin. However, this precipitate also contained other proteins and proteolytic enzymes. The origin of soluble mucus from the gastric mucosa and not from saliva was established by its presence in the stomach of Tom, the man with occluded esophagus and gastric fistula (M2, W18). It may be precipitated with 1.5 volumes acetone from the filtered juice, in the form of an upward floating and rapidly shrinking "mucin clot." By neutralizing gastric juice to pH 8–9, soluble mucus becomes depolymerized and loses its ability to precipitate in this form upon addition of acetone. Instead, it precipitates like the other mucosubstances contained in the dissolved mucin. It is not found electrophoretically, peak G2, in the trichloroacetic acid filtrate of the same juice (M1, M2).

2.2.2. *Dissolved Mucoprotein*

When 1.5 volumes acetone is added to the trichloroacetic acid filtrate of the gastric juice, an abundant flocculent precipitate forms, which contains all the components of dissolved mucin with the exception of soluble mucus. If this precipitate is taken up in dilute alkali and then acidified with dilute HCl down to pH 3.5, a fine flocculent precipitate forms, which we named "dissolved mucoprotein" (G27, G36). It was later renamed "glandular mucoprotein" (G9, G38) because of its close relationship to the fundic glands of the stomach. This material contained much protein; its nitrogen content was 12.61 ± 0.44% and its tyrosine content 7.50 ± 0.65% by the Folin-Ciocalteu reaction. The reducing substance content was 6.38 ± 1.48% before and 12.5% after hydrolysis (G9, G27, G36) (see Table 4). Werner (W9) determined the composition of this mucoprotein fraction and found that it contained 11.2% N by Kjeldahl, 8.8% hexosamine, 4.8% uronic acid, and 2.0% sialic acid.

The mucoprotein fraction concentration in gastric juice correlated with HCl and pepsin (G27, G52, G53) secretion, increased on vagal stimulation (G25, G27–G29, G35, G48, G52), and was absent or appeared in traces in juices of patients with pernicious anemia (G11, G38) and those with advanced atrophic gastritis (G11, G22, G28).

One aspect of the biological activity of the glandular mucoprotein fraction was its relationship to Castle's intrinsic factor (G10, G37). A daily dose of 50–100 mg of this material, when given to pernicious anemia patients with small oral doses of vitamin B_{12}, brought about a striking hematopoietic response. This activity was due, as we now know, to the content of the proteolytic degradation product of intrinsic factor (G12, G14, G18, G20, S22).

In 1961, Schrager (S10) found a correlation between the mucoprotein and pepsin concentration in gastric juice and concluded that the dissolved mucoprotein represents just pepsin. Schrager's conclusion represents a gross oversimplification of a complex biochemical problem, which does not take note of contributions of the last 12 years made in this area. As far back as 1950 we demonstrated, in collaboration with Pugh and Wolff (G52), that some correlation does exist between the concentration of the mucoprotein and pepsin in gastric juice. But that this was an

TABLE 4
CHARACTERISTICS OF GLANDULAR MUCOPROTEIN AND MUCOPROTEOSE IN GASTRIC JUICE

	Glandular mucoprotein	Mucoproteose
Composition:		
Nitrogen	12.6%	3.7%
Carbohydrates	13.5%	61.2%
Hexoses	5.4%	32.1%
Hexosamine	4.7%	18.9%
Uronic acid	2.2%	1.7%
Carbohydrates/protein ratio	1.4	14.9
Sulfates/carbohydrates % ratio	2.7	0.7
Electrophoretic mobility (Tiselius)	-6.9×10^{-5}	-0.5 to -1.0×10^{-5}
Probable cellular derivation	Mucoid neck cells of fundic glands	Surface epithelium (pyloric glands?)
Variations in health and disease	Increased, following vagal stimulation; abolished or decreased, in gastric atrophy	Decreased in gastric hypersecretion and following histamine stimulation

insignificant correlation was already clear at the time. This is further substantiated by unpublished data of Schwartz and Rich from our laboratory, listed in Table 5, which show lack of statistically significant correlation between variations of pepsin and mucoprotein in the same individual at various stages of gastric secretion and in various individuals under similar conditions of gastric stimulation.

When we processed dissolved mucoprotein in 1955 from gastric juice of various human subjects, and under conditions of variable stimulation, this material showed some variations in protein and hexose contents. Therefore, we stated at that time: "When we looked for explanation of these variations by elution of the paper electrophoretic strips, we found that the leading fast boundary corresponding to glandular mucoprotein contained much pepsin . . . . This pepsin apparently has been inactivated

by treatment with alkali during chemical fractionation of "glandular mucoprotein," so that it could not be detected in this fraction. Also other authors found previously that pepsin forms a kind of complex binding with mucoproteins . . . which cannot be dissociated by electrophoresis . . . . The complex binding of glandular mucoprotein to large amounts of pepsin accounts for rather high nitrogen and low carbohydrate content of our mucoprotein fraction and explains the variation in its chemical composition and why the dose of this substance needed for

TABLE 5[a]

RELATIONSHIP BETWEEN PEPSIN AND "GLANDULAR MUCOPROTEIN" CONCENTRATION IN 33 INDIVIDUALS PRIOR AND DURING STIMULATION OF GASTRIC SECRETION BY HISTAMINE AND INSULIN

Specimens	Pepsin ($PU_{Hb} \times 10^4$/ml) (means and ranges)	Glandular mucoprotein (mg/100 ml) (means and ranges)	Ratio pepsin to mucoprotein (means and ranges)
	23 histamine tests (0.1 mg/10 kg subcut.)		
Fasting	92(2–184)	89(42–163)	1.03(0.11–2.25)
After histamine			
20 min	113(7–176)	91(12–150)	1.24(0.34–2.34)
40 min	107(13–185)	79(19–147)	1.35(0.13–2.85)
60 min	101(10–192)	81(36–151)	1.25(0.07–2.58)
	10 insulin tests (16–20 U. i.v.)		
Fasting	135(115–152)	67(15–153)	2.01(0.83–10.10)
After insulin			
20 min	173(101–232)	83(27–149)	2.08(1.23–8.59)
40 min	270(109–373)	172(42–240)	1.57(0.96–4.78)
60 min	283(208–380)	191(97–251)	1.48(1.09–3.92)

[a] Unpublished data from our laboratory.

obtaining hematopoietic response in patients with pernicious anemia was in a relatively high range" (G12).

This corroborates the concept of Webster and Komarov, who wrote in 1933 (W6a): ". . . It seems more probable that pepsin, though, as Northrop states, itself a protein, is secreted by the gastric glands in a combination with a glycoprotein and possibly other proteins."

The association between acidic mucoproteins of the gastric juice and pepsin became more apparent with data obtained from the electrophoretic work in various laboratories. It was discussed by Grossberg *et al.* (G71), who found pepsin polydispersity along the electrophoretic partition, and was again stated by Komarov in 1953 (K25). Pugh *et al.* (P17) and Mack *et al.* (M1, M2) also encountered difficulty in separating

dissolved mucoprotein from pepsin on free boundary electrophoresis, and Norpoth arrived at similar conclusions, using electrophoresis on paper (N5a). Similar difficulties in separating acidic mucoprotein from pepsin on continuous paper electrophoresis were reported by Wada *et al.* (W2).

Since 1960 it has become evident (G18) that "glandular mucoprotein" fraction represents a complex of materials which include the following:

TABLE 6[a]
RATIO OF HEXOSES TO TYROSINE IN "GLANDULAR MUCOPROTEIN" AND "MUCOPROTEOSE" FRACTIONS OF GASTRIC COLLECTIONS FROM 15 INDIVIDUALS

		Hexoses[b] to Tyrosine[c] ratio			
		Glandular mucoprotein		Mucoproteose	
Cases	Specimens	Mean and SD	Range	Mean and SD	Range
1–7	Pooled fasting and histamine collections	0.63 ± 0.22	0.30–0.78	12.80 ± 3.10	11.07–15.17
8–15	Fasting	0.52 ± 0.13	0.40–0.71	9.36 ± 2.50	5.44–12.91
	After histamine				
	20 min	0.56 ± 0.20	0.31–0.90	11.30 ± 2.68	8.25–14.60
	40 min	0.53 ± 0.23	0.21–0.95	14.34 ± 3.54	7.86–18.35
	60 min	0.64 ± 0.23	0.32–1.00	13.69 ± 4.98	8.20–22.60

[a] Unpublished data from our laboratory.
[b] Hexoses determined by Shetlar-Badin trytophan-borate method.
[c] Tyrosine determined by Folin-Ciocalteu method.

TABLE 7[a]
HEXOSAMINE TO TYROSINE RATIO IN MUCOPROTEIN H (= MUCOPROTEOSE) AND MUCOPROTEIN V (= GLANDULAR MUCOPROTEIN) FRACTIONS OF DISSOLVED GASTRIC MUCIN COLLECTED UNDER SIMILAR CONDITIONS FROM HEIDENHAIN POUCHES OF 4 DOGS

Mucoprotein H		Mucoprotein V	
Mean	Range	Mean	Range
1.13	0.91–1.28	0.44	0.36–0.51

[a] Calculated from data of De Graef (D2a).

(1) acidic mucosubstances, in the form of sulfated aminopolysaccharides and sialomucins, (2) some pepsin, not precipitated by trichloroacetic acid, in some kind of complex formation with mucosubstances, (3) one or more of the peptic degradation products of serum albumin, and (4), degraded intrinsic factor and, related to it, "secondary vitamin B_{12} binder" (see Section 6). This complex material differs, however, from the complex contained in the "mucoproteose" fraction of dissolved

mucin which has justified the usefulness of this simple fractionation technique as shown by our data listed in Tables 3 and 6, as well as by those of DeGraef (D2a) on dog Heidenhain pouches (Table 7).

2.2.3. *Dissolved Mucoproteose*

This third fraction of the dissolved mucin is precipitated by acetone from the supernatant, after removal of dissolved mucoprotein from the solution (G26, G27, G36). The material forms a resin-like and clumping heavy precipitate, which, on drying, is an amorphous chalky white

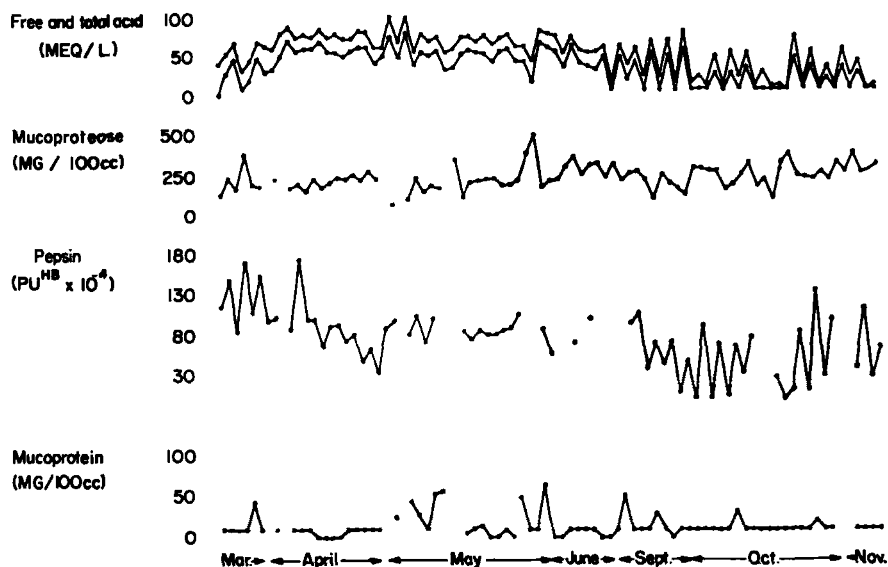

FIG. 18. Day to day analysis of fasting specimens of Tom's gastric juice. Note the roughly inverse relationship between mucoproteose and acid. From Wolf and Glass (W18).

powder. It contains only 5.7–7.3% N, as measured by the Kjeldahl method, 3.9–4.2% of tyrosine, as measured with the Folin-Ciocalteu reaction, but as much as 24–55% of reducing substances when tested by Dische's indole reaction. In addition, it yields a purple color with the Ayala "sensitized" diphenylamine reagent. Mucoproteose contains much fucose, hexosamine, and some galactose and mannose, when tested by Dische's cysteine reactions (G11). Werner (W9) found 8.2% N, 15.1% hexosamine, 2.1% uronic acid, and 3.1% sialic acid in the mucoproteose. His chromatographic analysis indicated presence of galactose and fucose

in large amounts in the mucoproteose fraction. As shown in Tables 3 and 4, mucoprotein and mucoproteose differ markedly. In addition, they also display marked individual differences in their composition, as shown by unpublished data of Rich and Schwartz from our laboratory. Material formed on autolysis of visible surface mucus in the incubator at 37°C was similar in composition to mucoproteose (G26). Norpoth (N5) confirmed that the ratio of protein to reducing substance was 7:1 in mucoprotein and only 1.5:1 in the surface mucus.

As stated in 1953 (G11): "Dissolved mucoproteose is not a chemical entity, but a complex of intermediate products of digestion of gastric mucus. For this reason, the peptide moiety of this fraction may show transitions between the first and further split products of enzymatic degradation of proteins (proteans, primary and secondary proteoses, and even peptones). This is why one part of the mucoproteose fractions is dialyzable, and disappears from the electrophoretic partition of the dialyzed gastric juice."

Mucoproteose concentration, in contrast to mucoprotein (Fig. 18) is inversely correlated with the HCl and pepsin output and volume of gastric juice (G27, G35, M52, W7). It is lowest at the peak of the secretion of fundic glands, and highest in gastric juice of patients with atrophic gastric mucosa. These results indicate that the mucoproteose fraction is partly derived from surface epithelium and possibly from cardia and pyloric glands, and that it represents the enzymatic degradation product of the visible mucus. Different responses of glandular mucoprotein and mucoproteose to histamine and peripheral vagal stimulation are well demonstrated on dog pouches by DeGraef (D2a). After histamine, while glandular mucoprotein remains unchanged, mucoproteose drops markedly. After carbachol, mucoprotein raises markedly while mucoproteose remains unchanged (Fig. 18a).

As we know at present, the mucoproteose fraction includes the following materials: (1) most of the gastric fucomucins, as well as some of the blood group substances, i.e., most of the neutral polysaccharides of the dissolved mucin fraction linked to their peptide moiety, (2) γ-globulin and probably also β-globulin, which pass into the gastric juice from the serum (G16, G42, H11, H20, H55) and probably account for the presence of mannose in this fraction (G11), (3) some of the peptidic degradation products of serum albumin and visible mucus, which are partly dialyzable (G16, K2), (4) native intrinsic factor, the related "primary vitamin B_{12} binder," and "tertiary vitamin B_{12} binder" related to the neutral mucosubstances (G14, U1, U2).

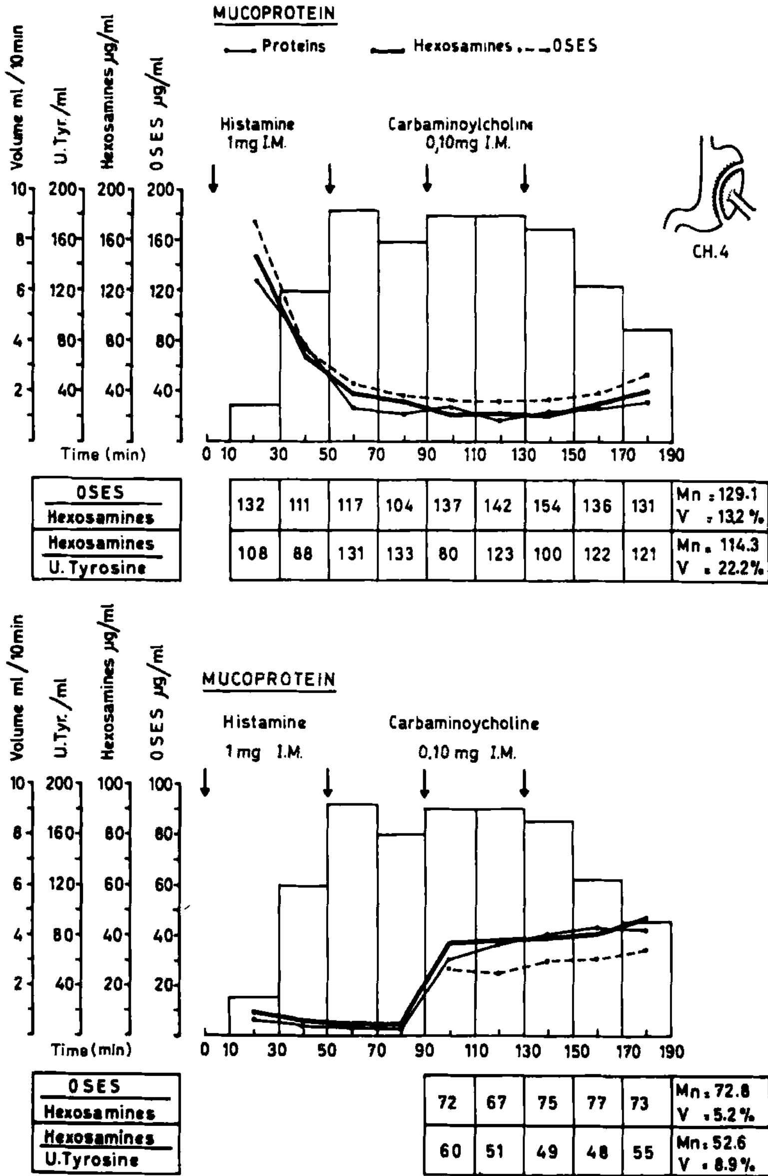

FIG. 18a. Contents of proteins (tyrosine) (●——●), hexosamine (●——●), and hexoses (●— — —●) in mucoprotein H (= mucoproteose) and mucoprotein V (= glandular mucoprotein) fractions of dissolved gastric mucin following stimulation of dog Heidenhain pouch by histamine (1 mg. i.m.) and carbaminoylcholine (0.1 mg. i.m.). From De Graef (D2a).

2.3. Methods for Quantitation of Mucus and Its Fractions

Quantitation of mucosubstances in the gastric juice has encountered difficulties, in view of their complex nature and variations in composition. Many investigators initially attempted to quantitate gastric mucin by measuring only certain physicochemical features related to the presence of mucosubstances in the gastric juice, such as the volume or viscosity of mucus and gastric juice turbidity. This purely visual estimation of mucus in the gastric content or volumetric determination after centrifugation or sedimentation has even been used recently in physiological research (H21a, N2, W11). It is absolutely inadequate, however, for scientific work because dehydration of the gel and syneresis shrink the mucus volume and, conversely, its hydration and inhibition of water increase the sol phase of the visible mucus. Mucus volume also depends upon pH level and water and electrolyte concentration of the environment. This is also true for the determination of the dissolved mucin by estimating the viscosity of the filtered gastric juice, or by measuring the speed of filtration (S21). This is inadequate, since the viscosity depends not only on the dissolved mucous components of gastric mucin, but also on contamination of gastric juice with viscous constituents of the saliva and bile, on gastric juice pH, and on the salt content.

The fact that dissolved mucin produces turbidity with acetic or trichloroacetic acid (G2, S40) has been the basis of other techniques. However, the opacity produced by various mucosubstances at the same concentration differs markedly under similar conditions, and may be caused not only by mucosubstances, but by proteins contained in gastric juice as well, lessening the value of these techniques. This applies to other methods (B35) which measure opacity development upon addition of alcohol to the total filtrate of gastric juice after sulfosalicylic acid precipitation. Here, the acid precipitation partly removes some of the products of mucus digestion, and the method is standardized on submaxillary mucin with physicochemical features different from those of gastric mucin.

Determination of total nitrogen content of the gastric juice, introduced by Wolff and Junghans (W22) and used later by others (D4), is inadequate for quantitation of gastric juice mucosubstances because proteins, peptides, and amino acids contribute to this measurement. Other authors precipitated gastric juice with methyl alcohol or acetone and determined the amount of alkali bound by the precipitate (M4–M6). These methods determined only the buffer capacity of the precipitate,

which, in addition to mucosubstances, also contains precipitated proteins.

The method of Baltzer (B7) yields better results. It consists of precipitating mucin with a mixture of sulfosalicylic acid and acetone and determining the reducing power of the precipitate. The obtained value is corrected for protein content, determined nephelometrically by sulfosalicylic acid precipitation.

Another method is the iodometric procedure described by the reviewer (G6, G7), where dissolved mucin was precipitated with 1.5 volumes of acetone at 40°C from trichloroacetic acid filtrate of the gastric juice. Mucin, in the precipitate, was quantitated by iodometric titration with the use of a standardization curve of electrodialyzed mucin solution and methylene blue as indicator. The mucin values of gastric juice determined by this method were 20–210 mg%, those of saliva 50–300 mg %. The method was subsequently used by many investigators, yielding satisfactory results (K6, K20, M25, M52, T33, W20, W21); it was considered to yield accurate results (Przylecki, cited in K20) with 6% error (Labby *et al.*, cited in T33).

The iodometric method was then modified (G23, G24) by changing the final concentration of trichloroacetic acid from 8% to 3.3%, leaving more proteose-like substances in the filtrate, and quantitating the mucosubstances in the filtrate colorimetrically by the Folin-Ciocalteu reaction with the phenol reagent, instead of iodometric titration. This colorimetric reaction determines the tyrosine and tryptophan content after alkaline hydrolysis of the total dissolved mucin.

Following this, the dissolved mucin was further fractionated in our laboratory by isoelectric precipitation of the dissolved mucoprotein fraction from its alkaline solution lowering pH < 3.5, leaving the second dissolved mucin fraction, mucoproteose, in solution (G27). The details of this fractionation technique are shown in Figure 19.

The results obtained were as follows. Concentration of the mucoproteose fraction in gastric juice was 35–700 mg/100 ml (G27). Highest values of mucoproteose were found in anacid gastric content, in patients with gastritis, chronic alcoholism (G8, G22), gastric cancer (W19), or gastric retention after vagotomy (G48). Its concentration was low following alcohol, histamine, or caffeine stimulation (D2a, G1, G27, G63), but usually increased slightly following i.v. injection of insulin (G28, G35, P14). The concentration of the other component of dissolved mucin, dissolved mucoprotein, was 0–460 mg/100 ml gastric juice; it was found at high concentration in the fasting contents of the juvenile hypersecretory stomach or of duodenal ulcer patients (G25, G30). After

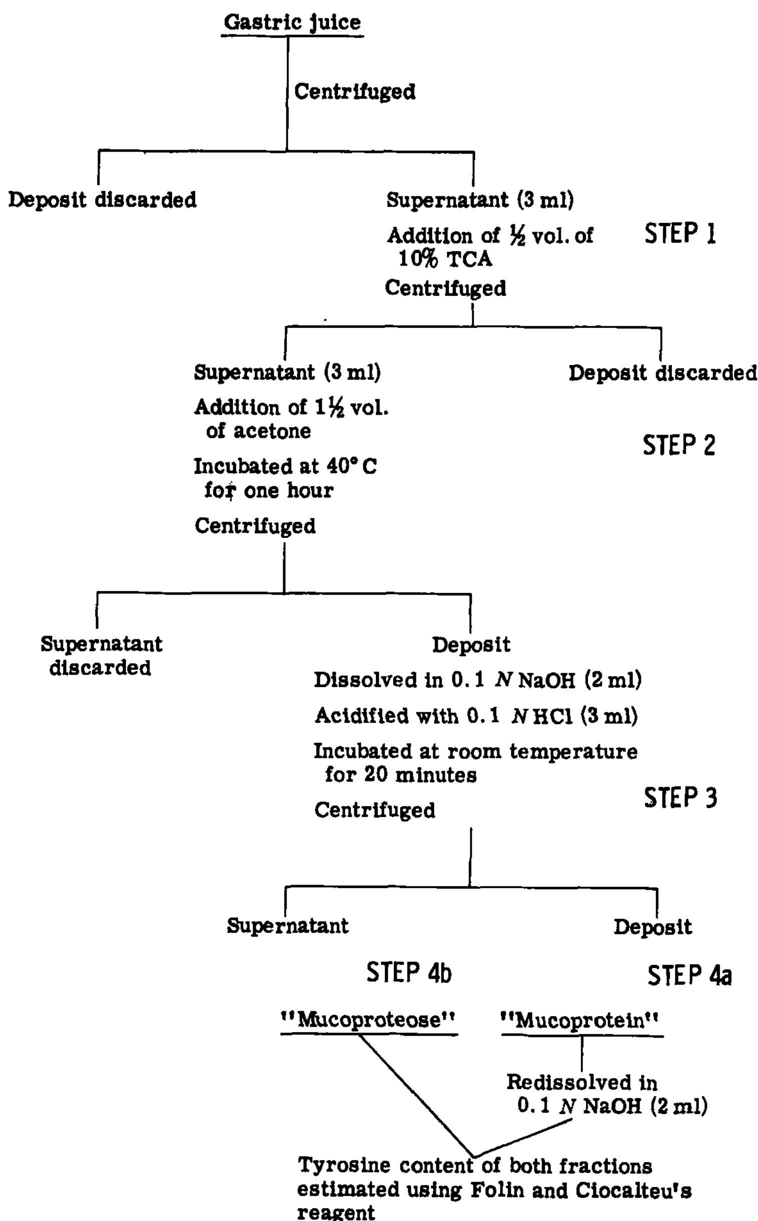

FIG. 19. Diagram of the fractional precipitation method of Glass and Boyd for the isolation of gastric mucoproteose and mucoprotein. From Schrager (S10).

central vagal stimulation with insulin, mucoprotein concentration in gastric juice rose as much as 4-fold, concomitantly with the appearance of the hypoglycemic reaction (G27–G29, G35, I1, I2). In the vagotomized human stomach, the stimulating effect of i.v. injection of insulin on mucoprotein secretion was abolished (G48).

This fractionation method was subsequently used successfully by other investigators (B11, D2a, G1, G64, H39, H40, I1, I2, N6, N7, P14, S6–S9, W7). Schrager (S10) 12 years later severely criticized it and considered that measurement of mucosubstances by determination of their peptide moiety was an improper technique for measuring the gastric juice mucosubstances. He simultaneously estimated the tyrosine and hexosamine contents of the mucoprotein and mucoproteose fractions and found no quantitative correlation between the two. He therefore concluded that tyrosine measurement was not adequate for quantitation of these fractions (see Section 2.2). These results are contradicted by recent data of DeGraef (D2a) obtained on Heidenhain pouches which show very satisfactory correlation between mucoprotein and mucoproteose assay by means of tyrosine and hexosamine quantitation (see Fig. 18a).

The unpublished data obtained in our laboratory by Rich and Schwartz on a population much larger than Schrager's show individual variations however, in the values of mucoprotein and mucoproteose calculated on the basis of tyrosine and total hexoses content (Table 6). This is easily understood, when one considers the complexity of both these "prototype fractions" discussed in Section 2.2.

It has been thought (H38, H46, H47) that methods based on quantitation of the polysaccharide moiety of gastric mucin would be more specific for the assay of mucosubstances. This leads, however, to many difficulties as well, and is not acceptable if one does not subject the mucosubstances to prior fractionation by chemical or physicochemical means (see Section 2.4).

Determination of reducing substances in native or filtered gastric juice, without acid hydrolysis, gives only relative values and is not a true reflection of the actual amount of hydrolyzable polysaccharides present. This limitation therefore applies to older methods based on this assay, as utilized by various investigators (B1–B3, K8, V1). Conversely, results obtained after hydrolysis do not duplicate well and depend on the duration of hydrolysis, acid concentration, and ratio of acid to the polysaccharide substrate present. In addition, various mucosubstances have different reducing-substance content (G11, G27) and changes in their ratio will create changes in the total reducing-substance value of the

gastric juice. Presence of proteins in gastric juice will also cause an error.

Various authors assayed only one polysaccharide component in gastric juice to measure the content of mucosubstances in gastric juice. Komarov *et al.* (K26a, S23) determined the amount of mucin by estimating uronic acid by the naphthoresorcinol method. Zaidi and Mukerji (Z1) measured the uronic acid content of gastric juice with carbazole. Piper *et al.* (P13), for this purpose, quantitated hexoses in the gastric juice with the use of the anthrone reagent, Polosa *et al.* (P15) using galactose and mannose standards, and Berg *et al.* (B14b) with the orcinol method and readings at 425 and 540 mμ. Schrager (S10) and Robert *et al.* (R7, R8) determined hexosamine content of gastric juice by the Boas technique.

Other workers determined the entire carbohydrate spectrum of human gastric juice in an attempt to evaluate the composition of gastric mucosubstances. Richmond *et al.* (R4) and Hoskins and Zamcheck (H50) studied a large number of individual gastric juices; in our laboratory (G55) a large pool of normal gastric juices was studied for hexoses, hexosamine, fucose, sialic acid, uronic acid, and total carbohydrate content. Results obtained in normals and patients with various gastric diseases are summarized in Tables 8 and 9.

There were marked variations in the ratio of various sugars between individual juices. The mean figures of Richmond *et al.* (R4) demonstrated that total hexoses formed about 32% of the total sugars in pernicious anemia, but 41–43% in duodenal ulcer and cancer of the stomach. Hexosamine constituted about 28% in cancer of the stomach, and in gastric ulcer on the average 45% of the total sugars. Individual differences between various cases were still greater. Differences in the ratios of hexosamine to hexoses were noted by Menguy and Masters (M28) in dog gastric juices. Thus, it is inadequate to evaluate the total concentration of mucous substances by assaying only one sugar, as was done in the past by quantitating hexoses (B14b, P13), hexosamine (R7, S10), or uronic acid (Z1) in gastric juice for this purpose. These shortcomings explain the differences between the findings obtained by different methods (see Fig. 20). A typical example is the study of the effect of various stimulating and blocking agents on the output and concentration of gastric mucosubstances. Plummer *et al.* (P14), using the tyrosine method (G27), found a considerable drop in mucoprotein concentration and a slight rise in mucoproteose concentration following administration of anticholinergic drugs, while Piper, who determined only total hexoses in gastric juice (P13), concluded that there was a rise in mucus concentration with little change in the actual output of mucus under similar conditions.

TABLE 8
CONTENT OF CARBOHYDRATES AND PROTEINS IN GASTRIC JUICE

Materials	Samples	Measurement unit	N	Proteins	Total carbohydrates	Hexoses	Hexosamine	Fucose	Sialic acid	Uronic acid	Authors, years, ref. nos.
Human native gastric juice	From 10–16 normals	Means in mg/100 ml		330.0	87.9	32.1	32.7	13.8	7.3	2.0	Richmond *et al.* (1955); (R4)
	From 19–26 duod. ulcers			212.0	84.5	34.7	30.2	9.7	8.1	1.8	
	From 7–12 gastr. ulcers			288.0	101.3	36.0	45.9	16.7	9.8	1.9	
	From 8–11 gastr. cancers			482.0	187.2	81.2	52.4	30.7	20.8	2.1	
	From 5–6 pern. anemias			242.0	187.9	60.0	74.3	33.3	18.2	2.1	
Human dial and lyoph. gastric juice	Pool from 13 normals & duod. ulcers (fast. & aft. hist.)	% of w/w			28.4	10.4 13.1 [a]	8.7	5.1	2.2 2.8 [a]	0.4	Glass *et al.* (1956); (G55)
	From 32 patients (fasting juice)	Means in % of w/w with % deviat. from means	9.4 ± 49	64.0 ± 18		25.0 ± 44	10.6 ± 39	5.9 ± 47	2.1 ± 48		Hoskins and Zamcheck (1963); (H50)
	From 4 collections in same pt.	Means in % of w/w with % deviat. from means	11.6 ± 5	82.0 ± 6	28.8	16.8 ± 32	6.9 ± 16	3.8 ± 13	1.3 ± 24		

TABLE 8 (*Continued*)

Materials	Samples	Measurement unit	N	Proteins	Total carbohydrates	Hexoses	Hexosamine	Fucose	Sialic acid	Uronic acid	Authors, years, ref. nos.
	From 13 normals	Means in mg%		280.0		(1) 231.0 (2) 245.0					Berg *et al.* (1960); (B15)
	From 18 superf. gastritis			280.0		(1) 391.0 (2) 518.0					
	From 8 atr. gastr.			270.0		(1) 298.0 (2) 335.0					
Dog gastric juice	Heidenhain pouch	% w/w					14.4	3.0	3.0	3.7	Werner (1953); (W9)
	Acetylcholine stimulated mucus from Heidenhain pouch	µmole/ml	47–138				1.6–3.2				Horowitz and Hollander (1961); (H44)
	25 samples from 6 Heidenhain pouches	Range in % w/w Range in mg/100 ml	1.6–11.9 24.8–121.6			16.0–122.4					Van Geertruyden *et al.* (1964); (V2)

[a] By two different methods.
(1) Read at 425 mµ (for galactose).
(2) Read at 540 mµ (for mannose).

TABLE 9

MOLAR RATIOS OF VARIOUS CARBOHYDRATES IN GASTRIC MUCOSA AND GASTRIC JUICE (WITH HEXOSAMINE = 1) AS RECALCULATED FROM DATA OF VARIOUS AUTHORS

Material	Specimen	Hexo-samine	Hexoses	Fucose	Sialic acid	Uronic acid	Authors, year, ref. no.
Human native gastric juice	Means from 10–16 normals	1	0.98	0.46	0.12	0.06	Richmond *et al.* (1955); (R4)
	Means from 19–26 duod. ulcers	1	1.14	0.35	0.16	0.06	
	Means from 7–12 gastr. ulcers	1	0.78	0.40	0.12	0.04	
	Means from 8–11 gastric cancers	1	1.55	0.65	0.24	0.04	
	Means from 5–6 pern. anemias	1	0.81	0.50	0.14	0.03	
Human dialyzed and lyoph. gastric juice	Pool from normals and duod. ulcers (fast. and after histamine)	1	1.19	0.64	0.15	0.04	Glass *et al.* (1956); (G55)
Human dialyzed and lyoph. gastric juice	Means from 18–32 patients	1	2.40	0.61	0.11		Hoskins and Zamcheck (1963); (H50)
Human gastric mucosa		1	0.67[a]	0.40	0.13		Masamune *et al.* (1948); (M13a)
		1	0.90[a]	0.50–0.54	0.02		Kawasaki (1958); (K11)
Dog gastric juice	From Heidenhain pouch	1		0.23	0.12	0.23	Werner (1953); (W9)
	From Heidenhain pouch (acid and anacid)	1		0.25	0.07–0.12		Horowitz *et al.* (1961); (H48)
Pig gastric mucosa	From fundus and body (1)	1	0.56[a]	0.26		0.25	Werner (1953); (W9)
	(2)	1	0.53[a]	0.45		0.14	
	From pyloric canal (1)		0.77[a]	0.25		0.09	Werner (1953); (W9)
	(2)		0.69[a]	0.30		0.04	

[a] Calculated as galactose.

(1) 55% ammonium sulfate saturation.

(2) 70–80% ammonium sulfate saturation.

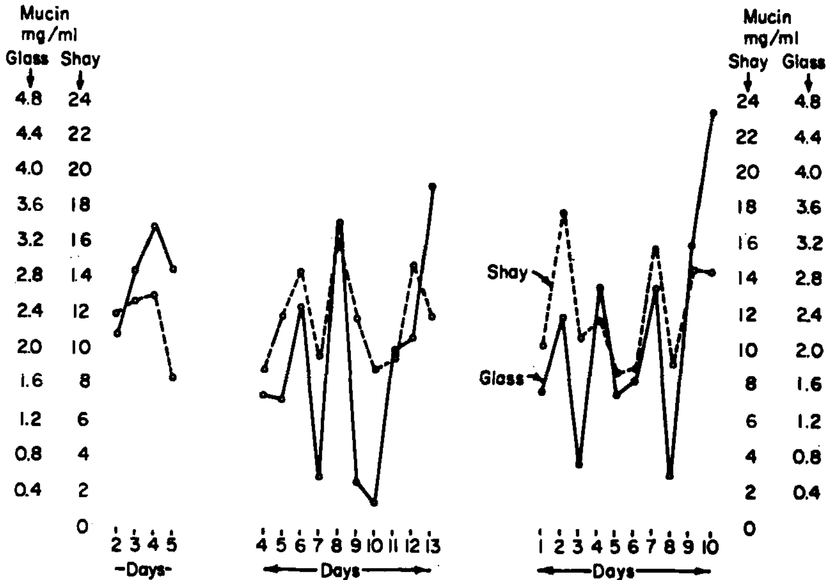

FIG. 20. Comparison of results of two methods for the chemical estimation of gastric mucin. From Wolf and Wolff (W21).

2.4. Composition of Gastric Mucosubstances

Gastric mucosubstances have been generally classified as belonging to the epithelial mucin group. This categorization is correct only if it is understood that "epithelial" refers not only to the gastric surface epithelium, but also to the secretory epithelium of the neck of the fundic glands and to the epithelium of pyloric glands, wherefrom the major part of gastric mucosubstances is derived.

Gastric mucosubstances belong to several classes, which are as follows.

2.4.1. *Acid (Sulfated) Mucopolysaccharides*

These materials are also called *aminopolysaccharides* by Kent (K15, K16) or *mucopolyuronides* by Blix (B22). They contain uronic (hexuronic) acid, usually D-glucuronic acid. Only one other uronic acid occurring together with D-glucuronic acid, namely L-iduronic acid, has been found in chondroitin sulfate B (dermatan). In addition to uronic acid, most of these materials, with the exception of hyaluronic acid, contain sulfates (in ester form). They also contain hexosamine as glucosamine or galactosamine, frequently in acetylated form.

Aminopolysaccharides have a high negative charge, due to presence

of uronic acid and sulfates, and are highly polymerized substances, with the disaccharides linked in straight unbranched chains. In the human body including the stomach, the acid mucopolysaccharides are loosely bound by hydrogen or salt linkage with peptide chains or proteins, forming mucopeptides or mucoproteins. The ionic bond can be easily dissociated by changes in pH or ionic strength of the solvent.

In the early twenties, Levene and Lopez-Suarez (L8–L8c) concluded that the sulfated acid mucopolysaccharide found in the gastrointestinal tract is mucoitin sulfuric acid, which they had isolated from hog gastric mucosa. Although the expected proportion of nitrogen, glucosamine, glucuronic acid, and acetyl groups in this material should be equimolar, the ratio of N:S they found was 4:1. Then Komarov (K22) reported isolation of the mucoitin sulfuric acid from canine gastric juice, with a yield of 22–46 mg per liter and having N:S ratio of 1:2. He isolated two preparations from alkaline gastric mucus consisting of what was thought to be a mucoprotein, including mucoitin sulfuric acid. These materials contained 13.3% N, 1.28–1.42% uronic acid (by the naphthoresorcinol method), 6.0–6.2% reducing substances (after acid hydrolysis), and 14.5–14.7% sugar (expressed as glucose).

Meyer *et al.* (M37) isolated two different types of carbohydrate material from hog gastric mucin. One of them had equimolecular amounts of glucosamine, hexuronic acid, acetyl groups, and sulfate, which was therefore thought to be mucoitin sulfuric acid. The second was a neutral polysaccharide, which will be discussed below. Wolfrom and Rice identified (W23) the hexuronic acid in this preparation as D-glucuronic acid.

Werner (W9) also prepared mucoitin sulfuric acid from pig gastric mucosa and pig gastric mucin by Meyer's method. The composition of these materials corresponds grossly to that of Meyer. Werner (W9) found large amounts of mucoitin sulfuric acid in the body of hog stomach, while the pyloric part (canalis) contained very little. His analytical figures for the mucoitin sulfuric acid prepared from hog gastric mucosal scrapings were similar to those obtained by Meyer, namely, 4% N, 33% hexosamine, 1% fucose, 26% glucuronic acid, and 11% sulfate. Composition of extracts from pig mucosa before and after proteolytic digestion, submitted to stepwise ethanol precipitation (20–80%), was studied by Werner (W9). He concluded that the acid mucopolysaccharides in dialyzed digests of mucosal scrapings of the stomach form about 22% of total carbohydrates. He also noted that there was far less mucoitin sulfuric acid in gastric mucosa and secretions than fucomucan. It was subsequently shown by Smith *et al.* (S25–S27) that mucoitin sulfuric acid is probably a mixture of heparin and two chondroitin sulfates.

Since that time, the existence of mucoitin sulfuric acid has been in doubt.

The presence of radioactive sulfates was demonstrated by Bélanger and Crevier (B13, C28, C29) in the mucoprotein of rat gastric juice, after injection of sulfate-S^{35} into rats. This material had the fastest electrophoretic mobility, and S^{35} could be detected also in the dissolved mucoprotein fraction prepared by our method (G27) from rat gastric juice. The authors' original concept, that this material was derived from the mucoid neck cells of the fundic glands, was later revised by Bélanger (B12). With better resolving power of autoradiographic films, it became apparent that the labeled sulfate-S^{35} was derived from the transitional (proliferative) layer of the surface epithelium at the bottom of the crypts, and not from mucoid neck cells (B12). This was in line with Florey's previous findings (F5, F6) and corroborated by recent histochemical work of Gerard on dogs (G3).

Various authors reported presence of other acid mucopolysaccharides in hog gastric mucosa (cit. H43), such as "mucoitin" (T1) having no sulfates, or "gastroitin sulfuric acid" allegedly containing galacturonic acid according to Sato (S2, S3) (perhaps iduronic acid?). Cresseri (C26) also isolated an acidic mucopolysaccharide from human acid gastric juice (mucoitin sulfuric acid?) with a yield of 7.4–7.9 mg/100 ml, when assayed by a turbidimetric method with acetyltrimethylammonium bromide.

In early work, we found only less than 0.4 mg uronic acid/100 ml acid gastric juice (after correction for hexose color contribution), using the carbazole method (G55). In more recent work from our laboratory Ibanez (see G19), using the Gregory modification of Dische's carbazole method, found relatively significant amounts of hexuronic acid in the first effluents from the Amberlite IRC-150 column (citrate buffer 0.2 *M*, pH 3.2–3.5), ranging from 3.4 to 10.0% of the total carbohydrates eluted by this buffer. When corrected for galactose contribution to color development, the values read, in most instances, below 1% of the total carbohydrates, with one exception where it amounted to 4.4%. Horowitz and Hollander (cit. H43) found approximately 0.2% uronic acid in organic solids of resting and of acetylcholine-stimulated gastric secretion of the dog.

The biological activity of acid mucopolysaccharides is discussed in Section 9. They are instrumental in hemagglutination inhibition of viruses, in enhancing virulence of bacteria (S25–S27), and in antilipemic (C1, C2, P4) and lipotropic activity (C4). They were also reported to have strong antipeptic activity (K21, K24) which we (unpublished) found, however, to be rather low.

2.4.2. *Fucomucins*

Fucomucins (Blix) represent another type of neutral glycoprotein. They consist of a complex formed by a protein moiety with a carbohydrate polymer called "fucomucan" by Werner (W9) and consisting of *N*-acetylglucosamine, *N*-acetylgalactosamine, galactose, and fucose (see Fig. 21). The polysaccharide moiety comprises 2/3 of the glycoprotein, the rest being protein. The two are firmly attached to each other by

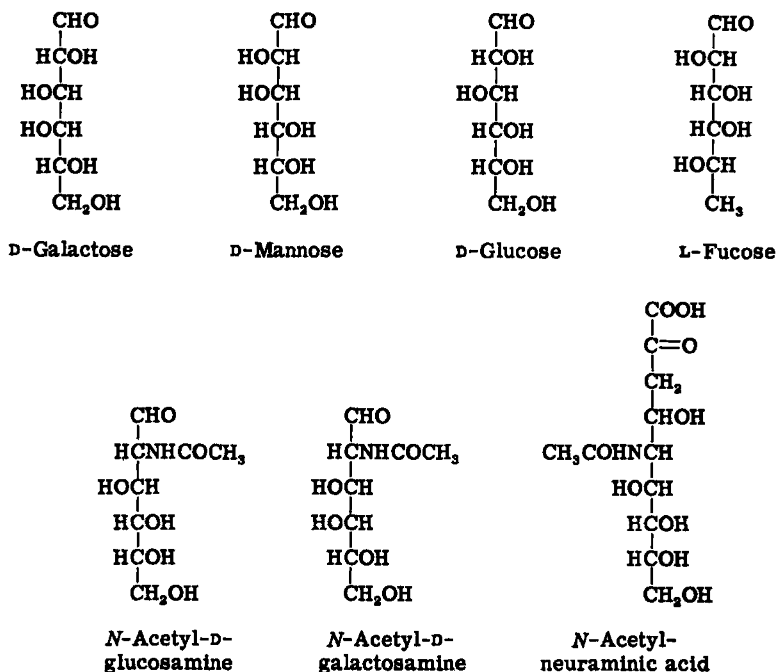

FIG. 21. Sugar constituents of animal glycoproteins. *N*–Acetylneuraminic acid is shown as an example of the sialic acids. From Spiro (S34).

covalent linkage. The structure of this polysaccharide is thought to be a basic polymer chain, from which short multiple side chains of fucose, hexosamine, and oligosaccharide residues branch out. A typical example of fucomucins is the blood group antigens (S35). Though they vary slightly in composition, they retain the basic features of a "fucomucin"-type of neutral glycoprotein. Their peptide part constitutes 20–30% of the total material. It is remarkably rich in threonine, contains only small amounts of tyrosine, phenylalanine, and histidine, and, according to Werner, no (or very little) methionine (W9) (see Section 5).

The composition of neutral glycoproteins and blood group substances

isolated from the gastric mucosa of man (yield = 0.03–0.16% of starting material) by Kawasaki and Masamune *et al.* (K11, M13a) was 1.8–2.5 moles of N, 0.67–0.90 moles of galactose, 0.40–0.54 moles of fucose, and 0.02–0.13 moles of sialic acid per 1 mole of hexosamine. Meyer and Bendich (see K1) found similar ratios of fucose and N to hexosamine for hog mucin and hog mucous lining, respectively.

Neutral glycoproteins appear to be large molecules. The molecular weight of glycoproteins from submaxillary or cervical mucin is assumed to be in the 1–4 million range (see S34). In human gastric juice, neutral glycoproteins form the first effluent peak on gel filtration on Sephadex G-200 column (G45). They precede all other materials excluded from this gel, suggesting a molecular weight of at least 200,000 (G45).

Contrary to Levene, Schmiedeberg (cited in K25) assumed that the polysaccharide component of body fluids and secretions is not mucoitin sulfuric acid, but a material he called "hyaloidin type I." He reported it to consist of one acetyl group, two hexoses, and two hexosamines, and to be devoid of uronic and sulfuric acid radicals. Meyer *et al.* (M37) isolated two polysaccharides from commercial hog mucins; one they identified with Levene's mucoitin sulfuric acid, the other they called "neutral polysaccharide" and identified as an acetylglucosamine-galactoside, devoid of uronic acid and sulfates. Thus, the concepts of Levene and of Schmiedeberg became reconciled.

Meyer (M34, M35) classified the neutral polysaccharide of hog gastric mucin as "mucoid," since it contained over 4% hexosamine. This probably corresponded to the mixture of the carbohydrate moieties of fucomucin and sialomucin, whereas his acidic mucopolysaccharide (mucoitin sulfuric acid) consisted perhaps of a mixture of acidic aminopolysaccharides (mucopolyuronides) and possibly some sialic acid.

The neutral polysaccharides of the human gastric juice and mucus form the carbohydrate part of what Komarov (K25) called "mucoprotein 2" or "neutral mucoprotein," while his "mucoprotein 1" or "acid mucoprotein" contained "mucoitin sulfuric acid."

2.4.3. *Sialomucins*

Sialomucins [Odin (O2)] or sialoproteins [Werner (W9)] are another type of mucosubstance found in the stomach. Their carbohydrate moiety is composed of equimolecular amounts of acetylhexosamine (usually galactosamine) and sialic acid. The latter is a group name for several materials derived from the same basic substance, neuraminic acid. In human mucosubstances, the *N*-acetylneuraminic acid is the main form of sialic acid (F1, F2). In hog stomach, the sialic acid consists of 80%

N-glycolylneuraminic acid, according to Attafelt *et al.* (A11), the rest being acetylneuraminic acid. Sialic acids are relatively strong, very reactive, and labile. In sialomucins, they are attached as an end group to hexosamines (galactosamine), similarly as fucose is attached to fucomucins. They may be split off sialomucins by acid hydrolysis or some bacterial enzymes such as sialidase (neuraminidase) from *Vibrio cholerae* or *Clostridium perfringens*, as well as by influenza virus (see B22). Removal of sialic acid by sialidase does not affect the susceptibility of mucus to proteolytic degradation (H49).

The sialomucin (sialoprotein) consists of acidic carbohydrate (sialic acid + hexosamine) and protein. Peptide chains are linked to the mucopolysaccharide by a covalent linkage, so that both carbohydrate and protein moieties cannot be dissociated, except by drastic treatment resulting in denaturation. Sialomucins belong to the acidic glycoproteins because of high negative charge. After removal of sialic acid by sialidase treatment, the isoelectric point of the sialomucin is markedly elevated. The content of hydroxyamino acids in the protein moiety of sialomucins is characteristically high, as shown by Hashimoto *et al.* (H3).

Werner (W9) found 3.0% sialic acid in dog gastric juice, as compared with 14.4% hexosamine, 3.7% hexuronic acid, and 3.0% fucose per dry weight of nondialyzable gastric solids. Chromatographic analysis showed hexosamine, galactose, and fucose. Anacid canine gastric secretion after acetylcholine stimulation (H43–H45) showed a sialic acid to hexosamine ratio of 0.07 and sialic acid to fucose ratio of 0.2, the acid gastric secretion 0.12 and 0.5, respectively.

Presence of sialic acid in the normal human stomach was also first reported by Werner (W9), who found 1.9–4.3% sialic acid per dry weight in various mucus and dissolved mucin fractions. A lyophilized pool of normal acid gastric juice analyzed in our laboratory (G55, G56) contained on the average 2.5% sialic acid (w/w). This figure was an average of determinations performed by two techniques, the Werner-Bial and Ayala methods. The ratio of sialic acid to hexosamine was 0.29 and to fucose 0.49 (w/w). Nondialyzable substances of human fasting gastric juice, as determined by Hoskins and Zamcheck (H50), included 2.1% sialic acid, with standard deviation of 48% from the mean value. The mean ratio (by weight) of sialic acid to hexosamine was 0.2, and to fucose 0.35.

Sialic acid-containing mucosubstances had a higher negative charge than fucomucins, which were more neutral on paper electrophoresis of gastric juice. As a result, we found sialic acid concentration highest in the most anodic area of the electrophoretic partition of the gastric juice,

where it formed 15.7–21.0% of total carbohydrates, as compared to the cathodic fractions where it formed only 2.6–6.7% (G19, G55, G56). The molar ratio of sialic acid to hexosamine in the two most anodic fractions was 0.86–1.41, while in the two most cathodic fractions the ratio was only 0.14–0.31, with intermediary ratios for the fractions between. The ratio of sialic acid to fucose shows a similar trend: the two most anodic fractions contained these materials in a ratio of 0.58–0.76, while in the two most cathodic fractions it was only 0.21–0.25, with intermediate values between.

According to Dische (D5), sialic acid and fucose may substitute for each other in various mucosubstances of the body. We observed a similar situation along the electrophoretic partition of human gastric juice (G19, G56). When the sums of fucose and sialic acid in each of the fractions were expressed in moles, they varied from 27.8 to 35.2%, although variations of sialic acid were greater by far.

Richmond *et al.* (R4) reported that sialic acid content in pernicious anemia and gastric cancer juice is about 2–3 times higher than in gastric juice of normals or patients with duodenal or gastric ulcer (see Table 8). Wada *et al.* (W3), using the diphenylamine reaction, found high values in gastric cancer juices to which they assigned diagnostic significance (Fig. 22). Since the diphenylamine reaction determines sialic acid content and yields readings similar to those obtained with the Bial method (G55), high diphenylamine reaction values obviously suggested sialic acid increase in gastric juice of these patients. However, since those with pernicious anemia and atrophic gastritis also demonstrate high sialic acid values, as shown by Richmond *et al.* (R4), this reaction is probably not specific for malignancy, but characteristic for histamine-fast anacidity. It has been argued that content of sialic acid in acid gastric juice is lower because sialic acid is split off from sialomucins at a low pH and is consequently lost during dialysis. This is not the case in gastric juice of patients with histamine-fast anacidity. It should be pointed out, however, that increased content of sialic acid was reported in gastric cancer tissue itself, and in the areas adjacent to cancer of the gastric mucosa (B9). One relatively purified sialomucin was extracted from gastric mucosa of cancer patients by Kawasaki and Masamune (K11, M13, M14), using ammonium sulfate and ethanol precipitation in the presence of barium sulfate. Here the molar ratio of various sugars was 2.5 moles *N*-acetylhexosamine, 2.2 moles D-galactose, and 0.65 mole of 1-fucose per mole sialic acid.

Relationship of sialomucins to cancer of the stomach is demonstrated by the following: (1) high concentration of sialic acid in the cancer

tissue and in the adjacent areas of the gastric mucosa (B9); (2) high concentration of sialic acid in gastric cancer juice, as well as in juice of pernicious anemia patients (R4); (3) high concentration of material reacting with diphenylamine, known to be sialic acid, in gastric contents of gastric cancer patients (W3); (4) presence of acid glycoproteins probably related to sialomucins in the cancerous stomach, as shown histochemically (M54).

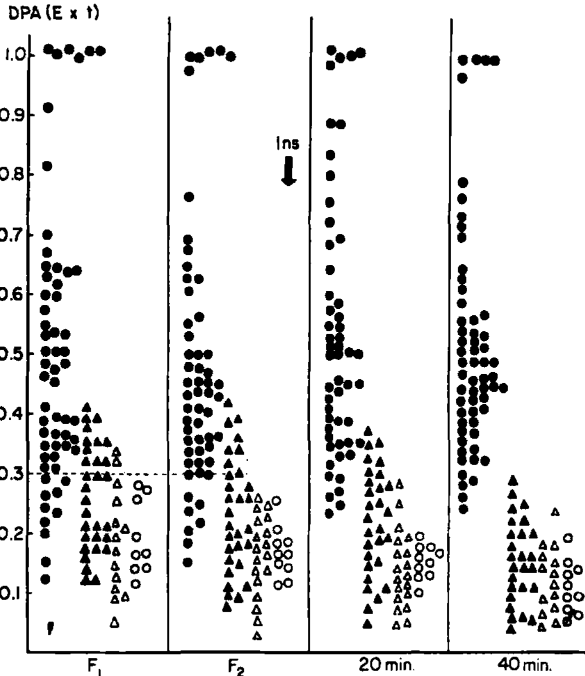

FIG. 22. Optical density of the diphenylamine reaction in the gastric juice in various gastric diseases and in normal controls.

KEY: ● gastric cancer; ▲ chronic gastritis and chronic ulcer; △ recent peptic ulcer; ○ normal controls. From Wada *et al.* (W3).

Subtle changes in pH, particularly within the acid range, may break some of the bonds of the carbohydrate moiety and liberate sialic acid. Submaxillary sialomucin loses its sialic acid at pH 3 and 37°C, and fucose and galactose are also slowly liberated from gastric fucomucins at pH 1.1–1.8 at 37°, according to Waldron Edward (W5).

2.4.4. *Glycoproteins of Dihexose-Hexosamine Type*

These glycoproteins are similar to serum glycoproteins and contain two equivalents of hexoses (mannose and galactose) and one of acetyl-

hexosamine (B22). Existence of this type of glycoprotein in gastric juice, though not well documented, is suggested by the following findings: (1) presence of mannose in the gastric mucin (G11) and gastric juice (B14), (2) passage of serum γ-globulins and of α-globulins into gastric juice (G17, H11, H20), both of which are known to contain this glycoprotein (B22, S34), (3) very high ratio of hexoses to hexosamine, as found in our laboratory in eluates from the paper electrophoretic partition of pooled normal acid gastric juice, in the area corresponding in mobility (G56) and localization to γ-globulin.

2.5. Biological Activities of Gastric Mucosubstances

The biological activities of mucosubstances comprise the following:

(1) Blood group substance activity (see Section 5)
(2) Possible relationship to intrinsic factor (see Section 6)
(3) Antianemia and nitrogen-sparing activity (see Section 9.4)
(4) Growth factor activity (see Section 9.5)
(5) *Lactobacillus bifidus* growth factor activity (see Section 9.6)
(6) Lipid absorption promotion (see Section 9.8)
(7) Lipid clearing from serum (see Section 9.9)
(8) Lipotropic effect (see Section 9.10)
(9) Virulence-enhancing action (see Section 9.13)

Other possible activities are listed in Sections 9.11, 9.12, and 9.14. It is certainly of interest that each of the classes of mucosubstances is probably endowed with its own biological activity, as follows:

Acidic (sulfated) mucopolysaccharides (aminopolysaccharides), which form only a small part of total mucosubstances, are associated with bacterial virulence-enhancing ability (S25–S27), as well as lipotropic (C4) and lipid-clearing effects (C1, C2, R11).

Fucomucins form the bulk of polysaccharides in gastric juice (and visible mucus), and are associated with blood group substance activity (Section 5), *Lactobacillus bifidus* factor (G78, S38) as well as rat growth (T31), nitrogen-sparing (P7, P8), and hematopoiesis-promoting ability in rats (C27). Sialomucins, which form a smaller group, are related to viral hemagglutination inhibition (B22). Finally, the dihexose-hexosamine types of glycoprotein probably penetrate gastric juice with the serum globulins, and are perhaps associated with antibody and gastrone activity (see Sections 7 and 8).

These four polysaccharides may form complexes between themselves which have not yet been resolved. The question as to whether these various polysaccharides are linked to similar basic peptide chains, or are bonded with various protein or peptide moieties, is still open.

3. Serum Proteins

3.1. Chemical Detection

The presence of serum proteins in gastric juice under pathological conditions has been suspected for 60 years, but its early documentation was inadequate because of the unavailability of satisfactory methods. As far back as 1903, Salomon (S1) reported the exudation of serum into the gastric lumen from ulcerated surfaces of cancerous gastric lesions. He reported the presence of large amounts of materials precipitating with Esbach reagent, which contains picric acid and has been used in the past for quantitation of albumin in urine and in gastric washings from patients with gastric cancer. Salomon also advocated this test as an aid in diagnosing gastric cancer. A few years later, Wolff and Junghans determined total nitrogen and protein content in gastric juice by the nephelometric method, after precipitating gastric juice with a reagent containing phosphotungstic acid in alcohol (W22). They found large amounts of nitrogen and abundant phosphotungstic precipitate not only in juices of patients with gastric achylia and gastric cancer, but of those with gastric hypersecretion as well. Therefore it was concluded that a high amount of protein precipitable with phosphotungstic acid, as well as a high nitrogen content in the absence of hydrochloric acid and pepsin in gastric juice, was highly suspicious for gastric malignancy.

About 30 years ago, Katsch (K8) described so-called *gastritis serosa*, supposedly characterized by an exudation of serum from the inflamed mucous membrane into the gastric lumen. He attempted to differentiate it from the *gastritis mucosa* by determining the total reducing substances as a measure of the carbohydrate content in gastric juice, and total proteins in gastric juice by Baltzer's nephelometric method (B) following sulfosalicylic acid precipitation. The large content of reducing substances was suspected to be pathognomonic for *gastritis mucosa*, and the large amount of proteins for *gastritis serosa*. Katsch (K8) found the latter in some infectious and toxic conditions of the stomach, as well as in achylia of patients with pernicious anemia. Despite some merits, these entities have, however, not been generally accepted.

More recently, Berg *et al.* (B14b) determined total proteins and total hexoses in gastric juice of normals and patients with superficial and atrophic gastritis, by the biuret and orcinol methods, as well as by paper electrophoresis followed by amido black and PAS staining. Normal gastric juice contained on the average 280 mg/100 ml of proteins and 230 mg/ml of hexoses, but juices of patients with superficial gastritis showed an increased carbohydrate content. Conversely, in patients with

atrophic gastritis, there was an increase in proteins and a strong albumin band was present in the electropherograms of gastric juice. This substantiated Katsch's concept of diagnostic differentiation of these two conditions.

Presence of proteins in gastric juice was also demonstrated by other methods. Martin (M9) precipitated proteins in gastric juice by ammonium sulfate at pH 3.6 or by a mixture of acetone and acetic acid. He described precipitated material as "gastroglobulin" because of its globulin features. This precipitate, however, resembled the dissolved mucin of Webster and Komarov (W6a) (see Section 2.2) and not serum globulins. Others (B10) precipitated gastric proteins by 50% saturation with ammonium sulfate at pH 6.6 and 37°C, and quantitated it by paper electrophoresis. Teichmann (T27) used the biuret technique to demonstrate and quantitate gastric juice proteins, whereas Norpoth *et al.* (N6, N7) precipitated gastric juice with alcohol, and determined the "protein content" nephelometrically. It is obvious that these methods were insufficient for quantitating gastric proteins and differentiating them from gastric mucosubstances. Results obtained were masked by the co-precipitation of mucosubstances and by peptide interference.

In 1933, Henning and Norpoth (H14), using a "dry drop" technique, found an increased protein ring on drying gastric juice on a slide in some patients with gastric atrophy, carcinoma, or gastritis. This they related to an increased concentration of proteins in the gastric juice.

Norpoth *et al.* (N7) then determined total proteins in gastric juice by the biuret method, total dissolved mucin by the tyrosine method (G27), and total acetylated hexosamine with the Ehrlich reagent. From these values, they attempted to determine the presence of abnormal (inflammatory) proteins in gastric juice. By deducting the proteins of the dissolved mucin from the total protein value, they found an increased amount of abnormal proteins, exceeding the upper limit of normal (700 mg/100 ml) in gastric juices of patients with gastritis, gastric ulcer, and pernicious anemia.

When Yamakawa (Y2) studied the gastric juice proteins in regard to resistance to alkali, formalin, and tryptic digestion, he found that most were more resistant to these agents than serum proteins. He opposed, therefore, the concept that proteins of gastric juice are derived from serum in which, as we now know, he was only partly correct.

3.2. Passage of I^{131}-Labeled Albumin into Gastric Juice

In 1957, Citrin *et al.* reported the first clear-cut demonstration of the passage of serum albumin into the gastric juice in a patient with Me-

netrier's disease (giant hypertrophic gastritis) with hypoalbuminemia and peripheral edemas (C18). When I^{131}-labeled albumin was injected i.v. as a tracer, a reduced albumin pool and an accelerated turnover were found; a large amount of protein-bound radioactivity was also detected in patients' gastric juice, which was precipitable with trichloroacetic acid. We found, by means of paper electrophoresis, that this juice (forwarded to us by the authors for the assay) contained large amounts of amido black-stainable protein materials, having serum albumin mobility. (This was the first instance that excessive passage of serum albumin into the gastric lumen was documented by paper electrophoresis of gastric juice.) Concentration of albumin was about 400 mg % which, at an assumed daily output of 2 liters of gastric juice, yielded an estimated figure of 8 g albumin (one half of the albumin daily turnover) passing into the gastric lumen in 24 hours.

More recently, Kimbel *et al.* (K17), Birke *et al.* (B17), and Wetterfors *et al.* (W10) studied the passage of I^{131}-labeled albumin injected i.v. into the gastric lumen in a large group of normal controls and patients with various gastric diseases. They found presence of I^{131} following injection in the gastric lumen in all cases, indicating physiological passage of serum albumin into the gastric juice. Data obtained by means of paper electrophoresis and autoradiography of electrophoretic strips supported this conclusion. It was suggested that 1.3 g albumin, i.e., 4.4% of the retained albumin dose, passes into the stomach in 24 hours, and that the gastrointestinal tract is one of the most important sites of the physiological catabolism of serum albumin.

Ullberg *et al.* (U4) injected I^{131}-labeled albumin into cats and found it to pass into the gastric lumen. Serial autoradiographies demonstrated the presence of labeled albumin in surface epithelium and the area of the gastric glands. Horowitz and Hollander (H45) injected I^{131}-labeled serum albumin into dogs and found radioactivity in the anacid mucus and its electropherograms, corresponding in location to the albumin band (Fig. 23). When this mucus was acted upon by the acid-pepsin containing gastric juice, the serum albumin was apparently digested by pepsin and was no longer detected by electrophoresis. In the same laboratory, I^{131}-labeled serum albumin or γ-globulin was injected i.v. into several human subjects and gastric juice was collected the following day for the radioactivity assay (C23). Radioactivity was detected in normal gastric juice after i.v. injection of both labeled albumin and globulin, which was considered to be proof of the physiological passage of serum proteins into the gastric juice. However, not enough evidence

was presented as to the fact that radioactivity in gastric juice was still associated with protein.

Ishimori in our laboratory injected I^{131}-labeled albumin i.v. into more than 20 patients with various gastric disease and controls to ascertain the passage of labeled albumin into the gastric juice at various time intervals after injection, extending up to 7 days. Methods used for

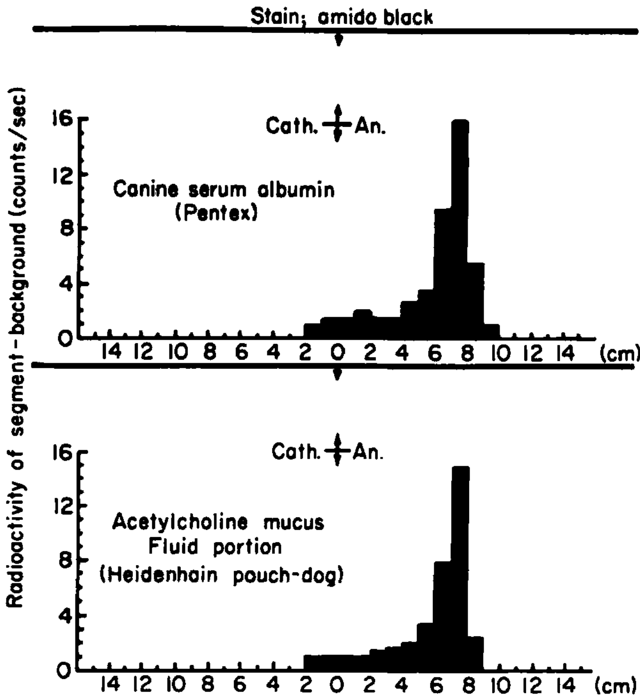

FIG. 23. Distribution of protein and protein-bound-I^{131}. Paper electrophoresis; Veronal buffer; pH = 8.6; $\Gamma/2 = 0.075$. From Horowitz and Hollander (H45).

identification of protein-bound iodine included: (1) paper electrophoresis of concentrated *in situ* neutralized gastric juice followed by differential staining, autoradiography, and radioactivity counting in cut segments of gastric juice electropherograms; (2) chromatography of gastric juice through an ion-exchange column which retained free I^{131} and passed through protein-bound iodine; (3) gel filtration on Sephadex G-25 columns, which reacted in a similar way; (4) dialysis, followed by radioactivity counting; and (5) precipitation of gastric juice by trichloroacetic acid and taking up by alkali, followed by dissolving the precipitate in

80% acetone. Results obtained indicated passage of serum albumin into the gastric lumen under normal conditions and its excessive leakage in patients with atrophy of the gastric mucosa and gastric cancer. Following histamine stimulation, passage of the I^{131} label into the stomach was markedly increased. This indicated removal of the label from albumin in the stomach by acid-pepsin digestion (unpublished work).

As a result of this massive protein leak into the stomach and the inability of the liver to increase production of albumin to keep up with albumin degradation in the gastrointestinal tract—hypoalbuminemia develops. This is the concomitant feature of the "protein-losing gastropathy" (E1), and is found in Menetrier's disease (C18, D1), gastric cancer (J4, S14), gastric polyps (D3), gastric atrophy (B14a), and some cases of peptic ulcer (T34).

The most valuable and convincing information on the passage of serum proteins into the gastric juice was yielded by paper electrophoresis (G16, G42, G76, H15, H19) and immunological techniques (F3, H20–H20b, H34, H41, H55, S22). The results of this extensive work of many investigators are reported in the second review of the author in this volume (pp. 389 and 419).

3.3. Mechanism of Protein Passage into Gastric Lumen

From the information just cited, it appears that serum proteins are normal constituents of gastric juice and can be demonstrated even in acid secretion if proteolysis is prevented by *in situ* neutralization of gastric juice.

The mechanism of the appearance of serum proteins in the gastric juice may be variegated. The possibility of simple transudation of the former into the gastric lumen was raised 30 years ago (H27). More recently Hollander and Horowitz (H35) considered the possibility that serum proteins may be an integral part of the primary mucinous secretion resulting either from the reincorporation of the serum proteins into the columnar and chief neck cells of gastric mucosa, by way of the interstitial fluid, or from their synthesis *in situ* by these cells (H31, H37). In addition to these two mechanisms, extracellular transfer of proteins into the gastric cavity by an active transport mechanism has also been considered, as well as the possibility of some kind of seepage of plasma proteins in the interstitial fluid among the cells (H35). The excretion of the heterospecies protein by the gastric mucosa (M38) occurs perhaps by one of these mechanisms.

The mechanisms involved in causing a massive protein leakage into the gastric lumen, in addition to gastric bleeding, are as follows (G42):

(1) exudation of serum from ulcerated surfaces of the gastric mucosa in gastric ulcer or cancer, (2) inflammatory exudation of serum from the gastric mucosa, as in gastritis, (3) excessive transudation of serum proteins as a result of increased vascular permeability to the albumin, and (4) leakage of lymph into the gastric lumen through obstructed, dilated, or eroded lymphatics. Several mechanisms may coexist and contribute to the penetration of large amounts of protein into the gastric lumen.

4. Peptides

4.1. Demonstration in Gastric Juice

The presence of peptides and amino acids in gastric juice was demonstrated many years ago by Nencki and Sieber (N3), Babkin (B1), and Komarov (K22). This became better documented only more recently with the use of modern physicochemical tools, such as paper partition chromatography, electrophoresis, and polarography. Demonstration of the products of proteolytic degradation of serum proteins and mucosubstances has also been made possible only by the use of these methods. The reader is referred to the second article by the author (G21) for pertinent information and to the article by Bouda (B29).

Kakei (K2) studied the ninhydrin reaction in gastric juices following Tazaki's report that ninhydrin-positive substances were present in the methanol precipitate of gastric cancer juices. When the pH was adjusted to 5, the ninhydrin reaction was always positive. It changed to negative, however, after the methanol precipitate was washed several times with methanol. Using high-voltage paper electrophoresis and polarography, Kakei found the ninhydrin-positive material in the supernatant of gastric juice after methanol precipitation; this corresponded to low molecular peptides and amino acids.

4.2. Gastrin

Another material of peptide nature which has been traced in the mucosa of the gastric antrum is gastrin. Its existence was first reported in extracts from pyloric mucosa by Edkins in 1905, who injected them i.v. into anesthetized cats and caused stimulation of gastric secretion (E2, E2a). For many years, gastrin was the subject of controversy and was suspected to act on the basis of its histamine content. For the last 25 years, however, the work of Komarov (K23), Uvnäs (U5, U6), Jorpes *et al.* (J11), and recently Gregory *et al.* (G67, G68) have completely rehabilitated gastrin. Now it is universally acknowledged as an antral

hormone of a peptide nature (G73). Purified gastrins, obtained by Uvnäs 20 years ago (U5, U6) and more recently by Jorpes *et al.* (J11), represent some progress in regard to Komarov's (K23) fractions. Whereas his preparations were concentrated to a dose of 1 unit in 2.5 ml, those of Uvnäs and Jorpes showed 1 unit of activity at a dose of 0.1 ml. (One gastrin unit produces secretion of 1 ml acid gastric juice in the anesthetized cat during 1 hour following i.v. injection.) These materials, however, were still quite heterogeneous and impure.

In the last few years, Gregory and Tracy (G69) developed a complex method for the purification of gastrin. It is based on treatment of fresh duodenal mucosa with picric acid, which precipitates proteins and stops enzymatic action, and extraction of crude material with 80% acid methanol or acetone. After removal of acetone and picric acid with ether, proteins are precipitated with trichloroacetic acid. The precipitate is soluble in dilute acid and is rich in gastrin. From this crude aqueous extract of mucous membrane, the bulk of inert protein is precipitated in hot solution at pH 8.5 in the presence of 2% sodium chloride, and the supernatant fluid is then saturated with sodium chloride at room temperature at pH 4.5. The precipitate, after dissolving as before in 0.1 normal HCl, is reprecipitated several times in hot solution at pH 8.5. It still contains large amounts of denatured protein. This is now removed by dissolving the powder in water containing parapepsin at pH 10, adding acetone to 80%, and reducing pH to 8.5. This causes precipitation of most proteins, while gastrin activity remains in the supernatant. The material is further fractionated on calcium phosphate gel column at an appropriate pH, resulting in retention of gastrin on the column. After washing, it is eluted from the column by diluted phosphate solution. The yield of active material is about 0.5 mg gastrin nitrogen from 1 kg mucosa. Intravenous administration of this material, at a dose containing 0.01 mg nitrogen of active material and equivalent to 20–30 g of native mucosa, causes a striking response in dogs on i.v., i.m., or subcutaneous injection. When administered to human subjects at a dose containing approximately 0.1 mg nitrogen, it causes marked acid secretion.

It is not as yet known whether gastrin enters the human gastric juice from the antral mucosa under normal or pathological conditions.

Harper *et al.* (H1) found no significant difference between the concentration of gastrin in antral extracts from cases of gastric ulcer and normal individuals. In duodenal ulcer, however, antral gastrin concentration was about twice that in gastric ulcer, and its concentration correlated with both the basal secretion and the response intensity to a maximal histamine stimulation.

Gregory and Tracy (G69a) have more recently described an improved and simplified method of fractionation and separation of purified gastrin from the hog antrum. Hog antrums were boiled in tap water, cooled, the fluid portion was drained off, strained, and stirred with DEAE floc. Then, gastrin was eluted together with inert proteins from the floc with 0.1 *N* sodium hydroxide, neutralized to pH 7 and brought back to pH 4 with glacial acetic acid. The precipitate which collected was then dissolved in water at pH 10. Dipotassium hydrogen phosphate and peroxide 3-isopropanol were added to the filtrate. The mixture was centrifuged, the upper phase (aqueous) containing gastrin was removed by centrifugation and addition of peroxide 3-ethyl ether and water. This procedure was repeated again, following which the solution was passed through a Sephadex G-50 column and eluted with ammonium bicarbonate. The included volume contained all the gastrin activity. After drying, materials were refractionated and 2 gastrin peaks were eluted. Both were ninhydrin negative, which indicated the absence of a terminal amino group in the molecule. Both gastrins contained one unit of aspartic acid, 6 of glutamic acid, 2 of glycine, one of alanine, 2 of methionine, one of tyrosine, 2 of tryptophan, one of proline, and one of phenylalanine. The minimal molecular weight for each gastrin, calculated from the amino acid composition was 2,114, whereas ultracentrifugal analysis gave molecular weights for both gastrins of approximately 1,335. Both gastrins were many times more potent than histamine in stimulating gastric acid secretion in dog pouches, when injected subcutaneously. In addition, both stimulated pepsin secretion, pancreatic flow and enzyme output, and gastrointestinal motility (G69, G69a).

5. Blood Group Substances

Yamakami and Landsteiner as well as Levine found that blood group antigens A and B are present not only in the erythrocytes, but also in a water-soluble form in sperm. Other authors [Landsteiner and Harte, Friedenreid and Hartman (see H2, K1), as well as Witebsky and Klendshoj (W15–W17)] found blood group substances A and B in various digestive secretions, including human gastric juice (K1a, R12). Witebsky and Klendshoj (W16) also reported the presence of O substance in the gastric juice of patients of O group type. Most Europeans also contain Le^a antigen in epithelial secretions, including gastric juice. Its concentration is low in secretors of ABO(H) antigens, and high in about 15% of the individuals who do not secrete ABO(H) antigens. From 3 to 10% of the population does not demonstrate the presence of Le^a antigens.

Mohn and Witebsky (M45) examined 22 specimens of gastric juice

for Rh factor. Of the 20 specimens obtained from Rh-positive individuals, 12 revealed the presence of Rh factor in the gastric juice and 8 did not.

5.1. Chemistry

Witebsky and Klendshoj (W15, W16) as well as Manski and Kozdroj (M7–M7b) determined the concentration of ABO substances in the human body fluids and secretions, including the human gastric juice. Yields of purified blood group substances isolated from 1 liter gastric juice were 123 mg substance B, 185 mg substance O(H), and 100–300 mg Le^a. Witebsky and Klendshoj obtained only a few mg of blood group materials from 38 other samples of gastric juice.,

Masamune *et al.* (M22) fractionated gastric juice by the method of Aminoff *et al.* (A2), using trichloroacetic acid–acetone precipitation, followed by precipitation with absolute alcohol and phenol extraction. Yields obtained were lower yet than those just cited: 70 mg blood group B was obtained from 3.35 g nondialyzable solids of gastric juices from individuals with blood group B type, 79 mg blood group O(H) from 3.9 g nondialyzable solids of individuals with O type, and 135 mg blood group substance A from 4.8 g nondialyzable gastric solids from patients with A blood group type. Kabat (K1) isolated relatively small quantities of blood group substances, 10–43 mg, from individual human stomachs, while Baer *et al.* isolated as much as 90 mg at one time. Hog gastric juice was a good source of blood group substances, which were easily obtainable by using Morgan and King's method (M51) with 90% phenol extraction, followed by fractional precipitation with ethanol.

A and O blood group substances from hog gastric mucin represent mucopolysaccharides of high molecular weight, containing carbohydrates and peptides (see K1, M49, M50). Following acid hydrolysis, the following sugars are liberated from blood group substances: (1) L-fucose, (2) D-glucosamine, (3) D-galactosamine, (4) D-galactose. Peptides containing various amino acids form an integral part of the blood group substances. About 11 amino acids, including alanine and methionine in large amounts, are found therein. As shown by Goebel (see K1) and Morgan and King (M51), their removal causes loss of blood group specificity. Methylation studies of blood group substance A demonstrated that L-fucopyranose and *N*-acetyl-D-glucosamine were present in terminal sugar units (S39). Additional groups were reported in other blood group substances.

The carbohydrate end groups in various blood group mucopolysaccharides, as described by Morgan (M50), are as follows: acetylgalactosamine, acetylglucosamine, and galactose on blood group substance A;

two galactose groups on blood group substance B; fucose and acetylglucosamine on blood group substance H(O); and fucose, galactose, and acetylglucosamine on Le[a] blood group substance. It has been suggested that the blood group substance H is the polymer of acetylglucosamine and galactose, with fucose located terminally as side chain. According to Morgan (M50), blood group substances contain repeating chains of these oligosaccharides. The carbohydrate spectra of various blood group substances are listed in Table 10.

Witebsky and Klendshoj found that the blood group substance B of human gastric juice contains 1.6% N and 75% reducing sugars, while substance O contains 2.8% N and only 40% reducing sugars (W15, W16). The data of Bendich, Kabat (K1) and others (A2) on hog gastric mucin indicate that blood group substance A of hog gastric mucin contains 5.9–6.6% nitrogen, 55–61% reducing sugar, 32–34% amino sugar, and 9.3–11.3% acetyl; its main constituents are, as stated before, L-fucose, D-galactose, D-glucosamine, and D-galactosamine. Hog gastric mucin of blood group O differs by lower content of nitrogen and slightly less of acetyl. Hog stomach A substance is a more potent antigen than human A substance from saliva (Witebsky, see K15). The basic difference in the composition of Le[a] and A substance, according to Morgan (M50), is that in Le[a] substance the ratio of glucosamine to galactosamine is about 3:1, while in blood group substance A these two amino sugars appear in equimolar proportion. Blood group substances are not precipitated by 10% trichloroacetic acid, 20% sulfosalicylic acid, or picric acid.

Morgan and his associates (A2, A8, A8a, G4, M51) analyzed blood group substances A, B, H, and Le[a] and obtained results as follows.

Blood group substance A was found (M51) to contain 44.2% carbon, 6.96% hydrogen, 5.7% nitrogen, and 8.8–9.1% acetyl. Content of hexosamine was 37%, of galactose 17%, and of fucose 18%. Of amino acids, the following were found: lysine, arginine, aspartic acid, glutamic acid, glycine, alanine, threonine, proline, valine, leucine, and methionine, of which threonine was the largest component.

Elementary analysis of B substance (G4) showed 41% carbon, 6.6% hydrogen, 5.7% nitrogen, and 7% acetyl. The same sugars and amino sugars were again found, including 17.9% fucose, as well as the same 11 amino acids. However, content of hexosamine was lower (20–22% only) as compared to 33–37% in blood group substances A, H, and Le[a].

Blood group substance H (A8) designated in the early literature by O, contained 41.4% carbon, 6.9% hydrogen, 5.3% nitrogen, and 8.7% acetyl. Its fucose content was 14% and that of hexosamine 31%. The same 11 amino acids were present. Le[a] substance showed 41.5% carbon,

TABLE 10

COMPARISON OF BLOOD GROUP SUBSTANCES FROM VARIOUS HUMAN SOURCES (% w/w)

Source	Blood group	N	Reducing substance	Galactose	Hexosamine	Fucose	Sialic acid	Authors, years, refs.
Gastric juice	A	4.7		26.6	30.8	14.0	3.0	Masamune *et al.* (1958); (M22)
	B	4.9		27.8	29.0	12.9	5.2	
	O	4.7		26.9	30.0	14.1	3.5	
	A, B, O	6.1		46.5	28.9			Manski and Kozdroj (1951); (M7)
	Le^a	6.0		46.4	28.7			
Stomach	A	6.8	48.0		25.0			Kabat *et al.* (1947); (K1a)
Saliva	A1	3.6–6.1	54.0–61.0		26.0–32.0	13.0–16.0		Kabat (1956); (K1)
	A2	5.5–6.1	54.0–60.0		26.0–30.0	16.0		
	B	3.3–4.5	50.0–68.0		19.0–29.0	13.0–17.0		
	O&Le^a	4.4	62.0		22.0–26.0	8.8–18.0		
Ovarian cyst mucin	A1	4.8–5.7	52.1–58.0	26.2–27.6	30.1–34.0	16.9–20.0	3.9–5.9	Gibbons *et al.* (1955); (G4a)
	A2	5.4	55.7	29.8	29.7	16.3	8.2	
	B	5.5	57.0	38.4	24.0	15.7	7.5	
	O	4.2–6.2	50.6–60.9	34.1–36.1	22.5–29.1	15.4–26.2	1.9–3.2	
	AB	4.9	55.0	28.8	27.0	17.7	2.9	
	A	5.7	56.0		37.0	18.0		Morgan (1956); (M49)
	B	5.7	50.0		20.0	18.0		
	H	5.3	54.0		31.0	13.0		
	Le^a	5.0	57.0		32.0	12.0		
	A	5.3	52.0		29.0	18.0		Morgan (1963); (M50a)
	B	4.9	51.0		28.0	18.0		
	H	5.3	45.0		24.0	19.0		
	Le^a	4.9	46.0		29.0	13.0		

7.1% hydrogen, 5.3% nitrogen, and 10% acetyl (A8a). There was only 12% fucose and, again, the same amino acids were present.

Sialic acid content of blood group substances was low and frequently not more than 1%. In some impure blood group preparations, however, the sialic acid content was as high as 7%. There was no sulfate or uronic acid present. The ratio of glucosamine to galactosamine in substance A was 0.7–2.1:1; in substance B, 1.5–3.8:1; and in Le^a substance 3.7:1.0. In blood group substance H, the range of variation was by far larger—2.5–15.0:1.0. On electrophoresis and ultracentrifugation, blood group substances separated by the Morgan group were reasonably homogeneous and showed molecular weight varying from 260,000 to 460,000 (C3, K12–K14), with a probable molecular weight for B substance of 1,800,000 (C3).

Structural details of various blood group substances were revealed by partial hydrolysis, chromatography, and results of periodate oxidation, treatment with various enzymes such as ficin (M50), and acetolysis (M11, M12, M15–M17, M19–M21, Y4, Y5). They are well described in various reviews on carbohydrates (K16, S39) and blood group substances (K1, M49, M50).

The extensive work of Masamune's school on structure of blood group substances also pointed to some chemical and physicochemical differences among blood group substances H, A, and B (M15–M17, M19–M22, T29, Y3–Y5). These are: (1) differences in glucosamine to galactosamine ratio among the group A, H, and O mucopolysaccharides, and (2) differences in sialic acid content between the group A and O, and group B substance, being highest in the latter. This resulted in higher electrophoretic mobility of the purified blood group B substances.

The composition of various blood group substances from human gastric juice, as reported by Masamune *et al.* (M22), Tiba (T29) and Yosizawa (Y3), varies only little. The N content is in the range 4.7–4.9%, hexosamine 29.0–30.8%, galactose 26.6–27.8%, fucose 12.9–14.1%, and sialic acid 3.0–5.2%. Presence of considerable amounts of sialic acid in these materials perhaps indicates their contamination with sialomucin.

Because blood group substances are similar in chemical and physical properties, they cannot be fractionated by electrophoresis (G38a) (see G21). Nor is there a known chemical method which allows their complete separation. According to Morgan (M50), evidence exists that H-active mucopolysaccharide is a basic substance in which A and B genes cause chemical modification of structure. It has been suggested that this structural change is responsible for the specific serological character of secretions from persons with various blood groups. However, differences may

exist in properties and chemical constitution of secretions from individuals having the same blood group type, none of which is as yet clear.

5.2. Correlation between the Carbohydrate Spectrum of Gastric Juice and Blood Group Status

Evans (E7) found higher fucose output and concentration in gastric juices of ABO(H) secretors, as compared to nonsecretors. Also Hoskins and Zamcheck (H50) demonstrated variations in the ratio of fucose to hexosamine in gastric juices with various blood group substance types. The highest ratio was present in juices of O(H) secretors, next in A and B secretors, and lowest in AB secretors and O(H) nonsecretors, which corroborated evidence presented by Evans (E7). They concluded that the blood group substance type and the AB(H) secretory capacity significantly affect the types of glycoprotein elaborated by the gastric mucosa. According to the reviewer, there is inadequate evidence to assume such an effect which may simply be due to various titers of various blood group antigens in gastric juice. The other alternative may be that genetically conditioned modifications in the structure of gastric mucosubstances may result in differences in composition of blood group antigens, which are derived from the former.

In work from our laboratory, Ibanez determined the composition of the first fractions eluted from the Amberlite IRC-50 column of gastric juice pools from individuals with various blood groups. The first fraction eluted with citrate of pH 3.2–3.5 is known (R4, R4a) to contain blood group substances.

Preliminary results were as follows. In secretors, the fucose to hexosamine ratio ranged from 0.50 to 0.89 and was highest in the AB secretors, next in A secretors, then in B secretors, and lowest in O secretors. While the mean ratio of fucose to hexosamine drawn from all secretor pools was 0.65, in the ABO(H) nonsecretor groups, who had blood group type O, A, or AB, this was 0.27–0.58, with a mean of only 0.46. However, this difference was statistically nonsignificant with P value above 0.1. Thus, although Hoskins and Zamcheck (H50) found that the secretors have higher ratios of fucose to hexosamine in gastric juice, we could not confirm the statistical significance of these findings. Nor could we corroborate the data of the Masamune school (M22) that the sialic acid content in fractions from patients with blood group substance B was higher than in the other groups.

Price Evans (E7) found a good correlation between the titer of blood group substances and the fucose concentration in gastric juice of persons with established blood group and salivary ABH secretor status.

A correlation did not exist in individuals of different secretory status, because contribution of fucose to gastric juice by the Le^a type of blood group substances was unknown.

Although there is statistically corroborated evidence from Evans' work (E7) that, in the particular phenotype and salivary secretory status, fucose content may be used for the determination of the blood group substance titer, we had some difficulty in accepting this correlation. Our work suggested that fucose represented a relatively fixed fraction of total carbohydrates of gastric juice (G55, G56), which amounted to 16.3–22.4% of total sugars. On the other hand, we found no correlation between the blood group substance titer in 77 gastric juices of various individuals and the total polysaccharide content of gastric juice, as determined by quantitation of the PAS-stainable material in the gastric juice electropherograms (G19, G39). Thus, it was difficult to accept the concept that variations in fucose content could be correlated with the blood group substance titer, especially since the contribution of the blood group substance carbohydrates to the total carbohydrates of the gastric mucosubstances was rather small.

5.3. Physiological and Pathological Aspects of Secretion of Blood Group Substances in the Stomach

Glynn *et al.* (G59a), using fluorescein isocyanate conjugates and anti-H and anti-Le^a sera, studied the patterns of distribution of blood group substances A, H, and Le^a in various parts of the human gastric mucosa. Blood group antigens showed different distribution in the cells of the fundic and antral mucosa. In the fundus and body of the stomach, blood group antigens accumulated in water-soluble form, principally on the surface epithelium, with some tapering off in the area of the mucous neck cells. On the other hand, the area of the chief cells contained no blood group substances. Parietal cells contained blood group substances A in persons with blood group A type, whether or not they were secretors of ABO(H) substances. This A substance, however, was not in water-soluble but in alcohol-soluble form. In the pyloric and prepyloric area, water-soluble blood group substances were located in all the epithelial cells of the foveolae and pyloric glands, close to the mucosal surface or in the deep parts of the pyloric glands, and far less in the intermediate strata of the mucous membrane. Anti-H blood group fluorescein conjugate did not differentiate between blood group substance H and the Le^a antigen. Alcohol-soluble blood group substances were found also in the gastric cancer cells.

In a joint investigational project, undertaken with J. A. Buckwalter

(B50, G43), in 400 specimens of gastric juice from 210 patients with various gastric diseases, we demonstrated that there was a higher incidence of blood group substance A and secretors of ABH antigens in the gastric juice of patients with gastric cancer and atrophic lesions of the gastric mucosa than in those with duodenal ulcer (Fig. 24). Conversely, the incidence of antigen A and ABO(H) nonsecretor status in the gastric

	Peptic ulcer	Gastric cancer	Gastric atrophy
Number of cases	113	33	29
Incidence of non-secretors of blood group substances AB (H)	31.8%	24.2%	20.7%

FIG. 24. Incidence of non-secretors of blood group substances AB(H) in the gastric juice of 175 patients with various gastric diseases. From Glass *et al.* (G43).

	Duodenal ulcer	Gastric ulcer	Gastric atrophy	Gastric cancer
Number of cases	82	31	29	33
Incidence of group substance 'A' in gastric juice	20.7%	25.8%	27.6%	30.3%

FIG. 25. Incidence of blood group substance "A" in the gastric juice of 175 patients with various gastric diseases. From Glass *et al.* (G43).

juice was highest in patients with duodenal ulcer (B50, G43) (Fig. 25). Similar relationships were found by Doll *et al.* (D6) for marginal ulcer.

We found no difference in the titer of blood group substance H and A in the gastric juice in peptic ulcer patients and those with other lesions (G43). This was true of both fasting and histamine-stimulated gastric juice, of native and lyophilized gastric juice, and of both A and H blood group substances of the stomach. Nor were significant differences found in the titers of the A and H blood group substances in the night secretions of patients with various gastric diseases. This failed to support the concept that a defective secretion of blood group substances weakens the

mucosal membrane's defense against digestion by gastric juice (see M27, M27a).

Evans *et al.* also concluded that the influence of blood group substances in disease is not found in the gastric lumen, but rather inside the tissues (E8). This author (E7) assumed that the increased susceptibility of various phenotypes to certain diseases may represent the interaction of an environmental factor and the blood group substances. This may

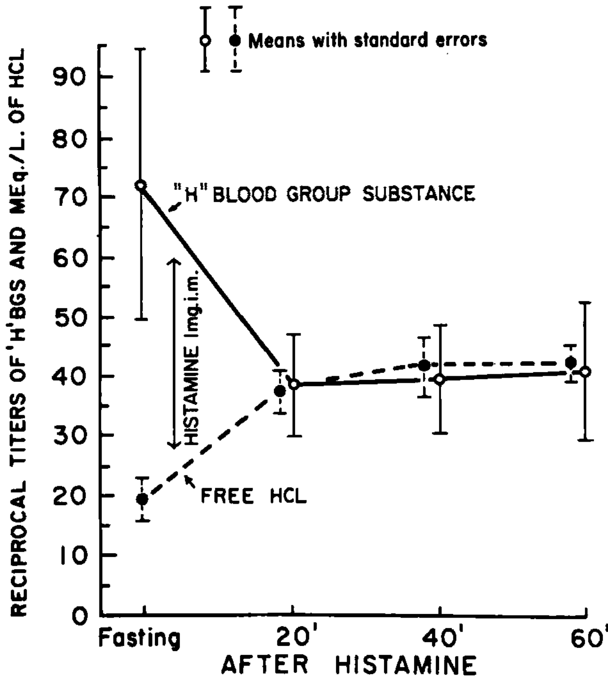

FIG. 26. "H" blood group substance and HCl concentration in gastric juice following histamine (54 cases). From Glass *et al.* (G43).

somehow incite the parietal cell mass to hypertrophy, occurring more in the presence of blood group substance H and Le[a] than in that of substances A and B. It may explain the gastric hypersecretion in secretors and nonsecretors with blood group type O.

We also determined the secretory patterns of blood group substances, HCl, and pepsin after histamine stimulation in 72 individuals with various diseases (G43). A statistically significant decrease was found in the titer of substances H and A after stimulation by histamine (Fig. 26). This was associated with a rise in acidity and frequently in pepsin concentration. Although the correlation of these data was not confirmed

statistically, we consider it as evidence that most blood group substances of the stomach are derived not from the parietal and peptic cells, but from other cellular structures, such as the surface epithelium, mucoid neck cells, and cardiac and pyloric glands. This conclusion corroborates the immunological work of Glynn *et al.* cited above (G59a) and has been recently supported by Evans *et al.* (E8), who studied 122 patients with blood group O type. These investigators determined blood group substance titer in the gastric juice, before and after augmented histamine test. They found that the mean titer of the agglutinogen fell significantly after histamine, whereas there was a statistically significant increase in the concentration and output of HCl. A significant decrease in fucose concentration was also apparent after histamine stimulation. From all these data, it may be concluded that blood group substances form a distinct entity in the nonparietal gastric secretion (E7, G43).

The canine gastric juice does not contain blood group antigens of a human ABO(H) system (H48).

6. Gastric Intrinsic Factor and Vitamin B_{12} Binders

6.1. Intrinsic Factor

For detailed information on intrinsic factor (IF) the reader is referred to the recent monograph on this subject by the reviewer, the bibliography of which includes over 1500 references (G20). Therefore only the most salient points will be discussed here.

The work of the past 35 years has confirmed Castle's initial concept (C5–C10) of a principle in human gastric juice necessary for normal hematopoiesis (G20). Its lack in the gastric content of pernicious anemia patients was recognized, as early as 1929 (C5, C9), to be the essential defect leading to the development of pernicious anemia, through failure of the reaction between extrinsic factor, now known to be identical with vitamin B_{12}, and intrinsic factor in normal human gastric juice. Other investigators confirmed that IF is formed by the fundus and body of the stomach in man (see G20) and by the pyloric end of the stomach in the hog (L2, M33), and that it is absent from saliva.

IF is thermosensitive (C6), nondialyzable, and nonultrafiltrable (H12b), but can be filtered through a Berkefeld filter. It has a relatively high molecular weight, as determined by gel filtration (G46), or enters into large molecular complexes with carbohydrates. The recent work from our laboratory on IF, using gel filtration on Sephadex columns, indicates that human IF, as it naturally occurs in human gastric juice, has a molecular weight of over 200,000, or rather that it forms a complex

with gastric mucosubstances of this molecular size (G46, G47). It is also resistant to storage and freeze-drying at neutral pH, but progressively loses its activity at low pH (C5, C8). It is resistant to alkaline pH up to 10.0 (see G20), but loses its activity at pH above 11.5 (C12, C13). IF is slowly inactivated by pepsin and loses less activity when bound to vitamin B_{12} prior to peptic digestion (G62). It is precipitable by ammonium sulfate at 46–60% saturation (C14, G61, P16, W12-W14), by cold ethanol at 70-95% concentration (L3–L5), and cold acetone at 40–80% saturation (G37).

TABLE 11
PROGRESS IN PURIFICATION OF INTRINSIC FACTOR[a]

Year	Authors	Material	Active dose
1929	Sturgis and Isaacs	Desiccated hog stomach	40 g
1949	Bethell *et al.*	Desiccated hog pyloric and duodenal mucosa	1–5 g
1950	Hall *et al.* Spies *et al.*	Extract from hog pyloric and duodenal mucosa	330 mg
1951	Glass, Boyd, Rubinstein, and Svigals	"Glandular" mucoprotein from normal gastric juice	50–200 mg
1953	Glass and Boyd	Intrinsic factor concentrate from hog gastric and duodenal mucosa	30–60 mg
1955	Latner *et al.*	Intrinsic factor preparation from hog pyloric and duodenal mucosa	1–4 mg
1957	Glass, Stephanson, Rich and Laughton	Intrinsic factor preparation from human gastric juice	2.2 mg
1957	Holdsworth *et al.*	Intrinsic factor preparation from hog pyloric and duodenal mucosa	1.0 mg
1959	Jacob, Williams, Howe, and Glass	As for Holdsworth *et al.*	0.4–1.0 mg
1960	Heatley *et al.*	As for Holdsworth *et al.*	0.3–0.5 mg
1960	Ellenbogen and Williams	As for Holdsworth *et al.*	0.3 mg
1961	Bromer and Davisson	As for Holdsworth *et al.*	<0.05 mg

[a] From Glass (G18).

Great progress has been made in the purification of IF. Whereas, 35 years ago, the dose of IF-containing material required for hematopoietic response in patients with pernicious anemia was about 40 g dried hog stomach, the most potent hog IF preparations processed during the last 2 years have shown activity at a dose as small as 40 μg (B33, B34), one millionth the amount necessary 35 years ago (Table 11). Progress in its

fractionation from human gastric juice has been similar. While initially about 50–75 ml normal human gastric juice weighing over 50–75 g were administered, today some of the most potent fractions are active at a dose below 0.1 mg (C16, G62a), which represents a 500,000-fold concentration (Table 12).

TABLE 12
IF-ACTIVE MATERIALS FROM HUMAN GASTRIC JUICE AND GASTRIC MUCOSA[a]

Year and authors	Method of processing	IF activity of most active materials	
		Dose (mg)	Assay[b]
1. 1951-1952, Glass *et al.*	Ppt. of g.j.[c] with trichloroacetic acid, filtrate ppt. with acetone, ppt. dissolved in NaOH; active fraction ppt. with HCl	50–100	H
2. 1953, Latner *et al.*	Preparative paper-electroph. of conc. g.j.	?	H
3. 1955, O'Brien *et al.*	Differential ultracentrif. of gastric mucosa	10	I
4. 1955, Glass *et al.*	As under 1	8	I
5. 1955, Johnson *et al.*	Chromatography of g.j. on Amberlite XE-64 col.	10	I
6. 1957, Glass *et al.*	Continuous electrophoresis of lyophil. g.j. on paper curtain	2.2	I
7. 1959, Caputto *et al.*	Chromatography of g.j. on Amberlite XE-64 col. with citrate-phosphate buffers	2.0	I
8. 1960, Heatley *et al.*	Chromatography of gastric mucosa on Amberlite XE-64 col. with phosphate buffers, followed by electrophoresis at pH 5.0, and ammon. sulf. ppt.	1.5 (?)	I
9. 1962, Welsh *et al.*	Modification of procedure described under 5 and 7	1.2	I
10. 1962, Gräsbeck *et al.*	Chromatography of g.j. on DEAE-cellulose and DEAE-Sephadex with phosphate-NaCl buffer and CM-Sephadex	≈ below 0.1 mg	I
11. 1963, Chosy and Schilling	Chromatography on Amberlite CG-50, DEAE-cellulose, and Sephadex G-75 cols., followed by ultrafiltration	≈ below 0.1 mg	I

[a] From Glass (G20).
[b] H = hematopoietic response; I = isotope assay.
[c] g.j. = gastric juice.

Purified IF concentrates from hog stomach are grossly of two types. The first has a molecular weight of 40,000–100,000 (A6, A7, B33, B34, G44, H25, J1), the other 5,000–7,000 (E4, E4a, L2–L5, W12–W14). Some of the IF materials of low molecular weight have been treated with proteolytic enzymes prior to purification, which may account, in part, for a decrease in molecular weight.

There also are at least two different IF materials in human gastric juice, namely, native and degraded IF (G14, G15, G20, G62a, U1). No IF concentrate has ever been prepared that does not contain peptides and carbohydrate moieties. Polysaccharides in the most purified preparations consist of 3–5% fucose, 5–6% (F2) and 8–10% (E3–E4a) hexosamine, 8–14% hexoses (F1), as well as small amounts of sialic acid (1–2.5%) (F1), consisting of 40% *N*-acetyl- and 60% *N*-glycolylneuraminic acid in hog IF, and acetylneuraminic acid only in IF from human sources (F1, F2). With few exceptions (B33, B34), most potent IF preparations processed from animal stomach have been highly heterogeneous on electrophoresis (E3, G44) or ultracentrifugation (E3, G44, J1).

No direct information is available on the cellular origin of IF. The indirect data are based on the study of the cellular pathology of gastric mucosa in pernicious anemia, as well as autoradiographic findings on vitamin B_{12} binding by gastric mucosa. Active and inhibitory IF preparations have been processed from the mitochondrial fraction of the human gastric mucosa (H7, O1, T25). At present, it appears that peptic, and possibly the mucoid neck cells in rats, and perhaps parietal cells of fundic glands of the fundus and body in humans, are related to IF formation (N3a) whereas in the hog the latter is of pyloric origin (H6, L2, M33).

Species specificity of IF, its site of origin in various species, as well as its pharmacology and pathology of formation are discussed in the aforementioned monograph on IF (G20). The nature and chemical composition of human and hog IF are not the same. The contention that IF is directly secreted into the gastric lumen by the glandular structures of the stomach is unproven. The reviewer has speculated (G20) that IF in man may not be a direct secretory product of the gastric glands, but is formed during the process of activation of the proteolytic enzymes in the human stomach. IF may be a by-product of the activation of pepsinogen or another protease zymogen to pepsin or other proteolytic enzymes. During activation of pepsinogen to pepsin, several peptides are known to be split off (V3, V3a). It is possible that one or more of them may contain terminal groups endowed with IF activity (G20), i.e., the ability to transfer vitamin B_{12} across the intestinal membrane (C8), and possibly

across other cellular membranes in the human body (G15). These reactive peptide groups then enter into a complex with some of the mucosubstances of gastric juice and B_{12} binding groups forming what is known as IF (G15, G20). This concept is purely hypothetical, however.

6.2. Vitamin B_{12} Binders

One of the most important features of IF is its vitamin B_{12}-binding ability, first discovered by Ternberg and Eakin (T28). It depends on many factors, such as pH, temperature, ratio of B_{12} concentration to protein, nature of the B_{12} binder itself, presence of inhibitors, as well as various techniques for measurement of B_{12} binding (G20, W12). The B_{12}-binding capacity of IF itself is unknown, since the latter has not been isolated. Nevertheless, materials have been obtained by purification of IF from hog gastric mucosa (A6, A7, C26, G65, G66, H25) as well as from human gastric juice (C16, G62a), which bind as much as 25 μg per mg of material. This is probably the upper limit of IF's binding capacity obtained so far. Purification of IF concentrates is usually associated with an increase in B_{12}-binding capacity (B33, B34, C16, G44, G62a, J1). No IF material has been found without some B_{12}-binding ability.

Binding capacity of gastric juice, depending on the saturation level and techniques used, ranged from about 15 mμg per ml on microbiological assay to about 40–70 mμg by dialysis or ultrafiltration (see W12). When assayed by electrophoresis at a high saturation level, gastric juice may bind over 200 mμg B_{12} per ml. Vagally stimulated gastric juice binds more B_{12} than gastric juice collected under fasting conditions or after histamine. Total B_{12}-binding capacity of gastric juice from patients with pernicious anemia, with atrophic gastritis, and with histamine-fast anacidity without pernicious anemia, is much lower than that of normals (G40). Welsh *et al.* (W8a) studied the total B_{12}-binding capacity of human gastric juice in 10 subjects following a single i.m. injection of histamine and in 2 individuals during continuous histamine infusion. The amount and concentration of total B_{12} binders peaked in the first half-hour collection after single injection of histamine. The output of total B_{12} binders did not correlate with that of hexoses and pepsin in the gastric juice.

The nature of the bond between IF and vitamin B_{12} is not known. Most investigators agree that sulfhydryl groups are not involved in B_{12} binding. Phenolic groups may be significant for this process (see G20). For the information regarding the nature of various B_{12} binders of gastric juice, as well as the effect of proteolytic degradation on B_{12} binders of the stomach, see the companion review of the author in this volume (p. 435).

7. Antigenic Materials in Gastric Juice and Gastric Mucosa

Hog mucin and partially purified intrinsic factor preparations from hog duodenum produce a typical anaphylactoid reaction in rats on i.v. administration characterized by edema of the paws, snout, ears, and genital region and hyperemia of internal organs (J5). Because of impurities in these materials, it has not been established whether the anaphylactoid reaction is due to intrinsic factor, which is rather doubtful, or to contaminating proteins, peptides, mucoproteins, or mucopolysaccharides in the crude gastric material. Subcutaneous injection of neutralized and dialyzed homologous gastric juice caused edema in the rat foot at the site of injection (H8). Oral administration of homologous gastric juice into the rat stomach was reported to cause a rise in acidity and diffuse edema and inflammatory lesions in the gastric mucosa (H8).

7.1. Antigenic Properties of Intrinsic Factor

Antibodies to intrinsic factor-active materials from human and hog stomach have been demonstrated in the sera of pernicious anemia patients by Taylor *et al.* (T9–T12) and Schwartz (S12, S13), and of rabbits immunized with these materials by Lowenstein *et al.* (L13). Antibody formation in rabbits is species specific, although antibodies have shown some cross-reactions. An antigenic factor common to human and hog material containing intrinsic factor probably produces formation of these antibodies (G75–G75b).

Intrinsic factor antibodies may be traced by: (1) precipitation reactions (T9–T12), (2) complement-fixation reactions between human and hog stomach antigens and rabbit antisera (L13, T9–T12), (3) hemaglutination by rabbit antisera of human red cells coated with intrinsic factor concentrate (S12, S13, T9–T12), (4) precipitation of human and hog intrinsic factor antigen by antisera, using the Ouchterlony double diffusion technique (T9-T12), (5) reactions of rabbit antiserum with red blood cells coated with hog intrinsic factor concentrate and bisdiazobenzidine (L13), (6) immobilization of the intrinsic factor-related B_{12} binder on starch gel or paper electrophoresis by pernicious anemia or rabbit antiserum (J7, J8, L13, T13), (7) inhibition of the stimulatory effect of human intrinsic factor by up to 60% of pernicious anemia sera on the B12 binding ability of serum proteins (A9a), (8) inhibition of B_{12} absorption-promoting action of normal human gastric juice in the human intestine by about 40–45% of pernicious anemia sera (T9–T12), and (9) inhibition of B_{12} binding to intrinsic factor-related B_{12} binder from gastric juice by the inhibitory sera of pernicious anemia patients, particularly

when this antiserum to intrinsic factor is added to gastric juice before B_{12} is added (A1, A1a).

Therefore, some autoantibody to human intrinsic factor probably exists in the serum of pernicious anemia patients, treated or untreated, as well as of immunized rabbits. Its physiological significance is not yet understood, and no information exists indicating the relation of these antibodies to the inhibitory factor in pernicious anemia serum, which inhibits maturation of megaloblastic bone marrow. The relationship of these antibodies to other gastric materials that inhibit gastric secretion and cause atrophy of the gastric mucosa (H13, S28, S30, S31) is also unknown, but raises interesting questions (see Section 8).

Circulating antibodies to hog intrinsic factor were also demonstrated in two pernicious anemia patients immunized with purified hog intrinsic factor concentrate (K5). Antibodies developed in their sera, which precipitated hog intrinsic factor in various immune reactions, immobilized it on electrophoresis, and depressed or abolished its enhancing effect upon vitamin B_{12} absorption in the intestine of other pernicious anemia patients.

In addition to these, another antibody to intrinsic factor may develop in a certain percentage of pernicious anemia patients, orally treated with hog intrinsic factor concentrate. As shown by Schwartz (S12, S13), this antibody is directed exclusively toward hog intrinsic factor concentrate and not human gastric juice or intrinsic factor derived from the human stomach. Apparently, it causes a topical antibody reaction at the mucosal level, blocking the promoting action of hog intrinsic factor concentrate on intestinal absorption of vitamin B_{12} in these pernicious anemia patients. This produces refractoriness to oral treatment with vitamin B_{12} and hog intrinsic factor concentrate in many pernicious anemia patients (S15).

7.2. Antigens in Parietal Cells

A complement-fixing antibody in the serum of pernicious anemia patients, diabetics, and those with iron-deficiency anemia has been detected against mucosal extracts of the fundus and body of the human stomach and the microsomal fraction of gastric mucosal extracts (D8, I4, M8, M48, R10, T13). Immunofluorescent studies have shown that activity is directed against gastric parietal cell cytoplasm (I5, R10, T13) and probably represents another type of antibody, which develops about twice as frequently as the one directed against intrinsic factor in pernicious anemia serum (Fig. 27).

Thus, in addition to an antibody to the human intrinsic factor or the B_{12}-binding principle, another antibody forms in pernicious anemia,

which is directed against some protein or mucopolysaccharide component of parietal cells. It is conceivable that the parietal cell and intrinsic factor antibodies are instrumental in an autoimmune mechanism which first causes atrophy of gastric mucosa, then loss of intrinsic factor, and finally development of pernicious anemia. A detailed discussion of these anti-

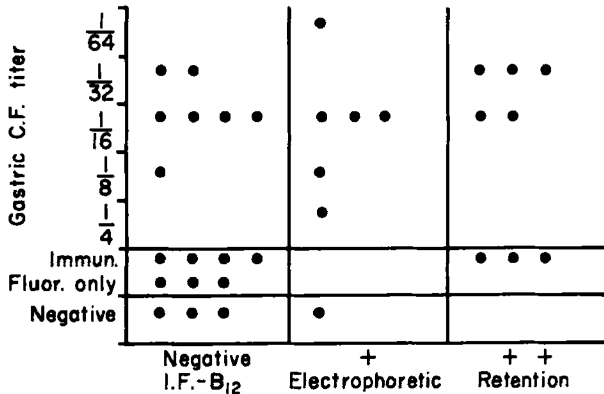

FIG. 27. Correlation of results of tests for parietal cell and IF antibodies in pernicious anemia patients, showing their independence. The parietal cell antibodies were tested by complement fixation test (CF) while the IF antibodies were assayed by electrophoretic retention test, i.e., immobilization of the intrinsic factor related B_{12} binder on electrophoresis. From Taylor *et al.* (T13).

genic materials in the gastric juice appears in the intrinsic factor review (G20). Hypothetical links between these antibodies and the gastric secretion-inhibiting materials in gastric juice, as well as gastric atrophy, are discussed in Section 8.

7.3. ANTIGENIC PROPERTIES OF PEPSINOGEN AND PEPSIN

Both pepsin and pepsinogen incite formation of antibodies in immune sera, which react with both pepsin and pepsinogen (A10). Kaminski concluded that pepsin at pH 1.0–4.5 produces three antigenic constituents (see G21). Antipepsinogen immune sera do not react with pepsin, indicating that pepsin and pepsinogen, though derived from the same species, are immunologically distinct from each other, from pepsin of other species, as well as from serum proteins. Lobachefskaya (see A10) found that the antibodies elicited by pepsinogen reacted with both pepsin and pepsinogen, which is contrary to the results of others. However, the antipepsin antibody reacts with pepsinogen and some cross-reactivity may be found between the two systems (A10). Horse and human pepsinogen cross-react with this antibody against the hog pepsinogen. The nature of the precipitation arcs in double diffusion technique demon-

strates that horse as well as human pepsinogen are similar, but not identical. The same is true in regard to human and hog pepsinogen. Possibly the antigenic sites on the pepsinogen molecule are different from those upon which the enzymatic activity of pepsin depends. There is also immunochemical cross-reactivity between pepsinogen and pepsin, when the immunodiffusion technique is used at pH 5.8.

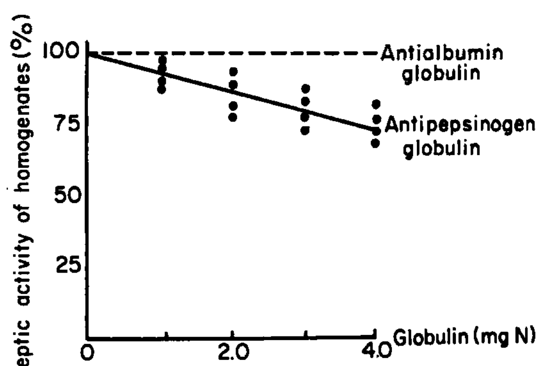

FIG. 28. Inhibitory effect of increasing concentration of immune rabbit globulin on peptic activity of homogenates of gastric mucosa. From Giron and Ramos (G5).

Following immunization with purified pepsinogen, only low potency immune sera were obtained in rabbit (A10). Tyrosylated pepsinogen was therefore used for immunization to enhance the titer of antibodies, which is caused by low antigenicity of pepsinogen (A10). This resulted in marked enhancement of antigenicity, indicating that tyrosine chains play a role in the antigenic properties of pepsinogen. Arnon and Pearlman found that immune sera directed toward the tyrosylated pepsinogen also react with pepsinogen, pepsins, and the tyrosine chains themselves.

Another antibody was experimentally produced in rabbits by injecting pepsin (G5). It was detected by the Ouchterlony technique and precipitation reactions, and may have some clinical significance in producing atrophy of chief cells. Repeated injection of this immune serum into rats also caused decrease in the volume of gastric juice, with lower concentrations of acid and pepsin. The controls injected with antibodies to egg albumin, nonspecific globulins, and saline did not show similar changes (see Fig. 28).

Because of pepsin's relatively low antigenicity, various immunoelectrophoretic studies were unable to detect the antibody to pepsin in serum of rabbits immunized with normal gastric juice (H20, H55). Only investigators administering large doses of gastric juice to the rabbit over

a prolonged period of time (S22) were able to produce high titer antibodies to pepsin and to obtain precipitation lines between antipepsin immune sera and the immunoelectropherograms of gastric juice (H20a, H20b).

8. Material Inhibitory to Gastric Secretion (Gastrone) and the Gastric Atrophy-Producing Factor

8.1. Gastrone

Brunschwig and his co-workers, in a series of papers between 1939 and 1942, reported the presence of a secretory depressant in human gastric juice (B36–B40, S16). When gastric juice or its alcoholic precipitate was injected into dogs with various types of gastric pouch and secreting acid in response to feeding, an inhibition of gastric acid secretion ensued. This was particularly striking after anacid gastric juice injection and still more so with juices of pernicious anemia patients. In Brunschwig's observations on dog gastric pouches, pernicious anemia juices caused on the average 82% inhibition of gastric secretion, that of gastric carcinoma—72%, that of patients with histamine-fast anacidity—36%, and normal human gastric juice—24%.

Code and his associates, in studies extending over a period of many years, corroborated and expanded these findings (B18–B20, C19–C22a, H42, L12a). They first developed a method for quantitative evaluation of the inhibition, using Heidenhain pouches which secreted steadily in response to histamine injected subcutaneously at 10-minute intervals (C21, C22). They also found that i.v. administration of alcoholic precipitate of achlorhydric gastric juice, especially from pernicious anemia patients, strikingly inhibits the response of these pouches to histamine, and that this inhibitory material was associated with the mucous fraction of gastric juice. Pepsin, however (B18–B20, C19), remained inactive. This inhibitory factor was found at high concentration in achlorhydric gastric juice of individuals without definite gastrointestinal disease and at a lower concentration in normal gastric juice. Minimal doses of precipitates which produced marked inhibition of gastric secretion were in the range of 10 mg. The inhibitory factor was also found in canine gastric juice, especially when obtained from Pavlov pouches after stimulation with mecholyl (L12, L12a). It was likewise present, but at a lesser concentration, in canine gastric juice from Heidenhain pouches after histamine injection (L12, L12a).

Others confirmed (K18) that achlorhydric gastric juice from patients with pernicious anemia had inhibitory activity on gastric secretion. They

obtained alcoholic precipitates from these gastric juices, which were then centrifuged, dried in air, ground to a powder in a Waring blendor, and administered in suspension form. While achlorhydric juice of patients with gastric atrophy was a source of potent inhibitory material, anacidity produced by X-ray radiation of the stomach did not yield a potent secretory depressant (Kirsner *et al.*, K18).

Code's group also found that hog gastric mucus, obtained by scraping the hog gastric mucous membrane and given intravenously in saline, exerted inhibitory activity on gastric secretion (B18, B19). A good quantitative relationship existed between the dose of the mucin injected and the per cent inhibition of gastric secretion.

This inhibitory material, which Code later named "gastrone" (C20a), could be precipitated by alcohol. Alcohol precipitates of saliva also exerted inhibition, but to a lesser degree than preparations of gastric mucin (C22a).

Extending this work, Livermore and Code studied the inhibitory effects of alcohol precipitates of canine gastric juice from vagally innervated and vagally denervated gastric pouches, collected after methacholine or histamine stimulation (L12a). One hundred mg of alcoholic precipitate of gastric juice from Pavlov pouches, after methacholine or histamine, caused higher inhibition (75%) than the same dose from Heidenhain pouches, following methacholine (54%) and histamine (25%) stimulation.

Furthermore, Hood *et al.* (H42) investigated the possible source of a gastric inhibitory factor in canine gastric juice. They found that it was derived from the antral mucosa. When Pavlov pouches were denervated by section of the vagal nerve, the inhibitory activity of these juices was greatly reduced.

More recently, Wolf's group expanded the work on this inhibitory material. Smith *et al.* (S29) used dogs with Heidenhain pouches, prepared according to the Dragstedt technique; they collected gastric juices for 2-hour periods after meat feeding. Inhibition of gastric secretion in dogs could be obtained with as little as 1 mg/kg of dialyzed and lyophilized pooled normal human gastric juice, injected i.v. In 80% of tests, a 59% inhibition from the control level was obtained with gastric content and saliva, but not with albumin, synovial fluid, bovine corpus vitreum, and fluid from ovarian cyst, known for its high content of blood group substances. In some animals, a prolonged period of inhibition was noted, occasionally lasting 3 days. Boiling for 15 minutes at neutral pH did not inactivate the inhibitory material. Acidification to pH

1.5 and incubation at 37°C as well as boiling at pH 1.0–1.5 completely abolished the inhibitory effect of human gastric juice. In studying the effects of this inhibitory material on gastric secretion, the authors noted that pepsin production was not inhibited, while gastric juice volume and its acidity declined markedly. Thus, the inhibitor demonstrated selective inhibitory activity upon the parietal cells.

Menguy and Smith modified the assay of the inhibitory material in the gastric juice and in saliva (M29–M30b). Shay rats were used for this purpose 4 hours after pyloric ligation, and the test substance was injected i.v. Rat gastric secretion was inhibited by 2–6 mg normal human dialyzed and lyophilized gastric juice and by 6 mg saliva in these experiments. Injection of 6 mg human gastric juice had greater inhibitory effect than 2 mg of the same material. Peptic ulcer juice demonstrated significantly greater inhibitory activity than normal gastric juice at equivalent doses, whereas gastric cancer juices had the least effect. Peptic activity in rat stomach was not affected by the injection of test material, nor did control injection of serum albumin or saline inhibit gastric secretion.

Greater activity of peptic ulcer over normal or gastric cancer juice was attributed here to elaboration of the secretory inhibitor in the gastric antrum, known to be more stimulated in patients with hyperchlorhydria than in those with low HCl output. Menguy and Smith explain the discrepancy with results of the Brunschwig and Code groups (who found higher inhibitory activity in anacid gastric juices as compared to normal or hyperacid juice) by calculating the inhibitory effects, not per volume of native gastric juice as the other authors did, but per weight of nondialyzable solids of the gastric juice. Higher inhibitory values obtained with these juices by previous authors were allegedly due to higher concentration of lyophilized solids in the achlorhydric gastric juice of patients with histamine-fast anacidity than in acid native juice.

Others (P1) in the same group studied the dose-response to secretory inhibitor in rats. One mg of dialyzed and lyophilized gastric juice of normal subjects, as compared with control rats, decreased the secretory output of the acid in pylorus-ligated rats by 42%, 2 mg by 73%, and 4 mg by 87%. Inhibitory activity was retained in 66% acetone or ammonium sulfate precipitate, as well as following 30-minute boiling, trypsin digestion, or charcoal adsorption. Oxidation with 10% hydrogen peroxide for 20 minutes at 4° caused marked decrease of the inhibitory activity to about one half of its original potency.

In further work, Menguy *et al.* (M29) compared the inhibitory ef-

fects upon the gastric secretion of saliva from patients with various gastric disorders. Highest inhibition was obtained with saliva of patients with gastric ulcer and gastric carcinoma, lesser inhibition with normals, and lowest with saliva from duodenal ulcer patients. The explanation for these findings is as yet unclear.

Katzka and Riss extended these rat studies (K9) and found that inhibitory material was present in gastric juice of normals and patients with pernicious anemia and in human saliva, but was absent from normal human serum, hog intrinsic factor concentrate, and purified blood group substance A. Gastric juice fractionation by starch block electrophoresis yielded inhibitory material in the protein fraction remaining at the origin, which was the largest fraction in pernicious anemia gastric juice.

Other authors have also studied the effects of human gastric juice injection on gastric secretion in pylorus-ligated rats (K30, M42–M44, W4); 10 out of 17 rats showed marked decrease of acidity (on the average by 65%) following injection of normal acidic gastric juice, as compared to saline controls. Anacid juices of cancer patients caused higher inhibition (79–99%), similar to extracts from the stomach mucosa of patients with gastric cancer (97%). Acidic cancer juices were less active than normal acid juices and caused 53% inhibition. The "glandular mucoprotein" fraction of normal gastric juice was active in only 1 of 11 experiments, causing 36% inhibition. The trichloroacetic acid precipitate of the same gastric juice was active in only 2 of 8 experiments, while the supernatant from the acetone precipitation of the trichloroacetic acid filtrate was completely inactive. The mucoproteose fraction from anacid gastric juice was most active, and 5 of 8 rats developed anacidity following mucroproteose injection. Total decrease of acid output was 77%. Serum mucoprotein caused a 57% decrease.

For the last 4 years, in a joint research project with C. F. Code, we attempted to come closer to an evaluation of the nature of gastrone. The study included the distribution of activity on the electrophoretic partition of pooled acid and anacid human gastric juices, as well as in effluents from the gel filtration of these materials on Sephadex columns of various porosities. Preliminary findings have been recently published in abstract form (K31).

We found that gastrone activity in human gastric juice is associated with at least two materials: (1) carbohydrate of sialomucin, and (2) protein, exhibiting the mobility of γ-globulin. It is affected by acid-pepsin digestion, but is retained in some split products of inhibitory materials (K31). The significance of these findings is discussed in Section 8.3.

8.2. Gastric Atrophy-Producing Factor

Smith *et al.*, expanding their initial work (S30, S31), found that dogs given several i.v. injections of dialyzed and lyophilized pooled normal human gastric juice have become permanently achlorhydric (S28). This occurred initially without histological lesions of parietal cells, but at a later date developed into gastric atrophy. Strikingly enough, this mucosal atrophy extended to the vagally innervated gastric pouches and the intact stomach of the dog, but not to the denervated Heidenhain pouches. The inhibition developed following semiweekly injection of 1 mg nondialyzable gastric solids per kg weight of the dog, and persisted in some cases as long as 9 months after injections were discontinued. As little as 8 injections over a period of 4 weeks caused the onset of atrophic lesions in some dogs. Twenty-four injections over a 3-month period resulted in achlorhydria in all but two of the dogs treated, and in marked atrophic changes in the gastric mucosa.

More recently (H13), six dogs received two large doses each of normal dialyzed and lyophilized human gastric juice (4 mg/kg weight) at 1-month intervals, mixed with Freund's adjuvant and administered intradermally at multiple sites. Two control dogs received Freund's adjuvant only. Histamine-fast achlorhydria developed in all dogs 2–7 months after onset of injections. Surgical biopsies of gastric fundic mucosa performed at 2-month intervals showed atrophic changes on histological examination in all dogs, regardless of whether they received autologous, isologous, or heterologous material. Changes consisted of edema of interstitial tissue, decreased amount of mucosal cells, proliferation of connective tissue in the area of fundic glands, and finally mucosal atrophy. When injections were discontinued for 9 months, free acid reappeared in two dogs given autologous gastric juice, in one given isologous juice, and in one given the heterologous specimen.

All dogs were tested for precipitation reactions with injected gastric juice. Results demonstrated appearance of the precipitant approximately 6 months after the start of injections in only 1 control, but in as many as 14 of 20 sera of dogs injected with gastric juice. Precipitation reactions performed with coated gastric mucosal extracts yielded 14 of 19 positive tests in dogs treated with gastric juice, but in only 1 of 7 controls (H13).

It is not known whether the substance which causes atrophic gastric mucosal lesions in dogs is identical with the gastric secretory inhibitor of Brunschwig *et al.* (B36–B40), i.e., gastrone of Code (C20a). Some doubts arise, however, since the atrophy-causing material is resistant to boiling

and acidification, whereas these procedures decrease the inhibiting effect of gastrone upon acid secretion. Hennes *et al.* (H13) concluded that an immunological mechanism is probably responsible for the appearance of atrophic gastritis in their experiments. An alternative possibility is that the continuing inhibition of the secretion of parietal cells may lead to atrophy of the gastric glands. Since no atrophy was found in Heidenhain pouches, it is possible that the vagus may have some relation to the development of this atrophy. Still another alternative may be the formation of antibodies to gastrin in dogs treated with gastric juice, which may depress the normal secretory response of canine parietal cells to gastrin, and so depress the normal acid secretion. Such a concept postulates the presence of gastrin in the gastric juice, which has yet to be established. Another possibility would be the formation of antibodies to some product of parietal cells, which results in atrophy (see Section 7).

Sircus *et al.* (S24) were unable to duplicate the results of Smith *et al.* (S28, S31). They used histamine-stimulated gastric juice from ABO(H) secretors and nonsecretors, which they dialyzed, lyophilized, and injected into dogs with Heidenhain pouches at a dose of 2 mg dried gastric juice/kg weight at 5–day intervals for 10–13 weeks. No significant change was observed in the acid response to maximum histamine stimulation in treated dogs, when compared to controls. There was no evidence of atrophic gastritis or destruction of parietal cells. Juices of various blood group types behaved similarly. Whether the discrepancy between these results and those of Smith *et al.* was due to administration of different gastric juices by the two investigators (fasting gastric juice by Smith's group and juices collected after augmented histamine test by Sircus *et al.*) is not known. It is possible that some destruction of the inhibitory factor occurred in highly acid gastric juice collected after histamine. The most recent work of these authors seems to indicate that similarly, negative results were obtained with fasting gastric juice as well (S24a).

Using rats and dogs, Kubo and Miyoshi *et al.* (K30, M42–M44) partially confirmed Smith's results. When gastric juice was injected i.v. into rats twice a week for 5–8 weeks, a slight decrease in gastric acidity and transient thickening of the Zeissl membrane in the gastric mucosa were noted. If injections extended over 15 weeks, a 50% decrease of acidity was found, thickening of the Zeissl membrane became more marked, and secretory glands separated from each other and atrophied slightly. In 7 dogs injected with gastric juice for more than 20 weeks, the gastric acidity was remarkably reduced to less than 50% of normal;

connective tissue in the lamina propria underwent proliferation; in three dogs a narrowing of the fundic glands and in two a distinct atrophy of the glands were observed. After cessation of gastric juice administration, the changes did not revert to normal. It should be noted that oral administration of human gastric juice to pylorus-ligated rats did not cause inhibition of gastric secretion.

The authors (K30, M44) also reported the presence of circulating precipitants in serum of dogs repeatedly injected i.v. with human gastric juice, using a relatively crude "ring test." This precipitation was positive in 13 of 17 sera from patients with histamine-fast anacidity, when tested against pooled human anacid gastric juice. It was also positive in 12 of 14 against hypoacid gastric juice, in 12 of 22 against normal gastric juice, and in 6 of 20 against hyperacid gastric juice. A total of 43 of 81 gastric juices showed a positive ring test. Correlation of the status of gastric mucosa on biopsy with the occurrence of the serum ring test demonstrated positive tests in 14 of 17 patients with gastric atrophy, proven by gastric biopsy; it was positive in only 1 of 5 subjects with normal gastric mucosa. The significance of these findings is not yet clear.

Miyoshi *et al.* (M44) studied *in vitro* the effect of gastric juice and its fractions on the parietal cells of fresh rat gastric mucosa, separated by a "needle technique." Parietal cells were suspended in a calcium chloride and sucrose solution. Following exchange on filter paper of the medium for "mucoproteose" solution, severe changes were found in the parietal cells. These included cytoplasmic atrophy, karyopyknosis, degeneration of mitochondria, and degranulation. Heating mucoproteose did not abolish its effects on parietal cells. When the "glandular mucoprotein" fraction was used, cytolysis and karyolysis developed. Pepsin caused changes similar to those observed with mucoprotein, and heating the mucoprotein fraction abolished its lytic effects. The significance of this interesting technique has yet to be evaluated.

8.3. Antibodies in the Gastric Juice

Presence of γ-globulin in normal gastric juice has definitely been proven by electrophoretic and immunoelectrophoretic work of various authors (G16, G42, H11, H20, H55). Especially high concentration of γ-globulin was found in gastric juice of patients with histamine-fast anacidity, associated with atrophic lesions of the gastric mucosa (G16). Since I^{131}-labeled globulin passes from the serum into the gastric juice (C23), as proven by the isotope technique, one can conclude that the γ-globulins found in gastric juice are derived from the serum. It is possible, therefore, that immune globulins present in the serum may also

pass into the gastric lumen, especially since the immune serum globulins do not belong to the large molecular macroglobulins, but mostly to the 7 S γ-, and β_{2a}-globulins.

The passage of antibodies into the gastric juice was reported by Balasz (B4, B5). He found that anacid gastric juice possesses a virus-neutralizing capacity, as studied in tissue culture, and contains *Escherichia coli* antibody, as determined by hemagglutination tests. The antibody level in anacid gastric juice depends on the antibody level of serum in the same subject. Although γ-globulins are much more resistant to proteolytic digestion than serum albumin (G16), it is possible that the antibody titer in acid human gastric juice is lower than in anacid juices. Loss of acidity and proteolytic power yields gastric juice richer in immune globulins.

This situation is similar to that observed in saliva, where the passage of serum antibodies into the salivary secretion has been demonstrated (E5). When antibodies were present in serum of some healthy subjects, antibodies to various bacteria in saliva were found with hemagglutination and bacterial agglutination tests. The presence of these antibodies in saliva may be correlated with the finding of γ-globulin in this secretion (E5).

Irvine *et al.* (I4, I5) and Doniach *et al.* (D8, R10, T13) found that sera of patients with atrophic lesions of gastric mucosa contain antibodies to parietal cells in the immune globulin fraction; they may certainly pass into the gastric lumen. The reviewer does not rule out that such a gastric juice, containing immune globulin directed against parietal cells, when injected into animals, may exert a blocking effect on the parietal cells of the recipient (dog or rat). The presence of such an immune globulin may be one of the important mechanisms of gastrone activity of this gastric juice in short-term experiments, as well as perhaps the cause of gastric atrophy on prolonged administration.

Thus, a hypothetical linking together of four rather disparate findings is possible: (1) presence of antibodies to parietal cells in serum of patients with advanced atrophic lesions of the stomach and pernicious anemia (D8, I4, I5, R9, T13), (2) high gastrone activity of anacid gastric juice (B18–B20, B36–B40, C21–C22), (3) concentration of an important gastrone activity in the γ-globulin fraction of anacid gastric juice (K31), (4) development of atrophic lesions of the gastric mucosa in dogs receiving prolonged injections of gastric juice (H13, S28, S30, S31). It is conceivable that all these elements are interwoven into a complex texture forming the sequence of events in the natural history of gastric

atrophy and in the development of gastric anacidity. The participation of the auto-immune mechanisms in these areas cannot be ruled out, neither their possible role in the "gastrone activity." This hypothetical concept requires much further work, until its significance is established or disproved.

This by no means disposes of the participation of the mucopolysaccharide materials from the gastric juice in the inhibitory gastrone activity, which seems to be relatively well established in regard to parietal cells secretion (G18, K31).

9. Other Biologically Active Materials

Several other biologically active materials have been described in the human and animal stomach. However, little information is available as to their nature or the mechanism of activity. The available data on these little-explored materials will be presented here, although the evidence submitted is not always adequate and convincing.

9.1. Erythroblast Maturation Factor

Osgood demonstrated that pernicious anemia serum delays maturation of megaloblastic bone marrow (see L1). The work of Callender and Lajtha (see G20) points out that addition of normal gastric juice potentiates the effect of vitamin B_{12} in counteracting this maturation-delaying effect of pernicious anemia serum. A B_{12} binder separated from normal gastric juice by electrophoresis or ammonium sulfate precipitation was also shown to enhance the B_{12} effect on maturation of the erythroblasts, when added to bone marrow (P2, P3). The relation of this maturation-promoting factor to Castle's intrinsic factor is not clearly defined (see G20).

9.2. Migration of Leucocytes and Maturation of Red Cell-Stimulating Factor

Addition of neutralized gastric juice from healthy individuals and animals, according to Feodorov *et al.* (F4), consistently stimulates the migration of leucocytes from a white cell film into a surrounding nutrient medium. In bone marrow cultures, this gastric juice produces increased mitotic activity in young red cells, accelerated maturation of erythroblasts, and speeded up formation of hemoglobin (F4). This factor or factors in gastric juice, supposedly produced in the fundic portion of the stomach, are present at highest concentration in gastric secretion

stimulated by milk and raw meat, and at lowest concentration after subcutaneous administration of histamine. In patients with posthemorrhagic anemia, its concentration in gastric juice decreases sharply; with multiple blood transfusion and in polycythemia, the concentration increases. As shown in Pavlov and Heidenhain pouches, the formation of this factor is under the influence of the vagus. Denervation of the spleen is reported to lead to its complete, though transient, disappearance from gastric juice.

9.3. Iron-Deficiency Anemia-Preventing Factor

Dominici *et al.* described the presence of a hypothetical factor in human gastric juice, lack of which is said to cause disturbance of the oxidative metabolism and iron-deficiency anemia (D7). Intramuscular injection of 5 ml normal gastric juice (neutralized to pH 7.5) and juices of patients with pernicious anemia or anemia of pregnancy caused, 5 hours after injection, a slight rise in temperature and marked drop in serum iron, blood sugar, and serum amino acids. However, injection of gastric juice from patients with hypochromic iron-deficiency anemia and achlorhydria did not cause a so-called normal "humoral reaction." Heating normal gastric juice to 60°C for an hour resulted in disappearance of this factor. Parenteral administration of normal juice, at doses of 10 ml, to patients with iron-deficiency anemia resulted in improvement of this condition.

9.4. Antianemic and Nitrogen-Sparing Factors

Mice and rats, rendered anemic by phenylhydrazine poisoning, were given oral mucin preparations from hog gastric mucosa, which significantly accelerated hematological remission (C27). This effect was related to a modification of intestinal flora or to an improvement of intestinal absorption of iron or vitamin B_{12}.

Piccinini reported a nitrogen retention-promoting effect in normal and hypophysectomized rats of hog mucin rich in blood group substances (P7, P8). Cresseri *et al.* noted retention of amino acid nitrogen in rats treated parenterally with mucin preparations and vitamin B_{12} (C27).

9.5. Growth Factor

Tomarelli *et al.* (T31) reported that rats grow more rapidly when feeding is associated with administration of hog gastric mucin. This effect may be due to its content of *Lactobacillus bifidus* factor. Findings were similar in children.

9.6. *Lactobacillus bifidus* Growth Factor

György (G77) demonstrated the presence of a factor required for the growth of *L. bifidus* in milk (especially human). However, it was also found in other body fluids, including human gastric juice (even from a pernicious anemia patient), and in hog gastric mucin and blood group substances A and B (G77, G78, S36, S37).

Tomarelli *et al.* (T32) separated, after acid hydrolysis and purification by charcoal adsorption and chromatography, a crystalline compound from hog gastric mucin which was highly active in *L. bifidus* assays. It was reported to be a disaccharide containing acetylglucosamine.

9.7. Gastrectomized Rat Survival-Promoting Factor

This was reported by Balfour *et al.* to be present in human gastric juice and to prolong survival of gastrectomized rats (B6). It has been suggested that a similar factor is of nutritional significance in man. It was suggested that the frequent inability of patients, following subtotal or total gastrectomy, to gain weight may be related to the decrease of this factor in gastrectomized patients.

9.8. Lipid Absorption-Promoting Factor

According to Peressini *et al.*, oral administration to infants of "glandular mucoprotein" causes improved fat absorption (P4). Blood lipids increase after feeding this material. The marked effect upon the lipids is not identical with that of vitamin B_{12}, nor is it due to the B_{12} absorption-promoting effect of this mucoprotein. The relationship of this factor to that of Balfour is not known.

9.9. Lipid Clearing Factor

This has been reported in hog gastric mucin and human gastric juice by Capraro *et al.* (C1, C2) and appears to be related to some acidic aminopolysaccharides.

These investigators administered normal gastric juice, as well as acetone-precipitated trichloroacetic acid filtrate of normal gastric juice to normal rabbits. They found a significant decrease in optical density of the blood plasma to which homogenized milk was added, when compared to controls. The same effects were obtained after administration of purified aminopolysaccharides containing large amounts of hexuronic acid, glucosamine, and only traces of sulfate, indicating a possible relation to hyaluronic acid. Since gastric juice contained material which had electrophoretic mobility similar to the latter, the authors assumed that

this aminopolysaccharide material present in gastric juice affected the lipid clearing of the plasma on oral administration.

Capraro's group also found lipotropic activity in rats and guinea pigs with mucin preparations from hog gastric mucosa (C1, C2). While heparin was active only on parenteral administration, the acidic aminopolysaccharides isolated from hog gastric mucin and related to hyaluronic acid were active on oral administration. While the administration of hog mucin to rabbits caused transient increase of plasma clearing power, the purified acidic aminopolysaccharides were 10 times more potent. This suggests that increase in clearing action is due to the aminopolysaccharide content of hog mucin. Others (B28) reported fat-dispersing activity of gastric mucin fed to a healthy infant in cow's milk.

9.10. Lipotropic Factor

Castellani (C4) reported that hog gastric mucin, when given by mouth or parenterally to guinea pig prior to administration of carbon tetrachloride, inhibited deposition of fat in the liver. It was also highly active in rats, where it prevented fatty liver caused by CCl_4 or a high-fat and low-protein diet. Similar lipotropic activity of hog gastric mucin preparations was reported by Goldeck (G60) in humans.

9.11. Gastric Motility-Inhibiting and -Stimulating Factors

According to Schmidt *et al.*, the mucoprotein fraction of human gastric juice slightly increased gastric motility, when given orally at a dose of 20 ml 1% solution (S9). Conversely, 20 ml 1% mucoproteose solution markedly inhibited gastric motility for 0.5–1 hour. Water or saline solution of the same pH caused only occasional disturbance of motility, lasting no longer than 10 minutes.

9.12. Adsorption of Histamine Promoting Factor

Holler *et al.* (H40) reported histamine-adsorbing capacity of the visible mucus and mucoproteose fraction of gastric juice.

9.13. Virulence-Enhancing Factor

This was discovered in regard to various *Streptococcus* and *Neisseria* species by Smith (S25) with the use of a hog mucin preparation. Smith *et al.* (S25–S27) and Record and Grinstead (R2, R2a) then demonstrated that this activity was not due to a single factor, but to a synergic combination of three factors: a viscous medium, a nonspecific particulate matter, and an important third factor. The latter consists of (1) heparin, (2) chondroitin sulfuric acid, and (3) neutral blood group mucoid,

whose activity is not connected specifically with either blood group A or H activity. Olitzki reported similar activity in human gastric and salivary mucin, and Smith in human stomach mucosal extract (see S25).

9.14. KIK Factor

In 1937, Kozawa *et al.* (K28) reported that gastric cancer juice contains material which, when injected i.v. into rabbits, decreases the red cell count. The active principle of this material, called the KIK factor, is a carbohydrate-rich protein (I8, I9). It is relatively thermostable, and 5-minute heating at 100°C neither destroys nor weakens its activity. It is precipitated at pH 4.5–5.0 by full or half saturation with ammonium sulfate, and is also precipitated with 25% sulfosalicylic acid or metaphosphoric acid, but not 5% trichloroacetic acid. The latter characteristic differentiates it from the toxohormone (see below). It is nondialyzable, insoluble in ether, ethanol, or methanol, adsorbed by kaolin at pH 6.0–6.5, and destroyed by pancreatin at pH 8, within 48 hours, but not by pepsin.

KIK factor was also studied in gastric mucin fractionated into our two fractions (S2, S3). Its activity was found to be distributed in both the mucoprotein and mucoproteose fractions. Hexose content of KIK in cancerous material was greater than in the noncancerous, and on paper electrophoresis the KIK factor from cancerous material revealed a higher anodic mobility in acetate buffer than the noncancerous material. In addition, the mucoprotein fraction precipitated from cancerous material by the Winzler method contained more hexoses and hexosamine than similar material from noncancerous stomach. These materials were far less purified, however, than the materials studied by Masamune *et al.*

The latter group (M13, M14) separated two types of KIK factor from cancerous gastric juice. The first was a mucopolysaccharide and the second a mucoprotein. Others thought it to be a mucopolypeptide. A similar factor from cancerous ascitic fluid (I9) contained 50–60% hexoses, 1.5% tyrosine, and 10% protein by biuret reaction. It formed a single peak at pH range 1.5–10.0 on paper electrophoresis, and at pH 8.6 moved with the mobility of serum γ-globulin.

Iwatsuru *et al.* (I9) obtained KIK material from the gastric juice by adjusting the pH to 7, adding 1 volume 10% trichloracetic acid, and dialyzing the filtrate. After concentration, followed by adjusting the pH to 4, and centrifugation, methanol was added at 90% concentration to the filtrate, and the precipitate obta'ned was dissolved in water and dialyzed. The procedure was repeated again and the crude material

obtained yielded 29–35% protein, 28–51% total carbohydrates, 10.6–14.1% hexosamine, and negligible amounts of hexuronic acid. A similar precipitate from noncancerous gastric juice contained less carbohydrates and proteins. Findings obtained with this material on continuous electrophoresis are described in the second review of the author in this volume.

Masamune *et al.* also studied the KIK factor in normal and cancerous gastric mucosa (M13, M14). KIK was purified by precipitating with barium salt and redissolving the precipitate in acetate buffer, followed by zone electrophoresis, phenol extraction, and sequential ethanol precipitation. Materials separated from cancerous and noncancerous gastric mucosa were very similar in composition, but differed in biological activity insofar as the material from cancerous stomach caused anemia in rabbits, and the noncancerous did not. Both cancerous and noncancerous purified KIK materials contained 11–12% nitrogen, 4.1–4.9% hexosamine, 5.1–6.4% hexoses, about 1% fucose, 1.5–2% sialic acid, and yielded a similar spectrum of amino acids, including aspartic and glutamic acids, serine, threonine, glycine, alanine, tyrosine, valine, methionine, phenylalanine, proline, arginine, and lysine. In addition, both KIK substances gave the same electrophoretic patterns, but were not homogeneous.

Thus, the pure KIK factor, if it exists, has not yet been isolated. It is thought that the anemia-inducing activity of the KIK factor from cancer stomach is due to some structural deviations in this material only, when compared with the noncancerous KIK substance.

9.15. Toxohormone

This material, described by Nakahara and Fukuoka (N1), is present only in cancer gastric juice. Upon intraperitoneal injection into mice, it depresses the catalase titer of the liver, kidney, and blood. The relationship of this material to mucopolysaccharides and mucoproteins of gastric juice is not well known. Toxohormone—like material obtained from normal stomach which is biologically inactive, however—was found to have lower polarographic activity than that from stomachs with gastric cancer (M18). Separation of materials with toxohormone activity by continuous electrophoresis on paper curtain and column chromatography is discussed in the following review in this volume.

10. Lipids, Nucleic Acids, and Phosphoproteins

Still other large molecular materials were reported to be found in gastric juice. They have been little explored and are as follows.

10.1. Lipids

Their presence in gastric secretions was reported by Ling and Lim (see H43) and Horowitz and Hollander (see H43). Little is known about their nature.

10.2. Nucleic Acids

They were found by Levene and Lopez-Suarez (L8–L8c) in the mucosal scrapings. However, Horowitz (H43) did not detect them in acetylcholine-stimulated canine gastric secretion and, if present, they could be derived from cellular debris.

10.3. Phosphoproteins

Yakhnina (Y1) reported that the rate of phosphoprotein circulation in gastric mucosa of cats injected with labeled phosphates was elevated upon histamine stimulation of gastric secretion. She considered it probable that phosphoprotein actively participates in gastric secretion.

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FRACTIONATION OF MACROMOLECULAR COMPONENTS OF HUMAN GASTRIC JUICE BY ELECTROPHORESIS, CHROMATOGRAPHY, AND OTHER PHYSICOCHEMICAL METHODS[1]

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Introduction

Human gastric juice represents a complex mixture of water, electrolytes including hydrochloric acid, and large molecular substances. The latter

[1] The investigative work upon which this paper is based has been supported over a period of years by research grants-in-aid: AM-00068, AM-04391, and AM-03778 from the National Institute of Arthritis and Metabolic Disease, P.H.S., and C-05601 from the National Cancer Institute, N.I.H., P.H.S., and grant # P-305(F) from the American Cancer Society which is gratefully acknowledged.

[a] The author acknowledges the helpful cooperation of those investigators and publishers who gave their kind permission to reproduce their figures.

include enzymes, mucopolysaccharides and mucoproteins, serum proteins and their degradation products, and many other biologically active materials. They are discussed in some detail in the preceding companion review by the author (G5).

While the study of the gastric electrolytes has been proceeding at high speed for many years, this is not true for the investigation of macromolecular components of gastric juice.

The number of technical difficulties has hindered the fractionation and identification of the gastric macromolecular substances. These are due to: (1) very low concentration of some components of great biological significance (such as intrinsic factor or some enzymes), (2) marked loss of some gastric juice components during dialysis and fractionation procedures, (3) abnormal aggregation of some gastric materials during concentration of gastric juice, without which most of the fractionation techniques cannot be performed, (4) depolymerization of some native materials in gastric juice by alkalinization, which is often used to prevent further proteolytic degradation of proteins in gastric juice, (5) inadequacies of available methods for isolating large molecular monomers from the polymers and complexes they may form in gastric juice, (6) complexity of techniques needed for identification of macromolecular materials.

Despite these difficulties, considerable progress has been made recently in fractionating large molecular gastric juice materials. This has been achieved mainly by the use of various physicochemical methods. They include: electrophoresis (free boundary, paper strip, paper curtain, cellulose acetate, starch block and gel, agar gel, and immunoelectrophoresis), immunodiffusion, column chromatography on Amberlite IRC-50, Dowex 50, Ecteola, and DEAE- and CM-cellulose as well as DEAE- and CM-Sephadex, partition paper chromatography, gel filtration on Sephadex, ultracentrifugation, and polarography. Of these, electrophoresis, column chromatography, and gel filtration were most successful in fractionating the complex mixtures and aggregates of macromolecules present in the gastric secretion. The application of these techniques to the study of gastric juice has been treated only casually or omitted in the recent monographs and reviews on electrophoresis and chromatography (B7, B9, H2a, P1, R2, W18, W19).

These various methods, as applied to macromolecular materials of gastric juice, and their results will be discussed here in more detail under separate headings. The review will demonstrate that much new knowledge has been derived from these investigations. However, the basic problems pertaining to identification of most of these materials are still

unsolved. Also, understanding is lacking of the structure of polymers and aggregates which these materials form in the gastric juice. The same is true for their products of degradation and their further fate in the gastrointestinal tract. Our state of knowledge of these macromolecules is still rudimentary, and much remains to be done in this area from a chemical, physical, and physiological point of view.

1. Electrophoresis

1.1. Free Boundary Electrophoresis

This method was first used in 1951 by Grossberg *et al.* (G32) in studying canine gastric juice obtained after sham feeding from dogs with gastric fistula and esophagostomy. Four negatively charged protein components and a small positively charged peak were distinguished with buffer of pH 6.0, all of which were heterogeneous. All exhibited a rather high degree of peptic activity, and all contained uronic acid and hexosamine at various ratios.

In 1952, Pugh *et al.* (P7) utilized this method for the study of human gastric juice and mucin fractions processed by chemical fractionation. This represented the first reported electrophoretic study of human gastric secretions. The authors used the Longsworth apparatus and acetate, phosphate, or barbiturate buffer of ionic strength 0.05 and pH ranging from 3.7 to 8.5. The "dissolved mucin" fractions were run at 0.6–1% concentration and nonfractionated gastric juice at 1–1.5% concentration with a potential gradient of 4.37–8.57 volts per cm for 2–5 hours. The electrophoretic pattern of human gastric juice was grossly similar to that obtained by Grossberg *et al.* (G32) in canine juice, although the first two peaks had somewhat faster and the fifth (slowest peak) slower mobility than those of Grossberg.

Crystalline pepsin of porcine origin showed a single large peak with negative mobility at pH 5.5 of 8.9×10^{-5}, and at pH 6.1 of $10.6–11.6 \times 10^{-5}$ cm^2 volt^{-1} sec^{-1}. These mobilities were only slightly higher than those observed by Grossberg *et al.* (G32).

The electrophoretic pattern of "mucoproteose" fraction prepared by dialysis and lyophilization showed one fairly well-defined peak with mobility of -0.6×10^{-5}, and another poorly defined peak with more negative mobility.

The electrophoretic pattern of "glandular mucoprotein" was distinctly different from that of mucoproteose, since it displayed a single, slightly asymmetrical peak of fast negative mobility, ranging from -8.5×10^{-5} to -7.2×10^{-5} in phosphate buffer over the pH range 6.0–7.3.

In acetate buffer of pH 5.0, its mobility was -6.7 to -7.3×10^{-5} cm^2 volt^{-1} sec^{-1}. In veronal buffer of pH 8.5–8.6, its mobility was surprisingly similar to that observed in phosphate buffers of pH 6.0–7.3. When mucoprotein was prepared by precipitation at pH 2.8 (and not at pH 3.5 which increases pepsin content in the precipitate) a single peak was still observed at pH 8.5 in veronal buffer, similar to other mucoprotein samples. However, this preparation in phosphate buffer at pH 6.0 had slightly higher mobility than the remaining samples.

Mack *et al.* (M1, M1a) extended this work and subjected human gastric juice to free boundary electrophoresis in an analytical Aminco

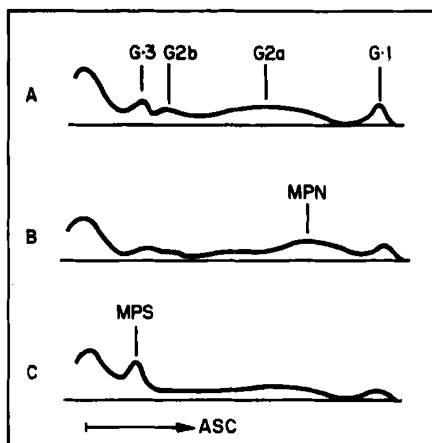

FIG. 1. A: The electrophoretic pattern of gastric juice from a healthy subject in 0.1 ionic strength veronal buffer at pH 8.6 and a protein concentration of 3.9. B: The same after addition of "glandular mucoprotein." C: The same as A after addition of mucoproteose. From Mack *et al.* (M1).

cell. They used either phosphate buffers of 0.1 ionic strength and pH from 5.1 to 6.9, or veronal buffer of pH 8.5. Six electrophoretic runs were performed on healthy subjects and 14 on duodenal ulcer patients, after dissolving dialyzed and lyophilized gastric juice at 1–5% concentration in the buffers used. Resolution in veronal buffer at pH 8.5 was better than that obtained with phosphate buffer of pH 5.1–6.9.

Substantiating the earlier observations of Pugh *et al.* (P7), they also noted that nondialyzable components of gastric juice had a higher anodic mobility in phosphate buffer of pH 6.9 than in veronal buffer of pH 8.5. Four electrophoretic components labeled G1, G2a, G2b, and G3 were resolved by this procedure (Fig. 1). Normal and duodenal ulcer juices did not differ in total number of components nor in resolution, but the

ulcer juices had higher first and lower third peaks than the normal ones. The component with the highest anodic mobility, G1, had slightly higher electrophoretic mobility than glandular mucoprotein processed from the gastric juice, but it moved more slowly than bovine pepsin. The number of peaks in group G2 varied from 1 to 3. The differences in size and resolution of boundaries obtained indicated nonhomogeneous material of relatively low molecular weight in the G2 area. Mobility of this major intermediate component was similar to that of the major component in saliva. The chance that G2 proteins in gastric juice represent only salivary contamination was ruled out by the presence of some of these materials in gastric juice of an individual having an occluded esophagus and a gastric fistula. The G2 protein components could be removed from gastric juice by trichloroacetic acid precipitation, which is known to precipitate the surface epithelial mucus physically dissolved in gastric juice, the so-called "soluble mucus." The slowest peak, G3, was fairly homogeneous and corresponded to a protein migrating at low mobility, which was electrophoretically identical with the mucoproteose fraction (see Section 1.9.2).

1.2. Paper Electrophoresis

1.2.1. *Various Techniques and Their Results*

In 1952–53 Norpoth *et al.* (N1), Henning *et al.* (H5, H5a), and Hiller and Bischof (H6) pioneered the application of paper electrophoresis to the study of gastric juice. The first author used the "dry drop method," which, as the name implies, consists of drying a drop of gastric juice on filter paper that is then submitted to electrophoresis. Electrophoretic patterns of gastric juice obtained by this method yielded 4–6 fractions. However, with this technique there was frequent distortion of electrophoretic patterns and only 50% of them showed fair resolution.

Hiller and Bischof (H6) detected 5 components on paper electrophoresis of dialyzed and concentrated fasting human gastric juice, which they named according to the nomenclature used in the electrophoresis of the serum. The first component ("albumin") formed 24.6–32.6% of the total protein, the second ("α_1-globulin") 8.2–9.5%, the third (α_2-globulin") 9.5–13.6%, the fourth ("β-globulin") 10.6–13.6%, and the fifth ("γ-globulin") 27.0–38.2%. In pathological gastric juices there was a frequent increase in fraction V, especially in gastritis.

Henning *et al.* (H5, H5a) improved these results by neutralizing gastric juice to pH 7.2 and concentrating it 7.5 times by 48–72 hour dialysis against Kollidon. Electrophoresis was performed at 2 mA and 110 volts in barbiturate buffer in the horizontal electrophoretic cell of

Grassmann and Hannig. Strips were stained with amido black, traced, and analyzed by Gaussian curves. Eighty-two gastric juices from 59 individuals were initially studied by this method.

Fasting secretion of normals usually contained about 4 bands, of which the first "fast" one contained 12–21%, the second 25–32%, the third 23–29%, and the fourth (of slowest mobility) 27–35% of total proteins. Electrophoretic mobility of the first band was similar to that of albumin, and that of band 4 to γ-globulin. Following stimulation of gastric secretion with histamine, the fourth band showed a consistent rise, so that it comprised 40–75% of total protein. The remaining bands were little changed, or showed a slight decrease in height. Patients with gastric and duodenal ulcer demonstrated no change from the norm, but occasionally additional positively charged components were discovered, in some cases, after histamine stimulation. In superficial gastritis, the slowest peak was usually increased, while other bands showed no abnormal change. In atrophic gastritis, the first peak, representing serum albumin, was markedly increased. A similar increase was found in the fourth peak which, according to the authors, corresponded to γ-globulin. In pernicious anemia, the electropherogram was supposedly similar to that of the serum and contained 4–6 bands, but band 4 (γ-globulin) was very high and amounted to 60% of total proteins. The albumin peak was also high. In some cases, other protein bands, having the electrophoretic mobility of α- and β-globulins, were also found. Electrophoretic patterns in 5 cases of anacidity without atrophic mucosal lesions were similar to those found in gastric atrophy. In gastric carcinoma, the first band with electrophoretic mobility of serum albumin was very high, as was the slowest band allegedly corresponding to γ-globulin. An intermediate component of α-globulin mobility was sometimes also present.

Henning et al. (H5a) refrained from drawing conclusions as to the nature of the components found in normal gastric juice. Nevertheless, they listed gastric components according to the serum protein nomenclature, though several gastric juices yielded a different electrophoretic pattern than serum. Although gastric enzymes were considered the causative factors in degradation of some protein bands in gastric juice, no bands were related to the degradation process. The authors apparently did not realize that the last band, increasing after histamine stimulation and in peptic ulcer, on horizontal electrophoresis represented not only γ-globulin, but also peptides resulting from degradation of proteins. They also did not associate some occasionally occurring cathodic bands with peptides. Instead, they were referred to as "paraproteins" related to γ-globulin.

Various authors (D2, G24, H5–H6, N1–N3, V1, V1a, W6, W7) applied to the electrophoresis of gastric juice and mucosa the standard technique used for electrophoresis of serum, i.e., horizontal unit and veronal buffer. The resolution obtained was not as good as that with serum (reviewer's comment).

A technique of vertical paper electrophoresis of gastric juice was developed in our laboratory, with the use of a Spinco-Williams cell and borate buffer (G19). Its advantage consists in sharpening the protein boundaries and in better resolution of cathodic bands, which are not resolved by the horizontal cell. The advantage of the borate buffer over veronal lies in the better resolution of carbohydrate materials in gastric juice and sharpening of mucoprotein bands. Gastric juice was either dialyzed and lyophilized (G19), or concentrated 10–15 times by 5–6-hour dialysis against Carbowax 6000 (I2). Fifty to 100 μl of 2% solution of lyophilized gastric juice in borate buffer of 0.24 ionic strength and pH 9 or 20–100 μl of gastric juice concentrated 15 times was applied in the middle of a Whatman No. 1 electrophoretic strip (3 cm wide) and submitted to electrophoresis at 120 volts and 0.4 mA/cm for 5½ hours at room temperature. Paper strips were stained with saturated (1%) solution of amido black 10B in a mixture of methanol and glacial acetic acid (9:1), which also stains the cathodic peaks not stained by bromphenol blue. Subsequently, materials were passed through five washing dishes containing methanol–glacial acetic acid solutions. Other strips were stained with SF light green (lissamin green) and periodic acid-Schiff (PAS) stain, according to the methods described.

The normal electrophoretic pattern of gastric juice, collected after histamine stimulation and resolved by the techniques described above (G3, G19), consists of 4–5 bands on the anodic side of the tracing and 4–6 bands on the cathodic side. The approximate distances of the various components, determined from the point of application at the center of the strip, are shown in Fig. 2. The usual total length of the tracing, 11–12 cm, serves as a basis for these distances. It should be noted, however, that there may be differences of 0.5–1.0 cm in the total length of the electrophoretic partition, depending on the room temperature, air convection currents, overloading of the electrical line, viscosity of the materials, and the precision with which the application is made (G3). These factors will obviously have greater effect on the mobility of the components localized at the anodic and cathodic ends of the tracings.

The leading anodic band of the normal tracing corresponds to pepsin, and for this reason we termed it band P. The other, more centrally located anodic bands, as well as the first cathodic band extending to

the point of application contain both proteins and carbohydrates and represent mucous substances of the gastric juice (Fig. 3). For this reason we called them bands M1, M2, M3, and M4. Component M3 can often be subdivided into two materials, M3a and M3b. Component M4 migrates toward the cathode by endosmosis, as shown by the extent of its cathodic travel when compared with that of materials not electrically charged, such as dextran (G19).

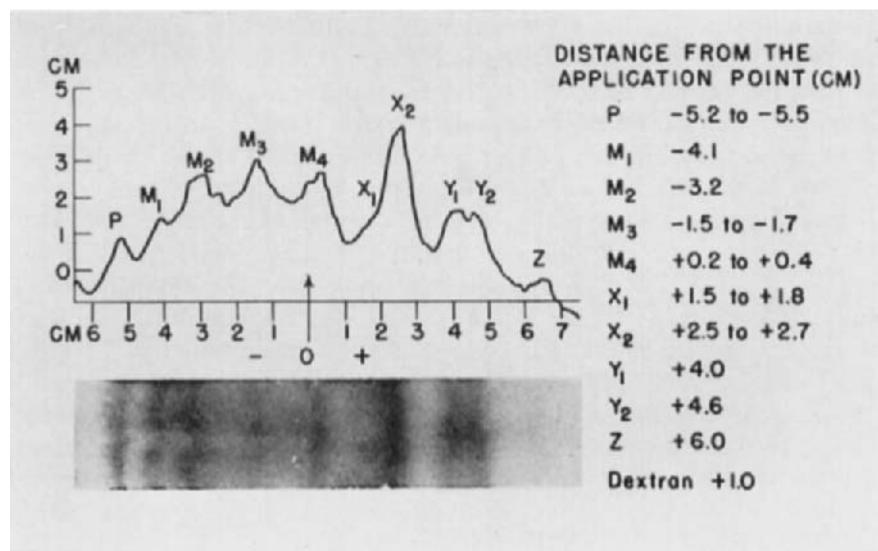

FIG. 2. Paper electropherogram of a pool of normal gastric juice. From Glass *et al.* (G19).

At the outset of this work, for want of an exact understanding of the cathodic bands, we called them X, Y, and Z. Bands X and Y were later found to consist of two subcomponents each, and these we labeled, respectively, X1 and X2, Y1 and Y2. Of these, only X1 was found to stain with protein stain (SF light green), while bands X2, Y1, Y2, and Z took up virtually no SF light green or carbohydrate stain or only in traces. On elution, the cathodic bands X2, Y1, Y2, and Z gave negative reactions for proteins and carbohydrates. Cathodic materials X2, Y1, and Y2 had mobilities similar to the peptic degradation products of serum albumin. Indeed, they are thus derived, at least in part, and represent non-dialyzable or slowly dialyzing polypeptides which contain organic bases (see Section 1.2.2.b).

The materials designated by the letter M in the electropherogram of

normal gastric juice contain polysaccharides and proteins and represent gastric mucoproteins and mucoids (G19). PAS stains the central area of the electrophoretic partition of gastric juice, mainly at the anodic side (Fig. 3), encompassing the areas in which materials M1, M2, M3, and M4 are contained. As shown by the intensity of the PAS staining, the highest concentration of polysaccharides was in the area of component M3b. Materials M2, M3a, and M4 contained polysaccharides also but to a lesser extent, while components M1 and X1 contained only very small amounts of polysaccharides. Pepsin (component P) and cathodic materials X2, Y1, Y2, and Z did not stain with PAS stain at all.

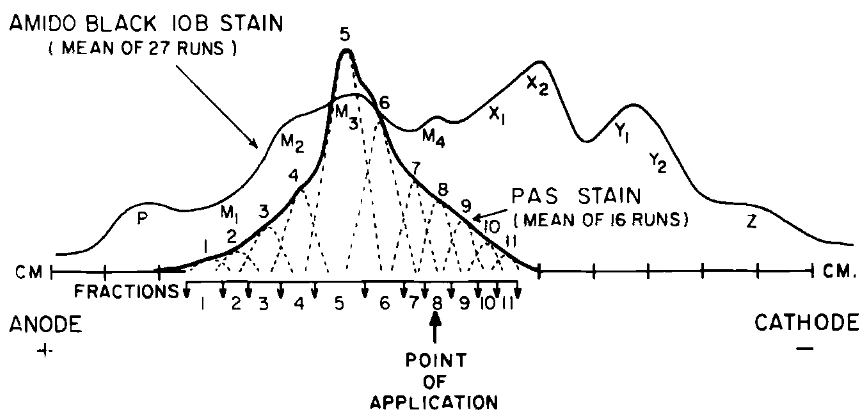

FIG. 3. Carbohydrates of a pool of normal human gastric juices fractionated by paper electrophoresis. From Glass (G3a).

Electrophoretic evaluation of the mucous substances of the gastric juice is complicated by the somewhat similar electrophoretic mobilities of serum proteins and their degradation products. Serum albumin has a mobility only slightly more negative than that of mucoprotein in M2 (Fig. 4). The first anodic degradation product of albumin has a mobility identical with that of component M3a (Fig. 4). The second anodic degradation product of serum is localized on the electrophoretic partition very close to component M3b (Fig. 5). Finally, the mobility of γ-globulin is identical with that of component X1.

The separation of these materials became possible later with the use of gel filtration on Sephadex G-75, G-100, and G-200 columns (see Section 5). The latter two columns also dissociate some neutral glycoproteins from their complexes with other gastric components (G12, G12a).

All the nondialyzable components, resolved by paper electrophoresis

in lyophilized gastric juice, were also detectable—and at a similar ratio of concentration—in the electropherograms of gastric juices dialyzed against Carbowax 6000 (I2). At times, we found some materials at higher

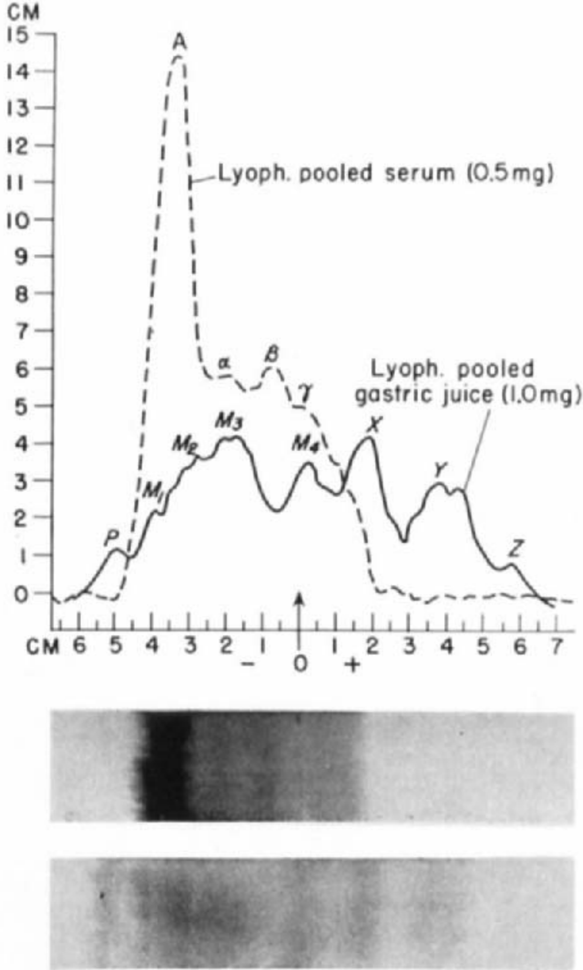

FIG. 4. Paper electropherogram of normal acid human gastric juice and serum. Borate buffer pH 9.0, Γ/2 0.24. From Glass *et al.* (G19).

concentration in Carbowax-concentrated gastric juice than in lyophilized gastric juice. This was especially true of some cathodic components, notably component X2 (Fig. 6). In other Carbowax 6000-concentrated acid-pepsin-containing gastric juices, we also found an acid polysac-

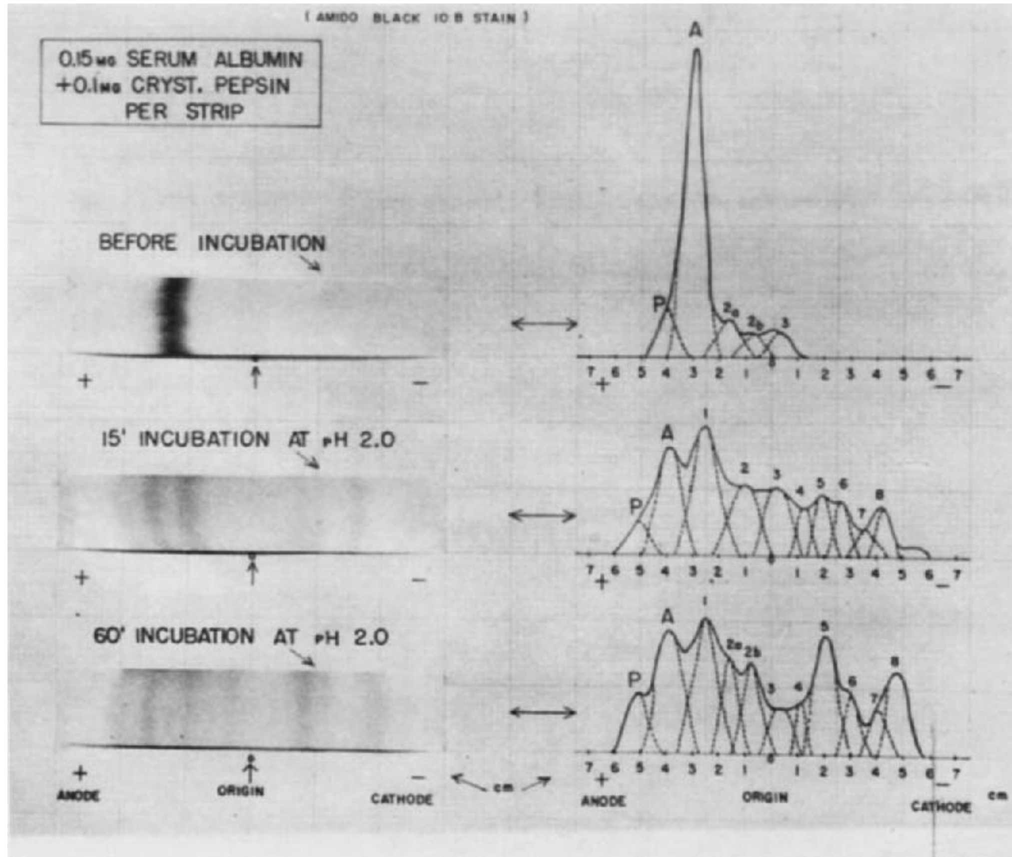

FIG. 5. Paper electrophoresis of serum albumin and its peptic degradation. From Glass (G3).

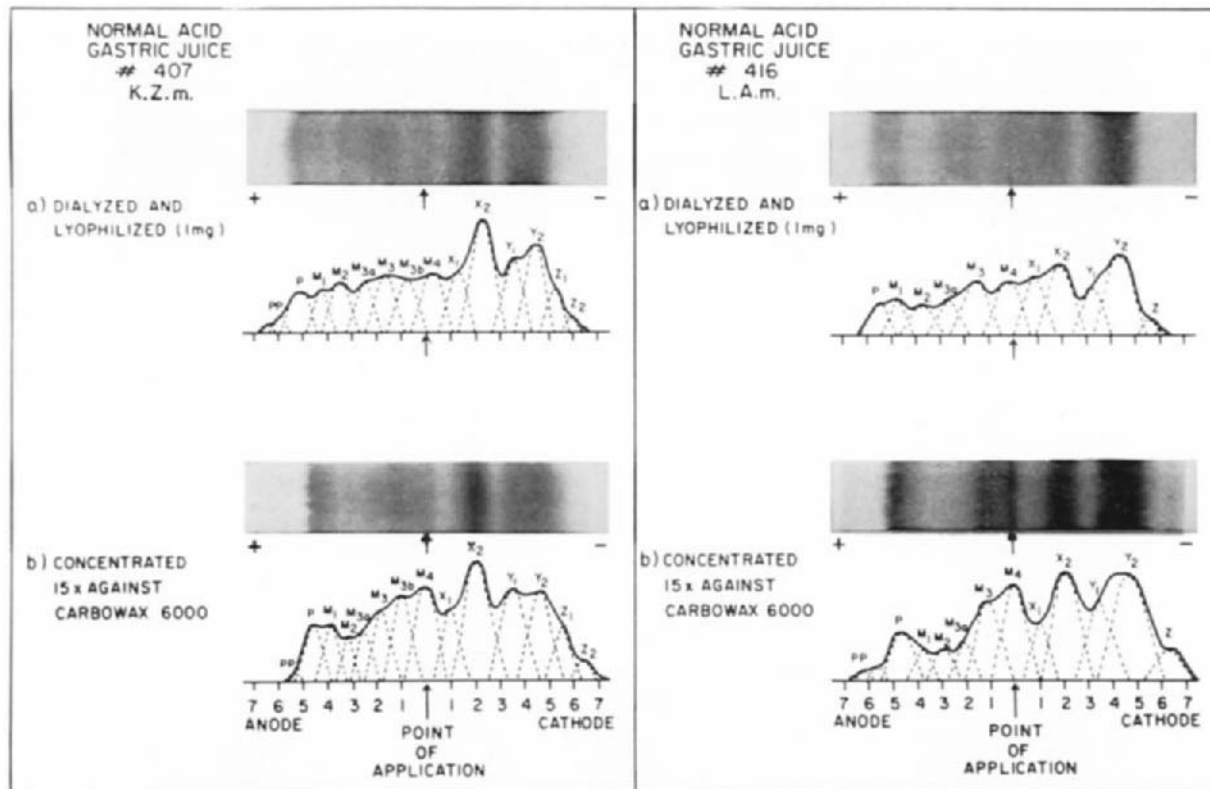

FIG. 6. Comparison of paper electrophoretic patterns of 2 normal gastric juices: (a) dialyzed and lyophilized or (b) concentrated against Carbowax 6000. From Ishimori and Glass (12).

charide which was absent when materials were extensively dialyzed and lyophilized. Most probably this fast moving component corresponds to the alcian-blue fastest staining component of the paper electropherograms of canine gastric juice obtained by De Graef (D1).

Another material with a higher negative charge than pepsin is not detectable in lyophilized gastric juices, but is found, in very small concentration, in Carbowax tracings (I2). It was termed PP (propepsin). This material was noted in earlier work on starch gel electrophoresis of acid gastric juices. It is either one of the negatively charged products of albumin digestion or represents mucopeptides whose high negative charge may be due to a high sialic acid or sulfate content. All these materials apparently dialyze out when gastric juice is dialyzed extensively against water, but not when it is dialyzed for a short time against Carbowax (see Fig. 6).

Generally, results with the Carbowax 6000 technique were comparable to the lyophilization technique with the added advantage of faster processing, making the method applicable to routine clinical use in many diagnostic applications.

Borate buffer, introduced in our laboratory for paper electrophoresis of gastric juice (G19) has since been used for this purpose by other authors (B12, C5a, D1, F1, G33, G34, H1, K2, V1a, W11) and also for electrophoresis on cellulose acetate strips (P3, P4). Instead of vertical paper electrophoresis units, some of these investigators used borate buffers with horizontal units (B13, F1, K2, V1a). The resolution obtained was certainly less satisfactory than that yielded by the vertical unit (V1a) and cathodic peaks were not resolved (B13, C5a, K2).

Fasel and Scheidegger (F1) fractionated gastric juice by horizontal paper electrophoresis at 50 volts/cm for 6 hours, using dialyzed and lyophilized gastric juice dissolved at 4% concentration in borate buffer of pH 9.0. The authors noted that peak M3 was more elevated in patients with gastric than with duodenal ulcer. This finding was later corroborated independently on cellulose acetate strips (P3, P4), where an increase in the corresponding peak 2 was noted in gastric ulcer.

Gullberg and Olhagen (G34) attempted to simplify the electrophoretic pattern of normal acid gastric juice, which is obscured by the proteolytic degradation of some of its components. Prior to gastric collection, they repeatedly introduced sodium phosphate buffer of pH 7.2–7.4 and ionic strength of 0.2 into the stomach to neutralize gastric juice *in situ*, under fasting conditions and during carbachol (carbamylcholine chloride) or histamine stimulation. After collection, gastric juice was concentrated

by ultrafiltration and submitted to vertical paper electrophoresis in borate buffer at pH 9.0. Without neutralization, gastric juices showed as many as 8 components, which was similar to our own results. The most anodic component had the mobility of crystalline pepsin. In the *in situ* neutralized normal gastric juice, the second component had albumin mobility. The albumin nature of this peak was verified by double immunodiffusion, according to the Ouchterlony technique. The third, slower anodic component contained most of the carbohydrates and corresponded to the bulk of gastric mucosubstances (M3 in our nomenclature). The fastest component—pepsin—was absent from gastric juice of patients with histamine-fast anacidity, from anacid basal fasting secretion, and from some acid juices neutralized *in situ*. The second fastest anodic component (albumin) was present at high concentration in gastric juice of patients with gastric achylia. It disappeared upon gastric juice digestion with pepsin at pH 1.0–2.0 (Fig. 5), as well as upon acidification of pepsin-containing normal gastric juice neutralized *in situ*. Following the latter procedure, some cathodic components appeared of a less important size than those found in the nonneutralized acid gastric juices of duodenal ulcer patients. Cathodic bands X, Y, and Z, except for component X1, were generally absent from the gastric juices neutralized *in situ*. Similar inferences were obtaned by Cornet *et al.* (C5a) and Hartmann *et al.* (H1) who used tris(hydroxymethyl)aminomethane buffers for gastric juice neutralization.

Verschure (V1a) introduced yet another modification into paper electrophoresis of gastric juice. He used nonconcentrated native gastric juice, to which radioactive I^{131} was added for labeling gastric juice proteins. A few microliters of this mixture were applied to Whatman No. 1 filter paper, which had been previously dipped in buffer solution containing 1% human albumin to avoid absorption of labeled proteins by the filter paper. Electrophoresis was performed in veronal buffer of pH 8.6. After air-drying, autoradiographs of the strips were prepared and traced in the densitometer. Eight to eleven components were found. According to the author, this method has the advantage of avoiding the aggregation of gastric proteins which occurs on concentration of gastric juice. Piper *et al.* (P3), who tried this method with cellulose acetate strips instead of paper, could not, however, confirm its superiority over the conventional method of paper electrophoresis.

The effect of acidification on electrophoretic patterns of gastric juices, collected from patients with pernicious anemia and histamine-fast achlorhydria, was studied by Brummer and Seppälä (B13). They used dialyzed and lyophilized gastric juice, a horizontal electrophoresis ap-

paratus, borate buffer of pH 9 and ionic strength 0.24, and a 16-hour electrophoretic run at 100 volts and 0.5 mA/cm. Paper strips were stained with SF light green, and components separated by electrophoresis were labeled according to the nomenclature of our laboratory. It should be emphasized that the resolution obtained by the horizontal cell did not permit exact superimposition of these tracings upon the electropherograms obtained with a vertical unit. With horizontal electrophoresis, bands X, Y, and Z are not resolved and materials M4 and X1, moving by endosmotic flow in a cathodic direction on vertical electrophoresis, have a more central localization.

When two aliquots of achlorhydric gastric samples were acidified to pH 1.2, incubated for 15 minutes, and neutralized to pH 7.0, weakening or disappearance of band M4 was found (B12) and frequent decrease in intensity of bands M2 and M3. In many instances the albumin band decreased as well, and a cathodic peptide band (X+Y+Z) appeared concomitantly. Since these changes were found in gastric juice from patients with pernicious anemia, not containing pepsin, these changes were considered the result of acidification. However, the presence of other proteases in these juices may challenge this conclusion (see G5). Similar results were previously reported by Kakei (K2), who found that the compressed pattern of anacid gastric juice may change into one resembling that of normal juice as a result of acidification.

Kakei (K2) dissolved lyophilized gastric juices at 5% concentration in borate buffer of pH 9, and submitted them to horizontal electrophoresis at 0.45 mA/cm and 5 volts/cm for 8 hours, following which the strips were oven-dried and stained with bromphenol blue, PAS, and ninhydrin. He also used horizontal paper electrophoresis and, like others, found only 5 main components in normal acid gastric juice, which he named P1 though P5, counting from the anodic end. Component P1, of fastest anodic mobility, was increased in duodenal ulcer and acid gastric juice, but was absent from anacid juices. Peaks P2, P3, and P4 were prominent in anacid gastric juice, P3 being especially high, whereas peaks P1 and P5 were very low or absent; this was similar to the findings of others using horizontal electrophoresis. By combining paper electrophoresis with polarographic assays, Kakei (K2, K5) concluded that components P5 were peptides. Some were dialyzable on prolonged dialysis and were also found in the supernatant of the trichloroacetic acid filtrate of acid gastric juice precipitated with acetone, i.e., in the "dissolved mucin" fraction. This peptide increased in concentration in gastric cancer with preserved HCl, but was absent from anacid juices. When gastric juice was dialyzed all peaks became sharper, but the height of cathodic

peaks 4 and 5 decreased, due to dialyzability of some peptides. This conclusion was reinforced by the fact that the ninhydrin-stained peaks disappeared almost completely after dialysis. PAS-stained patterns of anacid gastric juices, with and without cancer of the stomach, were similar, while in acid gastric juices carbohydrate content was lower. Kakei also reported that carbohydrate material from patients with gastric cancer with preserved HCl had slower anodic mobility than that from noncancer patients. The ninhydrin-stained peaks of cancer gastric juice electropherograms were more numerous and prominent than in noncancer juices. Kakei suggested the presence of abnormal peptide material in these cancerous gastric juices.

A similar technique was used by Wada *et al.* (W5–W7), who studied gastric juices from normal individuals, patients with cancer of the stomach and those with histamine-fast anacidity. Juices were dialyzed and concentrated against Carbowax 1500 to a protein concentration of about 3% and subjected to paper electrophoresis at 10 volts/cm, either in veronal buffer of pH 8.6 or acetate buffer of pH 4.5 of 0.1 *M* ionic strength. Normal electrophoretic patterns in veronal buffer again showed 5 main bands, tentatively named B1 through B5, when counted from the anodic end of the partition. A few smaller peaks were also noted in the middle of the partition. When electrophoresis was performed in acetate buffer of pH 4.5, two to three peaks were found on each side from the application point. Patients with gastric cancer showed larger than normal B2, B3, and B4 peaks, when run in veronal buffer, whereas peak B1 was lower than normal or absent, especially in acetate buffer of pH 4.5. Patients with histamine-refractory anacidity, without gastric malignancy, had electrophoretic patterns intermediate between those of healthy acidic controls and gastric cancer patients. To the reviewer, it appears that peak B1 of Wada, in veronal buffer, corresponds to the association of our peaks P and M1, which appear separated in acid gastric juice in borate buffer. This association has been shown (in Section 1.1) to occur on free boundary electrophoresis as well. Band B4 of Wada corresponds to the association of our bands M4-X1; B5 represents the sum total of cathodic peaks (X2, Y1, Y2, Z) in borate buffer. Wada's band B2 probably represents M2 in acid, and albumin plus M2 in anacid gastric juice, whereas B3 equals M3 bands. Similar correlations apparently apply to peaks P1–P5 of Kakei (K2, K5).

Others (S3) reported that the electrophoretic pattern of cancer gastric juice is characterized by an abundance of electrophoretic components having slow electrophoretic mobility, and disappearance of materials of fast mobility. In Sasai's studies, gastric juices from patients with gastritis

and gastric achylia showed similar electrophoretic patterns to gastric cancer juices (S3).

Yamakawa (Y1) studied 150 juices by free boundary and paper electrophoresis in veronal buffer of pH 8.6, phosphate buffer of pH 7.6, borate buffer of pH 5.8, and acetate buffer of pH 4.5. The normal electrophoretic pattern consisted of four to seven peaks, of which four were considered to be main components. The best resolution was obtained with the first three buffers. In gastric ulcer, the slowest component, B4, was increased whereas, in atrophic gastritis and gastric cancer, components B3 and B4 were high and additional subcomponents were noted.

Balazs and Fröhlich (B2), like Verschure (V1a), attempted paper electrophoretic analysis of gastric juice without prior concentration and dialysis of the samples used. They did not, however, use radioactive markers. Gastric juice (0.05 ml) was applied and electrophoresis was conducted at 1.5 mA per strip in borate buffer of PH 8.6 for 2–3 hours. Wet strips were dipped in acetonic ninhydrin solution (0.1 g/100 ml acetone), dried, and read in Analytrol. Normal acidic gastric juices, as well as those from ulcer patients, showed 3 peaks staining pale red, while the anacidic gastric juice showed 5 components staining very intensively, of which 3 were anodic, 1 cathodic, and 1 remained at the application point. The quality of the anacid juice resolution was satisfactory, while that of acidic juice was very inadequate as compared with tracings obtained by others using dialyzed and concentrated gastric juice (reviewer's comment).

Shinoda (S8), Yoshikawa (Y2), Rambaran *et al.* (see B9), as well as Betti and Di Leo (B6a) and Maservici (M3), also used paper electrophoresis in studying gastric juices. Their results were grossly similar to those reported in this chapter.

1.2.2. *Clinical and Physiological Applications*

The most important applications of paper electrophoresis of gastric juice are as follows.

(a) *Determination of serum proteins in gastric juice and in gastric mucosa.* This is of special significance for the detection of massive protein leakage into the stomach, diagnosis of protein-losing gastropathy, and screening for gastric cancer in cases of anacidity (G3, G9).

Most significant information on the passage of serum proteins into the gastric juice has been obtained by means of electrophoretic techniques, especially paper electrophoresis and immunoelectrophoresis (see also Section 1.8).

Henning *et al.* (H5a), on the basis of similarities of electrophoretic

mobility of gastric juice components and of serum components, arbitrarily assumed the fastest anodic band in the *in vitro* neutralized gastric juice to be serum albumin, the second fastest one—α-globulin, the third—β-globulin, and the fourth, slowest band—γ-globulin. Gastric juices of patients with atrophic gastritis, gastric atrophy, and pernicious anemia had increased total protein content and high first and fourth peaks in electropherograms. This the authors interpreted as an increase in serum albumin and γ-globulin. In some cases an intermediate band appeared having the mobility of α-globulins. The electropherogram of gastric carcinoma was very similar to that of the serum, which Henning *et al.* (H5a) considered to be due partly to increased permeability of the mucous membrane for the high molecular materials, and partly to the protein secretion into the gastric lumen by the tumor itself.

Similarly, Norpoth *et al.* (N2) reported a high albumin peak in gastric electropherograms of patients with anacidity and atrophic anacid gastritis. It disappeared in acid gastric juices. The addition of serum to normal acid gastric juice did not produce the characteristic albumin peak in the electropherogram of the mixture, which was explained as the result of the albumin degradation by acid-pepsin digestion. In acid juices from patients with gastritis, the slow-moving components close to the application point were greatly increased. The authors referred to them as abnormal proteins resulting from the inflammation of the mucous membrane. They failed, however, to consider them as protein digestion products (see below).

In 1958, we reported paper electropherograms of gastric juice from 6 patients with massive leakage of an albumin-like material into the gastric lumen (G22) (Fig. 7). Gastric juices were anacid in all instances. They included 2 cases of gastric cancer, 1 of Menetrier's disease, and 3 of atrophic gastritis (of which 2 had pernicious anemia). Similar reports were published in 1959–1960 from other sources, where the passage of serum albumin into the stomach was demonstrated by a similar method (C5, G34, J2).

Gullberg and Olhagen (G34) prevented the proteolytic degradation of serum albumin in the stomach, prior to gastric juice collection, by intragastric instillation of phosphate buffer at pH 7.2–7.4. They then used our technique for electrophoresis of gastric juice and agreed that, under normal conditions, the first peak corresponds to pepsin and the second to serum albumin, if albumin degradation in the stomach is prevented by *in vivo* neutralization. Albumin was identified by double immunodiffusion on agar gel, using the Ouchterlony technique.

In subsequent work from our laboratory (G3, G8, G9) a serum albumin

band was detected on paper electrophoresis in 40 anacid gastric juices. At a high concentration, albumin appeared as a prominent and heavy amido-black staining band in the anodic area of the partition in the area adjacent to mucosubstance M2, which demonstrated the electrophoretic

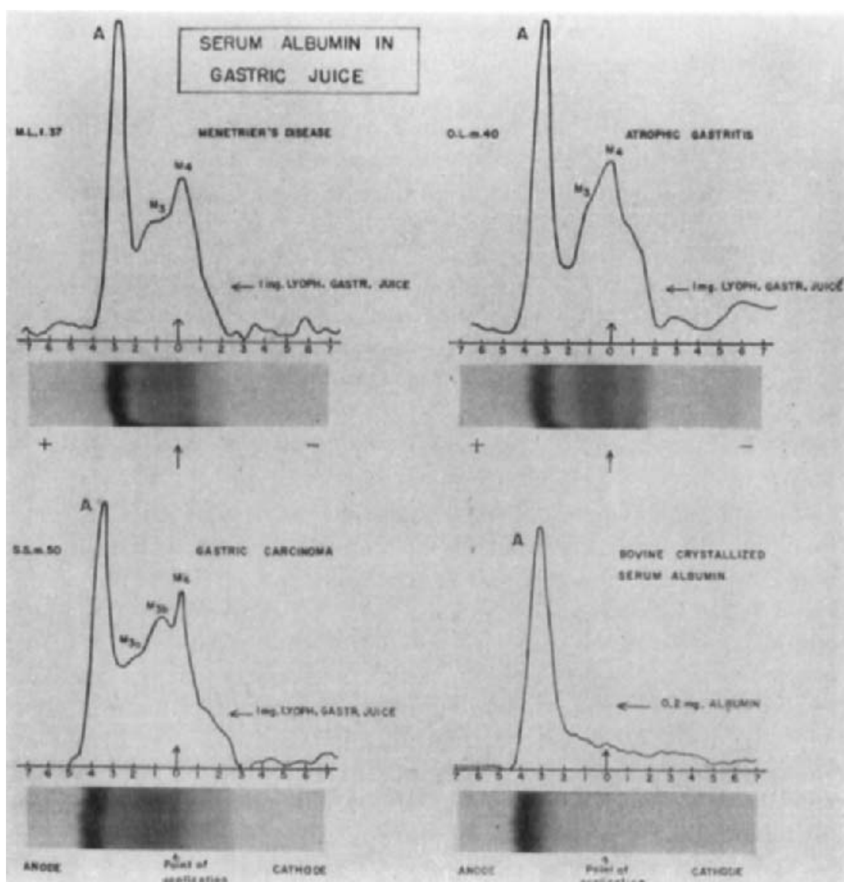

Fig. 7. Serum albumin in gastric juice. From Glass *et al.* (G22).

mobility of serum albumin. This material was precipitable with trichloroacetic acid, and the precipitate was soluble in 95% ethanol and 80% acetone, characteristic for the albumin precipitate. Addition of serum albumin to the gastric juices augmented the albumin band.

Serum albumin in gastric juice was quantitated by means of a standard curve prepared from commercial serum albumin at various concentrations and subjected to paper electrophoresis under identical conditions

(G3, G9). In half of these 40 anacid gastric juices showing a serum albumin band, concentration of albumin was relatively low, below 20 mg/100 ml, and amounted to 2–14% weight per weight of the nondialyzable solids. Eight of these juices were collected from patients whose gastric secretion was anacid under fasting conditions, but which contained free HCl and pepsin after stimulation. Here, the albumin concentration did not exceed 8% (w/w) of the nondialyzable solids, and was below 10 mg/100 ml gastric juice.

Massive leakage of serum albumin into the gastric juice was found in 19 subjects, including 10 cases of gastric cancer with histamine-fast anacidity, 1 of pernicious anemia, 1 of sprue, 5 of Menetrier's disease (giant hypertrophic gastritis) associated with anacidity and hypoalbuminemia, and 2 of steroid-treated rheumatoid arthritis (Fig. 8). Content of serum albumin was 15–63% (w/w) of nondialyzable gastric juice solids, and 20–482 mg/100 ml native gastric juice. In several gastric juices containing serum albumin, another material was found, situated at the cathodic site of the tracing, close to the application point, which had characteristics of γ-globulin. Immunoelectrophoretic identification of these two proteins by Ohara in our laboratory confirmed the albumin and γ-globulin-like nature of these two components (G3).

Others, on paper electrophoresis of anacid or *in vivo* neutralized gastric juices under normal and pathological conditions, found protein bands representing serum albumin and serum globulin (B1, B3, B13, C5, D3, G24, G25, H4, H5a). Whereas in the work of Cohen *et al.* (C5) serum albumin concentrations were 1.7–45.0 mg/100 ml, γ-globulin concentrations were 3–9 mg. When 24-hour output of gastric juice was calculated arbitrarily on the basis of 15-minute basal secretion, the authors concluded that the normal stomach may allow passage of 21–690 mg albumin and 33–284 mg globulin into the lumen in 24 hours. Brummer and Seppälä (B13) and Cohen *et al.* (C5) found albumin concentrations in anacid gastric juice of 0.3–26.1% of nondialyzable gastric solids, when determined spectrophotometrically at 635 mμ.

In collaboration with Skoryna (G18) we found that rats, with explanted gastric mucosa and resulting atrophy of the explanted segments, leak large amounts of serum albumin and γ-globulin into the secretion of the explant. This is a good experimental preparation for studying gastric protein leakage in rats. Demling *et al.* (D2), Götz *et al.* (G24, G25) and Scheiffarth *et al.* (S4) applied electrophoretic analysis to the study of the tissues, including those of the stomach (see also Section 1.8).

Götz *et al.* (G24, G25) studied paper electrophoretic patterns of gastric juice, gastric mucosa, and gastric wall, using a horizontal cell and

veronal buffer of pH 8.6. Only 2 of 10 juices demonstrated presence of materials stainable by protein stains, revealing to the reviewer the inadequacy of the method used. Gastric mucous membranes removed at operation were scraped off the gastric wall, homogenized, suspended in saline, centrifuged, and concentrated by dialysis against kollidon. On paper electrophoresis a very high albumin peak and a second high peak with β-globulin mobility were found. In addition, one or two "pre-albumin" peaks, two or three intermediate peaks of α-globulin mobility, and one γ-globulin peak were present. Electrophoretic patterns of the homogenized wall of the stomach were similar to those of the gastric mucosa, with the exception of the carbohydrate materials, which were localized principally in the albumin area and not close to the application point. The mucous membrane of the stomach of patients with gastric cancer differed in electrophoretic pattern from that of the normal control, since there was marked decrease of the albumin band associated with an increase of materials localized in the area of the α- and β-globulins (G24). Hollander *et al.* (H12, H14) found serum albumin in canine gastric para-mucus. Polarographic gastric proteins studies are discussed in Section 7.

In unpublished work from our laboratory by Ohara, Castro and Ishimori, gastric mucosal extracts were homogenized, dialyzed, lyophilized, and submitted to vertical paper electrophoresis. Their electrophoretic pattern, stained with amido black and PAS stains, revealed the presence of heavy protein bands with the mobility of serum albumin and of globulin, other protein materials of intermediate mobility corresponding to pepsinogen and other globulins and polysaccharides. The available data on the immunological identification of serum proteins in gastric juice will be discussed in Section 1.8 (Immunoelectrophoresis) and Section 2 (Immunodiffusion).

(b) *Detection of excessive degradation of proteins in the gastric juice.* This is clinically of significance in conditions associated with gastric hypersecretion (duodenal ulcer) (G3, G9) or administration of steroids (H8, H9).

As a result of the paper electrophoretic analysis of gastric juice using differential staining, in 1956 we assumed that cathodic peaks X, Y, and Z in gastric electropherograms most probably represent "nondialyzable peptide materials containing organic bases" (G19). This conclusion was based on the finding that these materials stain very slightly or not at all with protein-staining SF light green stain, do not stain with PAS, but can be visualized with amido black. At that time they were thought to represent products of peptic digestion of the protein moieties of gastric

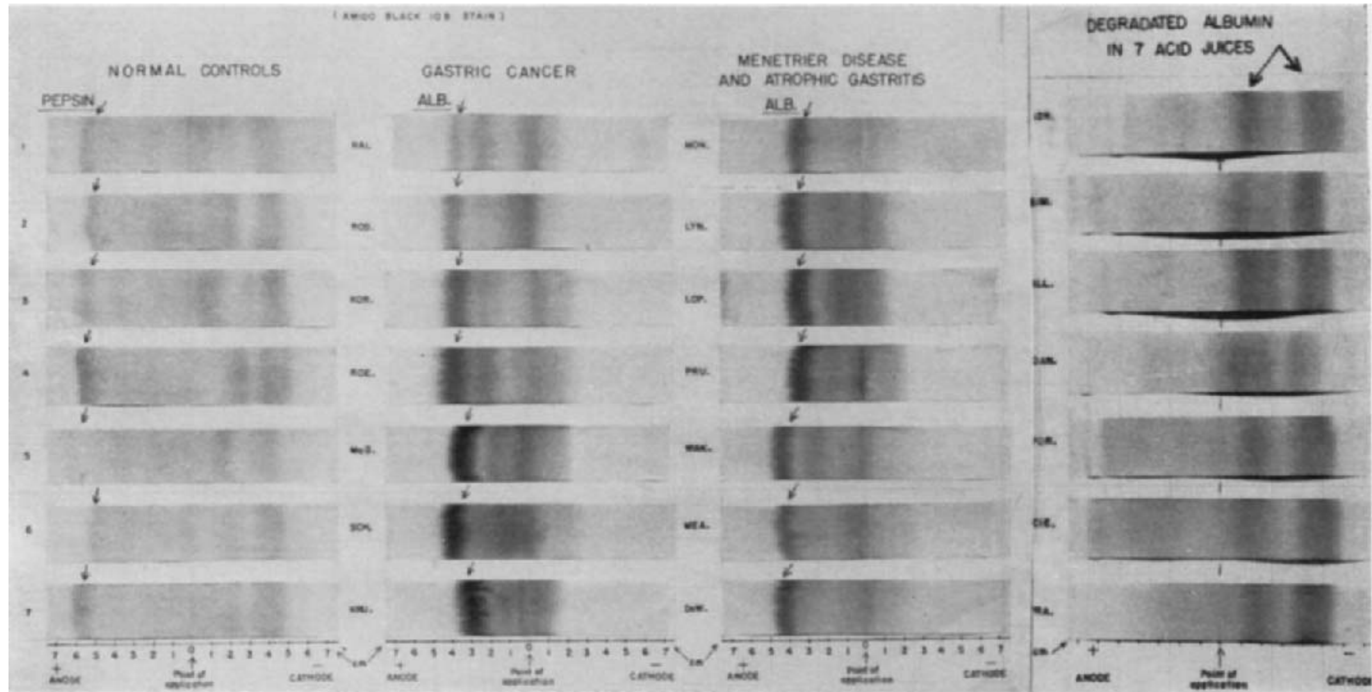

FIG. 8. Serum albumin in electropherograms of pathological gastric juice. From Glass and Ishimori (G9).

mucoproteins, and their absence from the stomach of pernicious anemia patients to be due to the absence of peptic digestion. Elution of these cathodic materials from electrophoretic strips, followed by chemical analysis, confirmed absence of carbohydrates in materials X2, Y1, Y2, and Z, as well as their nonprotein nature. This was proven by the absence of tyrosine and tryptophan, resulting in a negative Folin-Ciocalteu phenol reaction (G16).

In acid and pepsin-containing gastric juices collected without intragastric neutralization, especially from patients with duodenal ulcer, gastric hypersecretion (Fig. 8) and, after histamine stimulation (G3, G19) or cortisone treatment (H9), these peptides were present at a high concentration.

As Ohara and Ishimori have shown in our laboratory, the serum albumin band, after 15-minute incubation with pepsin at pH 1.5 becomes markedly reduced in size (G3, G9). Simultaneously several additional bands form, some of which are localized in the central anodic area and others at the cathodic side of the electrophoretic partition. With longer incubation, the serum albumin peak almost completely disappears and more degradation products form, having the electrophoretic mobility of components X2, Y1, Y2, and Z (Fig. 5). When serum albumin is incubated with human gastric juice containing acid and pepsin, degradation products again form with the mobility of cathodic components X2, Y1, Y2, Z1, and Z2. This was corroborated by similar findings of Gullberg and Olhagen (G34) and others (B13), following acidification of normal anacid gastric juice. When these cathodic materials were studied polarographically (see Section 7), they showed the presence of a single polarographic peptide wave (K1, K2, S1–S3). Some of these peptides, X2 and Z, had relatively low molecular weight, as shown by their dialyzability (G3) and retardation on Sephadex G-25 column (G12a).

When albumin was incubated in our laboratory with gastric juices of pH 3, which did not show peptic activity at pH 1.5, degradation products of this type did not appear. Instead the albumin band blurred, widened, and tended to spread more toward the anode, following which other albumin degradation products appeared, which were more negatively charged (G3).

(c) *Diagnosis of advanced atrophic lesions in the gastric mucosa* by means of the "compressed pattern," following histamine stimulation (G18a, G22). This compressed (G18a, G22) or, as recently renamed by French authors (C5a), "amputated" pattern simply indicates anacidity (G18a, K2, V1) in nonstimulated gastric secretion. However, its detection in the histamine-stimulated stomach has a significance similar to

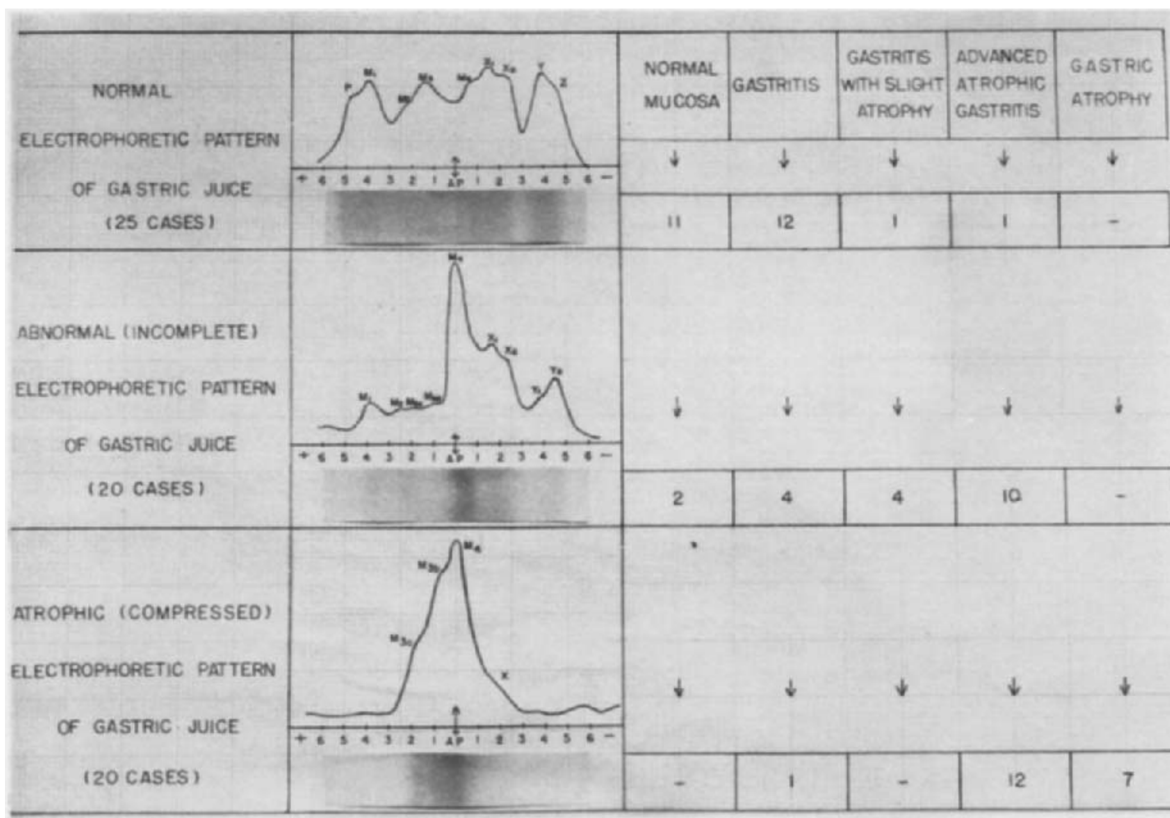

Fig. 9. Paper electrophoresis of gastric juice and histology of gastric mucosa in 65 individuals. From Glass *et al.* (G18a).

that of gastric biopsy in the diagnosis of atrophic lesions of the gastric mucosa (G18a, G22) (Fig. 9).

Van Geertruyden *et al.* (V1) studied Heidenhain pouches in 7 dogs. They obtained over 400 electropherograms of gastric juices with veronal buffer and horizontal unit, followed by staining with amido black. Results obtained were correlated with hexosamine. The authors concluded that the electrophoretic pattern depended principally on the gastric juice pH or the chemical composition of gastric juice and, therefore, that it is doubtful whether there is any electrophoretic pattern which can be considered diagnostic for gastric atrophy. This hasty conclusion represents an extrapolation of difficulties observed under physiological conditions in the analysis of gastric juice into the area of gastric pathology, for which the authors did not publish any personal data (reviewer's comment).

(d) *Study of polysaccharide content and distribution in the gastric juice.* We used paper electrophoretic fractionation of gastric juice in our laboratory for determining the composition of mucosubstances present in normal acid gastric juice (G15–G17).

About 350 electrophoretic strips were cut into 0.5-cm segments and after elution were analyzed for proteins, hexoses, hexosamine, fucose, sialic and uronic acids, and sulfates. Results obtained were correlated with the mean electrophoretic pattern of the same gastric juice pool stained by amido black and PAS stains, traced in Analytrol, and analyzed by means of Gaussian curves (Fig. 10).

Three main protein peaks were found localized in the areas corresponding to (1) pepsin, (2) mucosubstances M2 and M3, in which albumin was also located, and (3) M4-X1, where γ-globulin was present. Only one major carbohydrate area was found which coincided with that traced from the PAS-stained zone of the electropherogram, corresponding to the area of mucosubstance M3. The latter, on Gaussian curve analysis, showed the presence of many components, however, which extended from the application point to a point 3.5 cm from the anode.

The carbohydrate spectrum along the electrophoretic partition changed markedly, and the anodic area contained the highest concentration of sialic acid and hexoses and lowest of hexosamine and fucose. The central area which coincided with the carbohydrate peak contained the highest concentration of hexosamine and fucose and lowest of sialic acid (Fig. 11). The cathodic area had the highest concentration of hexoses and lowest of sialic acid, hexosamine, and fucose. Sulfates formed three peaks (Fig. 12), of which the highest was located in the most anodic

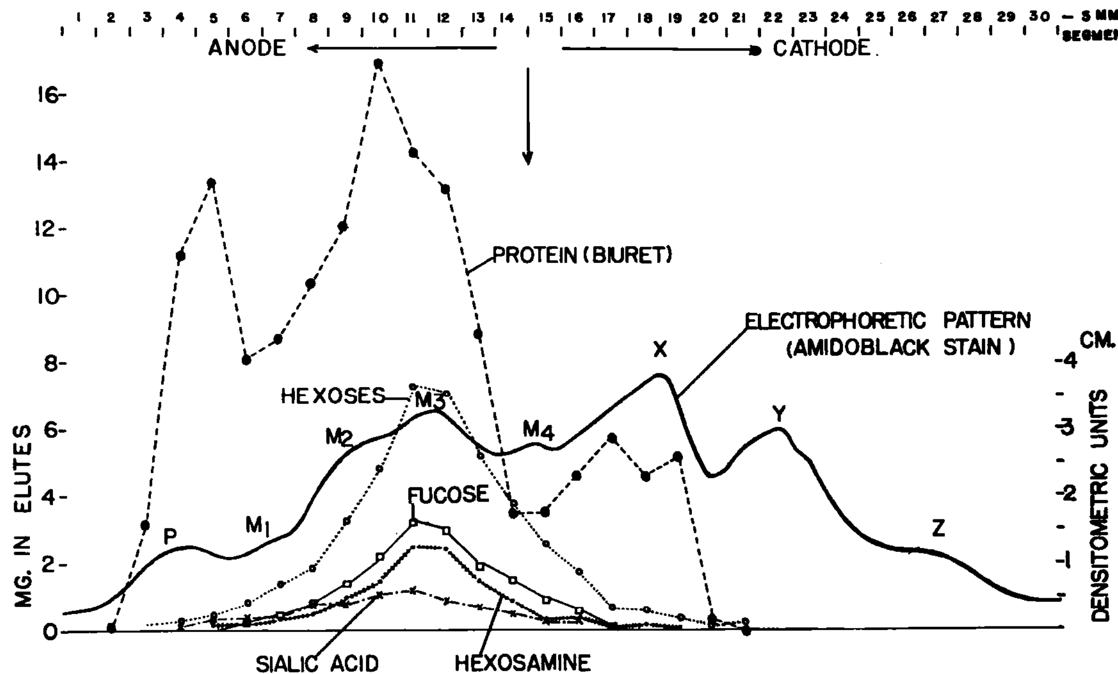

FIG. 10. Electrophoretic pattern (average of 27 runs) and composition of elutes from 30 identical segments 5 mm wide of 354 paper electrophoretic runs of a pool of human gastric juice (457 mg dry weight). From Glass *et al.* (G16).

area where no proteins or PAS-stainable carbohydrates were detected, and two others corresponding to two areas within the carbohydrate peaks, namely, M2 and M3. The hexoses formed in most segments 48–62.5% and only in the most cathodic area 76.6% of the total carbohydrates, the mean value being 56.7%. Fucose accounted for 16.3–22.4%

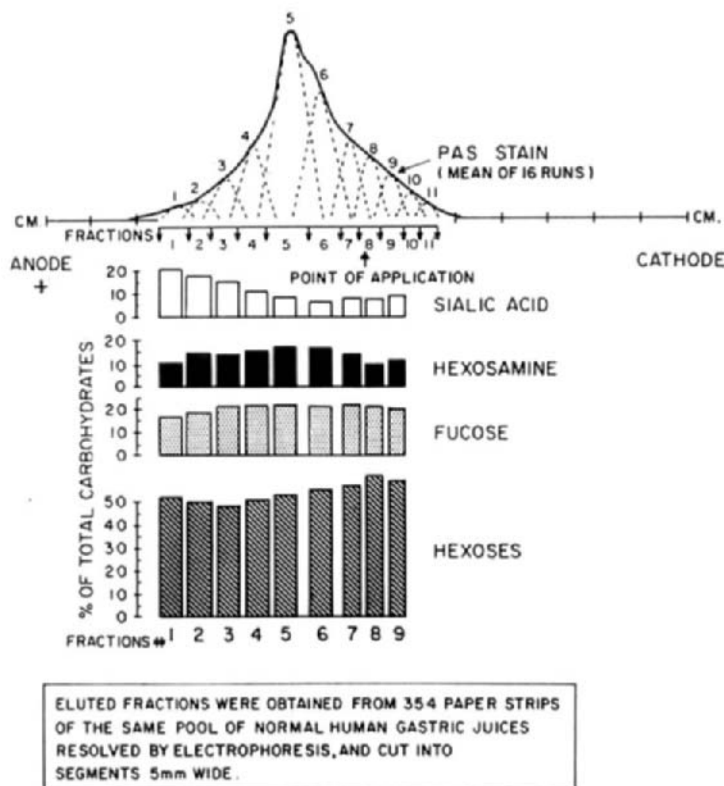

FIG. 11. Carbohydrate spectrum of a pool of normal human gastric juices fractioned by paper electrophoresis. From Glass (G3a).

of carbohydrates, the mean being 19.1%, and only in the most cathodic area was as low as 7.8%. Hexosamine formed 10.1–17.5% of total carbohydrates, having a mean value of 13.7%. Finally, sialic acid showed a continuously declining concentration with the gradient going from the anode to the cathode. It formed 15.7–21% of total carbohydrates in the three most anodic fractions, while in the two most cathodic fractions, it made up only 2.6–6.7% of the total carbohydrates, the mean value being 10.5%. Molar ratios of various carbohydrates eluted from con-

secutive fractions of the electropherograms of the pooled normal gastric juices are shown in Table 1.

The complexity of sugar distribution along the electrophoretic partition of normal acid gastric juice suggests the presence of several carbohydrate materials of different composition, complexing with protein or peptide chains. Thus, no single chemical carbohydrate analysis of the total nonfractionated acid gastric juice is capable of the quantitation of gastric mucosubstances. This is evidenced by composition of various

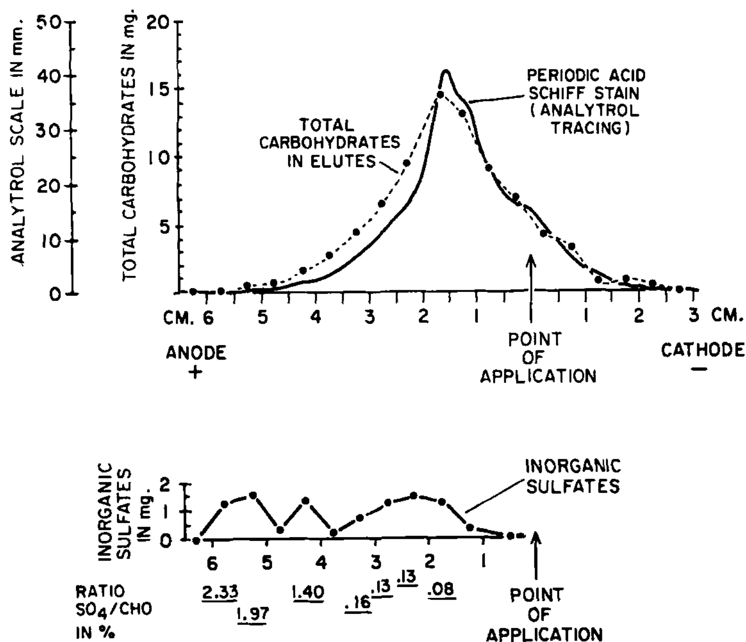

FIG. 12. Inorganic sulfates and total carbohydrates of a pool of normal human gastric juices fractionated by paper electrophoresis. From Glass (G3a).

mucosubstances as fractionated by paper electrophoresis and as listed in Table 2 and Fig. 11.

Berg *et al.* (B5a) correlated the results of gastric biopsies with chemical and electrophoretic analysis of gastric juice. They determined total proteins by the biuret method and total hexoses colorimetrically by the orcinol technique, and performed horizontal paper electrophoresis followed by amido black and PAS staining. Their work corroborated previous findings (G19) that carbohydrate materials staining with PAS were localized in the center of the electrophoretic partition of gastric juice with some extension to adjacent areas. They also observed that

gastric juice of patients with superficial gastritis had higher total carbohydrates by PAS staining and by orcinol reaction. Reading hexoses at various wavelengths suggested some changes in the carbohydrate composition, especially an increase in galactose content. In atrophic gastritis, the total hexoses were less markedly increased but the serum albumin band was very high. All this corroborated Katsch's old concept regarding "gastritis mucosa" and "gastritis serosa" (see G5).

TABLE 1

MOLAR RATIOS OF VARIOUS CARBOHYDRATES IN ELUTES FROM CONSECUTIVE CARBOHYDRATE FRACTIONS OF A POOL OF NORMAL HUMAN GASTRIC JUICES RESOLVED BY PAPER ELECTROPHORESIS

Fractions		Hexoses/ hexosamine	Sialic acid/ fucose	Sialic acid/ hexosamine
Anode	1	4.48	0.76	1.41
	2	3.73	0.58	0.86
	3	3.37	0.45	0.77
	4	3.33	0.31	0.49
	5	3.00	0.22	0.32
	6	3.24	0.21	0.28
	7	4.23	0.22	0.38
Cathode	8	5.83	0.23	0.54
	9	4.77	0.28	0.50
	10	4.33	0.25	0.31
	11	5.90	0.21	0.14
	Mean:	4.20	0.34	0.55

TABLE 2

CARBOHYDRATE SPECTRUM OF SOME FRACTIONS OF HUMAN GASTRIC JUICE RESOLVED BY PAPER ELECTROPHORESIS ON COLUMN CHROMATOGRAPHY

Materials	Fractions	Proteins	% Carbohydrate spectrum			
		Carbohydrates	Hexoses	Hexosamine	Fucose	Sialic acid
Electrophoretic partition of pooled human gastric juice[a]	P (pepsin)	13.1				
	M1 (mucosubst.)	3.9	53.5	11.6	16.3	18.6
	M2 (mucosubst.)	2.0	50.0	15.0	22.0	13.0
	M3 (mucosubst.)	1.0	53.3	17.4	21.7	7.6
	M4 (mucosubst.)	1.0	60.3	10.3	20.7	8.7
	X1 (γ-globulin)	4.5				
Chromatography of pooled human gastric juice on Amberlite IRC-50[b]	1st peak eluted with citrate buffer pH 3.2, 0.2 m	0.3	37.9–50.4	27.5–47.6	7.4–18.7	0–1.5

[a] Glass *et al.* (1956); (G16).
[b] Glass (1963); (G3b).

In unpublished work with Ishimori (G10) we analyzed the carbohydrate-staining materials in the gastric juice partition, according to two criteria: (1) the total amount of carbohydrates found on the electrophoretic partition of gastric juice, calculated in terms of the arbitrary integration units as recorded in the Analytrol instrument, and (2) the distance of the carbohydrate peak, calculated from the application point.

According to the first criterion, total carbohydrate material on the electrophoretic strip did not exceed 200 integration units per 2 mg material, with a lower value of around 60 in more than 200 gastric specimens from over 100 patients. In the great majority of duodenal ulcer patients, total carbohydrate content of the electrophoretic strip was within the normal limits (60–200 integration units per 2 mg material applied). An increased amount of carbohydrate was found on the partition in only 5 of over 30 of these cases, reaching as high as 400 units. The majority of duodenal ulcer juices had a total content of nondialyzable residue of gastric juice within normal limits, when calculated dry weight per weight; however, the yield of nondialyzable material per 1 ml gastric juice in duodenal ulcer was below that of the normal gastric juice. Therefore, 1 ml duodenal ulcer gastric juice will have less carbohydrate material in the nondialyzable fraction than 1 ml normal juice. On the other hand, total gastric juice output in duodenal ulcer patients is higher than in normals, resulting in unchanged total output of the carbohydrate material. Thus, carbohydrate-containing mucosubstances are simply diluted by the large volume of parietal secretion, which results in their decreased concentration. In 6 of 10 cases of gastric ulcer, total carbohydrates on the electropherograms of gastric juice were above the normal limit of 200 integration units, reaching as high as 300. In 3 of 17 gastric cancer cases, carbohydrate content was also higher than normal, reaching as high as 400 units. In 5 of 7 patients with histamine-fast anacidity, with or without pernicious anemia, the carbohydrate content was above the normal limits of 200, reaching as high as 360 arbitrary units. Since the yield from 1 ml anacid gastric juice of nondialyzable components is higher than in normals, carbohydrate content calculated per 1 ml gastric juice is markedly increased in patients with histamine-fast anacidity and atrophic gastritis. This corroborates the observations of Berg *et al.* (B5a) and Wada *et al.* (W6), who found high concentration of carbohydrates in these patients.

In roughly one half of our electrophoretic strips, the carbohydrate peak was localized 1–1.5 cm from the application point, corresponding to the localization of the mucosubstance component M3b. This pattern was shared by gastric juices from normals and some patients with

duodenal and gastric ulcer, gastritis, gastric cancer, and other miscellaneous conditions. In several patients with gastric or duodenal ulcer and gastric hypersecretion, however, the carbohydrate component shifted closer to the application point, so that its peak was only 0.5 cm toward

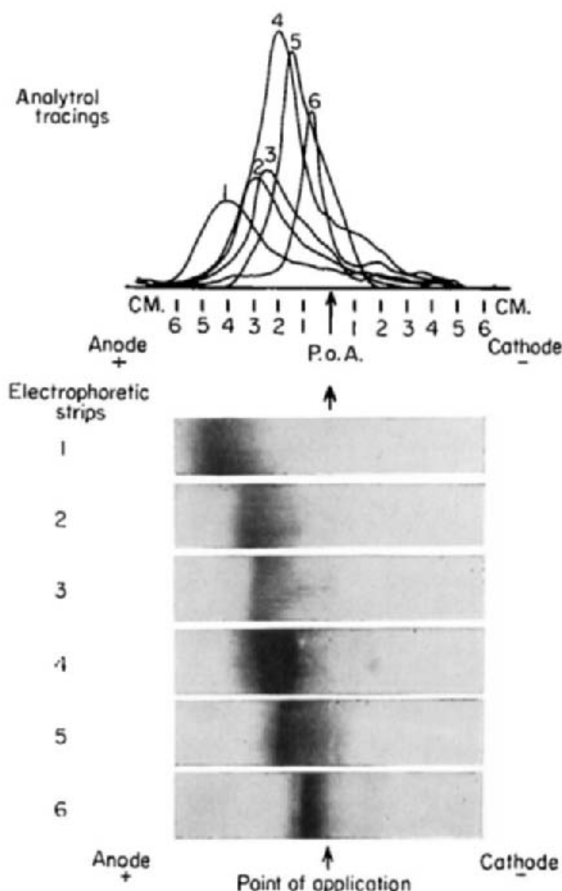

FIG. 13. Paper electrophoretic patterns of carbohydrates in various human gastric juices. From Glass (G3a).

the anode from the application point (Fig. 13). This shift in localization of the carbohydrate peak toward the application point was characteristic of hypersecretory stomach and probably due to the splitting off of sialic acid from sialomucins in the acid medium; this might have resulted in decreased negative charge of mucosubstances.

On the other hand, in 15 gastric juices, of which 10 were from patients

with histamine-fast anacidity, atrophic lesions of the gastric mucosa, or gastric cancer, we found the carbohydrate peak localized more toward the anode, most frequently 3.5 cm or more from the application point, in the area of component M2. The other 5 subjects had gastric ulcer or gastritis, with low output of HCl. Thus, high carbohydrate content with its more anodic localization on the electrophoretic partition was characteristic for atrophic lesions of the gastric mucosa, associated with gastritis or gastric ulcer or cancer. Conversely, low carbohydrate concentration with more central localization of the carbohydrate component on electrophoretic partition was observed mostly in hypersecretory stomach, associated or not with duodenal ulcer.

(e) *Localization of isotope-labeled materials along the electrophoretic partition*, such as labeled serum albumin (see H13, H18) or other proteins (VIa). This has been discussed in this section.

(f) *Identification of various fractionation products* obtained with physicochemical techniques, such as continuous curtain electrophoresis (G20, W4), chromatographic ion-exchange columns (W11), gel filtration on Sephadex columns (G3a, G12a, G14, K3), ultracentrifugation (G11), and enzymatic digestion of proteins and mucosubstances (G3). This is discussed in various sections of this review.

(g) *Study of pepsin in the gastric juice* and its quantitation, localization, and fractionation by paper electrophoresis will be discussed in Section 1.9.1.

(h) *Study of dissolved mucin fractions in the gastric juice* by paper electrophoresis will be discussed in Section 1.9.2.

(i) *Assay and fractionation of vitamin B_{12} binders and intrinsic factor* by paper electrophoresis will be outlined in Section 1.9.3.

1.3. Continuous Electrophoresis on Paper Curtain

This was applied to the human gastric juice in 1957 in our laboratory for the fractionation of intrinsic factor (G20). The results obtained are discussed in Section 1.9.3.

Takamura *et al.* and Wada *et al.* (W4) fractionated gastric juice of normal individuals and patients with histamine-fast anacidity and gastric carcinoma by continuous electrophoresis on paper curtain, and assayed the chemical composition and biological activities of the fractions obtained. Electrophoresis was performed in veronal buffer of pH 8.6 and ionic strength of 0.03, at 225 volts and 7.5–8.25 mA for 24 hours. As in paper strip electrophoresis, 4 major components were obtained, named by the authors (from anode to cathode) B1, B2, B3, and B4; B3 contained three minor components: A, B, and C. In addition, acidic gastric

juices produced B5, a small component which, on paper strip electrophoresis in veronal buffer, showed slight cathodic mobility. In juices from patients with histamine-fast anacidity, B2 and B3 were increased as compared to controls. In juices from gastric cancer patients, there was a marked decrease in component B1. Fractions from the curtain, upon collection, were analyzed for polarographic waves, and chemically for sialic acid, hexosamine, hexoses, and hexuronic acid. In acidic juices, the highest polarographic filtrate wave, as well as highest tyrosine and sialic acid concentrations, were observed in peak B1 of highest anodic mobility. Hexosamine content was highest in B4, but was also present in areas localized between fractions B1 and B2. Hexuronic acid was found, surprisingly, in component B4, of slow mobility. In gastric juices from patients with histamine-fast anacidity, and especially from patients with gastric cancer, fractions B2 and B3 showed high content of sialic acid, which, in acidic gastric juice, was also true for fraction B1.

Wada *et al.* (W8) have studied other biologically active materials by this method in gastric juices from normals and patients with gastric cancer, such as: pepsin, blood group substances, toxohormone, and KIK activity (see G5). This group (W8) reported following distribution of KIK factor and toxohormone on continuous electrophoresis: the former was found mostly in the first fraction having fast anodic mobility, B1, while toxohormone activity was present mostly in fraction B4 (the most cathodic fraction of the partition, if fraction 5 was not present). Blood group substances were distributed throughout the entire partition (U3, W8). In normal acid gastric juice, the highest blood group titer was localized at both the anodic and cathodic sides of the partition, being lowest in the central portion of the curtain. On the other hand, in patients with gastric carcinoma and histamine-fast anacidity, there was relative increase of blood group substance titer in the midportion of the partition, and decrease in the anodic portion. Distribution of blood group A and B substances was similar, both in control patients having acid gastric juice and in those with gastric carcinoma and histamine-fast refractory anacidity.

In studies performed in association with J. A. Buckwalter's group from Iowa City, we studied the distribution of ABO(H) blood group substances in the electrophoretic partition of gastric juice (G3a, G6). Several acid and anacid pools of gastric juice were subjected to continuous electrophoresis on paper curtain in borate buffer of pH 9.0, μ 0.06. The eluted fractions were assayed for blood group substances, as well as for protein and carbohydrate content. Results indicated that the gastric juice blood group substances were distributed diffusely on the paper

curtain throughout the entire anodic side of the electrophoretic partition. No blood group substances were manifest in the cathodic area of the partition. Our conclusions differ from the findings of Japanese authors (U3, W8), and may be due to the utilization of different electrophoretic techniques. While group substances H and A usually formed 2 anodic

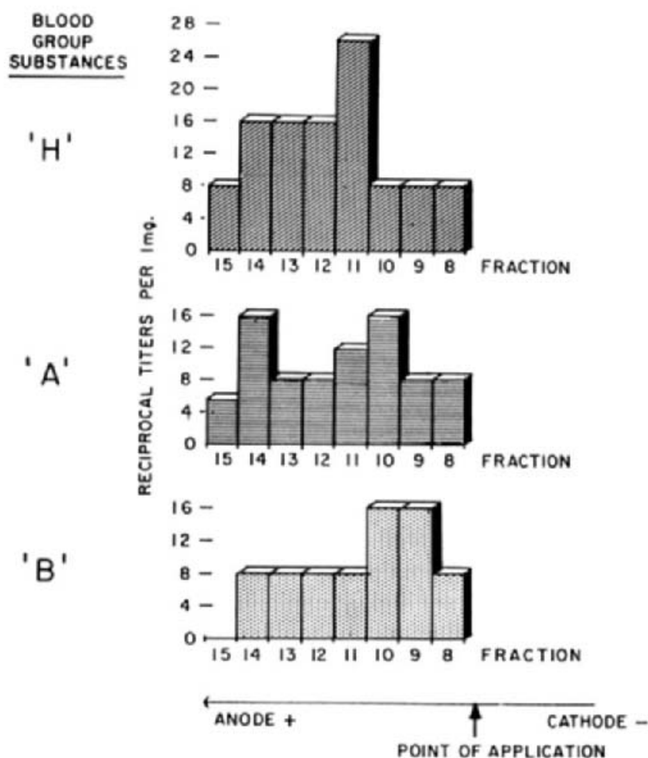

FIG. 14. Blood group substances AB(H) in the electrophoretic partition on paper curtain of a pool of normal human gastric juices. From Glass (G3a).

peaks, with a plateau between, blood group substance B was associated only with material of slow anodic mobility (Figs. 14 and 15).

Our findings cannot be accepted as proof of the fact that the electrical charges of blood group substances H, A, and B differ from each other. They may rather be considered as evidence that various blood group substances form different complexes with negatively charged mucosubstances in gastric juice. This may influence their electrophoretic mobility. Possibly blood group substance B, which, according to Masamune *et al.*

(see G5), is more negatively charged, forms an aggregate with a mucosubstance less negatively charged than that with which blood group substances A and H are associated. While blood group substances belong mostly to the fucomucan type of mucopolysaccharide, other mucosubstances contain sialic and uronic acids and belong to the sialomucin and

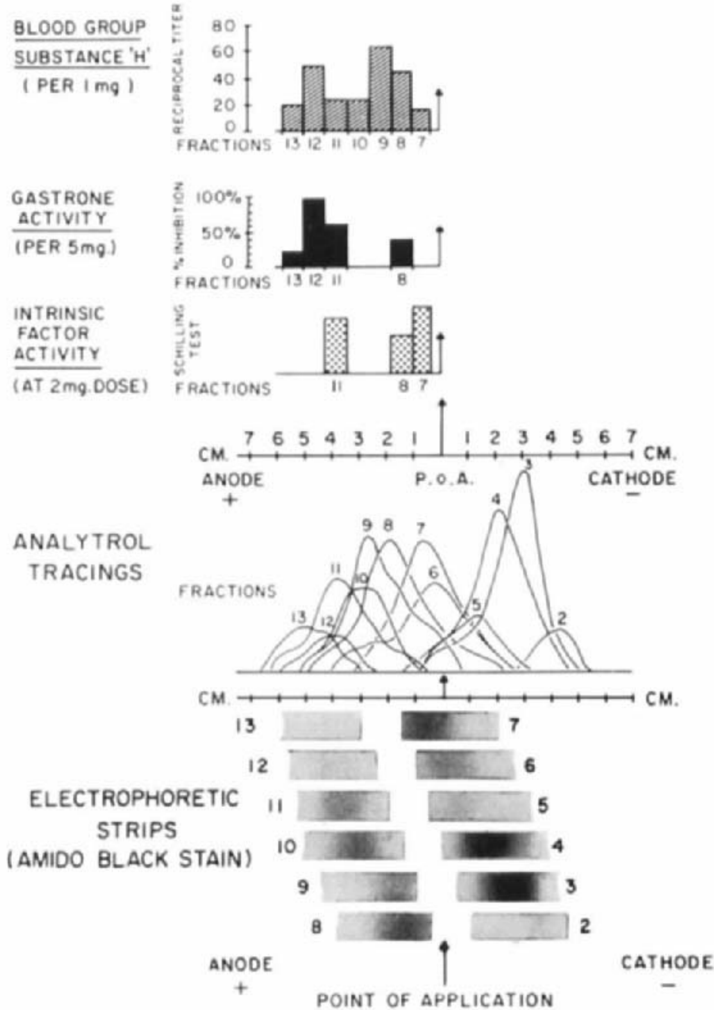

FIG. 15. Localization of biologically active substances, including blood group substances, in the electrophoretic partition on paper curtain of a pool of normal gastric juices. From Glass (G3a).

acid aminopolysaccharide classes, respectively. These latter materials may aggregate with blood group substance fucomucans, lending them more or less negative electrophoretic mobility.

Continuous electrophoresis on paper curtain was used in our laboratory for fractionation of "gastrone," the material inhibitory to gastric secretion (G3a, K8). In an unpublished study with C. F. Code's group, and Stephanson, Castro-Curel, and Carton we submitted gastric juices from normals, and patients with histamine-fast anacidity to electrophoretic fractionation on paper curtain in borate buffer of pH 9.0, μ 0.06. A total of 5 large acid and anacid gastric juice pools were fractionated. Every fractionation consisted of 6–10 runs during each of which 50–75 mg was fractionated in 16 hours. Effluents were collected and pooled correspondingly until enough material was available for processing. All continuous electrophoretic fractions were then assayed on Heidenhain pouches stimulated by intravenous (i.v.) infusion of histamine, as well as on pylorus-ligated rats. Other aliquots were subjected to paper strip electrophoresis followed by staining for proteins, peptides, and carbohydrates.

At least two zones containing the gastrone activity were found in the electrophoretic partition. One inhibitory area had high negative charges, did not show presence of proteins, but consisted mostly of carbohydrate-stainable material demonstrating sialomucin mobility. The other inhibitory area, especially from anacid juices, was localized slightly toward the cathode from the application point, stained strongly for proteins, and had electrophoretic mobility of β_{2a} and γ-globulin (see Section 1.3 and 5) (Fig. 16). These materials were further identified by Kubo and Dolinski in our laboratory by means of immunodiffusion and immunoelectrophoresis.

We also assayed for gastrone activity fractions separated by continuous and strip paper electrophoresis from anacid gastric juice before and after exposure to peptide digestion. Results obtained indicated that the polydispersity of inhibitory activity, on electrophoretic partition of acid gastric juice, is due to some extent to the peptic degradation of gastrone (K8).

Continuous electrophoresis was also used in our laboratory by Castro-Curel for fractionation of inhibitors to B_{12} uptake by liver tissue. The results obtained will be reported elsewhere.

Peeters recently studied the distribution of proteolytic enzymes and proteins on curtain electrophoresis by means of his modified continuous electrophoresis apparatus (P1a) and a new technique which he calls "electrochromatography." The viscosity of gastric juice neutralized *in situ* with phosphate buffer (pH 7.2) was decreased by liquefaction with

Ascoxal (a mucolytic drug). Then the gastric juice was concentrated by ultrafiltration and subjected to "star electrophoresis" in veronal buffer of pH 8.6, Γ/2 0.033. The curtain was stained after oven drying with amido black, azocarmine, or bromophenol blue. Up to 10 protein fractions were localized on the curtain, including pepsin, albumin, and contaminant protease (trypsin?) derived from regurgitated duodenal secretion. Peptic

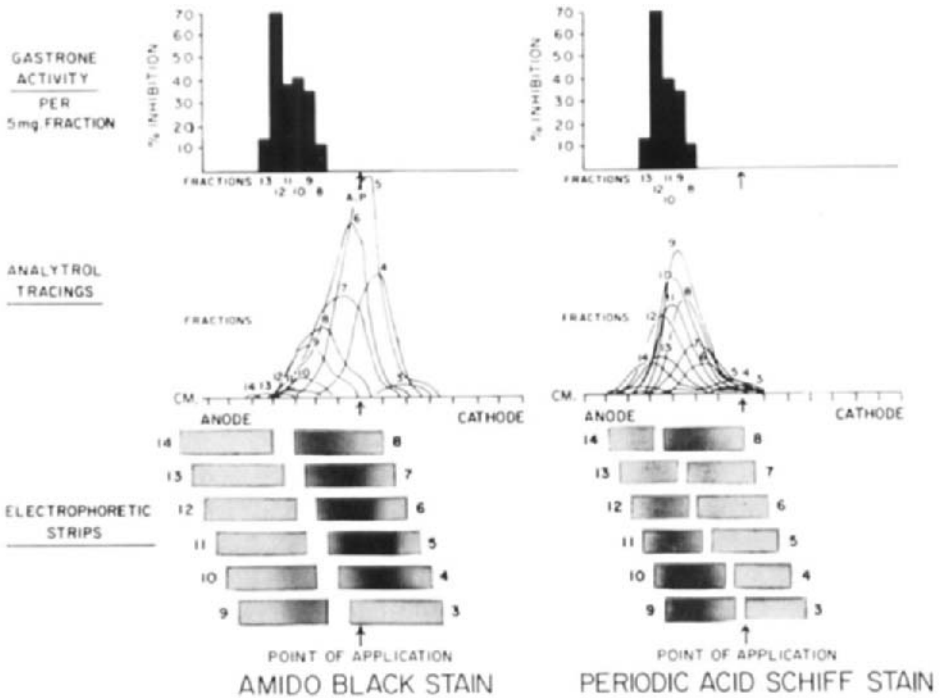

FIG. 16. Localization of the gastrone in the electrophoretic partition on paper curtain of a pool of anacid gastric juices. From Glass (G3a).

activity was demonstrated along the anodic border of the albumin zone, but not as a distinct fraction.

Peeters *et al.* (P1b), using his method of gastric juice electrophoresis on paper curtain, attempted to evaluate the significance of the electrophoretic patterns for the diagnosis of certain pathological conditions of the stomach. Gastric juice from 4 cases of peptic ulcer, 2 of gastritis, 3 of hyperacidity, and 7 from patients without chemical or radiologically detectable gastrointestinal lesions was collected and immediately neutralized to pH 7, then concentrated by ultrafiltration, and applied to the curtain. Curtain electrophoresis was carried out in a veronal buffer

for 3 hours, followed by staining of the curtain with amido black or bromophenol blue stains.

In this very limited study, with an unconventional technique, Peeters found various patterns in the same patient when examined at various time intervals. Fasting juice gave different results from the juice buffered in situ, which Peeters attributed to the regurgitation of duodenal juice into the stomach. Normal individuals, as well as those with gastric tumors and ulcers sometimes gave similar electrophoretic patterns. Some of the ulcerated tumors had, at times, a pattern similar to that of serum, but at other times, they gave an entirely normal gastric pattern. Peeters considered that the cause for the absence of a well-defined normal and pathological electrophoretic gastric juice pattern was the contamination with saliva and duodenal content, which represents a variable.

It appears to the reviewer that several important physiological and pathological issues have been overlooked by these authors, when they took very little notice of the notorious physiological variability of secretory patterns of gastric juice and of the physiological *in situ* degradation of gastric materials as the main cause of changes in electrophoretic patterns, while they shifted the emphasis on the reflux contamination of the gastric juice. Furthermore, the small number of pathological cases investigated, as compared to the experience of other laboratory groups working with gastric juice, and cited above, makes their conclusions at this point somewhat biased.

1.4. Electrophoresis on Cellulose Acetate

Piper *et al.* (P3, P4) applied the paper electrophoretic method of our laboratory to gastric juice electrophoresis on cellulose acetate strips. They collected gastric juice after augmented histamine stimulation, but gastric acidity prior to collection was neutralized *in situ* by intragastric instillation of sodium bicarbonate in 5–20% solution.

In 24 controls without gastrointestinal disease in whom gastric secretion was neutralized *in situ*, the electrophoretic pattern consisted of 3 major anodic bands and 1 band remaining at the application point. Furthermore, in about one half of cases, a small fifth band emerged, moving slightly toward the cathode. The most anodic band migrated with serum albumin mobility. Occasionally it was preceded by another small band, which moved ahead toward the anode. This "pre-albumin" band was found only in 7 of 24 cases, and only at low concentration. The authors did not decide whether this band corresponded to pepsin or to partly degraded albumin. When localization of each of these bands was deter-

mined in terms of the distance travelled by band 5 (albumin), band 4 migrated 69–84%, band 3 30–68%, and band 2 29% of this distance, while band 1 remained at the application site. Band 3 was frequently subdivided into two or three minor peaks. As did Gullberg and Olhagen (G34), they noted that the band with electrophoretic mobility of albumin was absent from the nonneutralized *in vivo* gastric juice and that it was replaced by 3 bands of lesser anodic mobility.

When Piper *et al.* incubated neutralized gastric juice with pepsin at pH 3.0 for 5–60 minutes, the albumin peak gradually decreased, while some more rapidly migrating albumin degradation products appeared. This was similar to previous findings of our laboratory (G3). Since Piper *et al.* used a horizontal cell, their other degradation products did not move to the cathode, as demonstrated in our laboratory (G3, G9) and by others (G34) with vertical electrophoresis, but remained at the application point. In pernicious anemia patients, in line with the previous findings (G22), the albumin band was increased, while the anodic band 4 was decreased and central band 2 was usually absent. Decrease in band 4 perhaps could be related to the decreased pepsinogen content in gastric juice of pernicious anemia patients. Piper *et al.* suggest that band 2 corresponds to intrinsic factor-related vitamin B_{12} binders, which is the reason for its disappearance in pernicious anemia. The present reviewer cannot agree, since this binder's concentration is so low that it falls below the resolution power of paper electrophoresis (G2). The same must apply to cellulose acetate strips whose resolving power is certainly not greater than that of paper strips.

In gastric juice of patients with gastric cancer, according to Piper *et al.*, the most important abnormality was an increase in band 5, i.e., a rise in the albumin concentration of the gastric juice. This was in line with earlier results obtained with paper electrophoresis (G3, G8, G9, G22). In gastric cancer, the albumin represented, on the average, 35.5% of total protein, in gastric ulcer patients 33.0%, and in normal controls 26.7%. The close overlap of albumin increase in patients with gastric carcinoma and gastric ulcer as well as lack of a clear demarcation line between the controls and the cancer group detract, according to Piper *et al.* (P3, P4), from the significance of this finding for gastric cancer diagnosis. In about 50% of gastric ulcer cases, a distinctly increased band 2 was found. This was localized slightly toward the anode from the application point. It was present in only 10% of individuals without gastric ulcer. The increase of this band in gastric ulcer was noted before by Fasel and Scheidegger (F1) and Kakei *et al.* (K5). Its mean size in gastric ulcer patients was 23.3%, in 14 controls and in 5 duodenal ulcer

patients 9.0%, and in 5 patients with pernicious anemia and gastric carcinoma only 3.9%. Piper *et al.* (P4) suggested that this increase in band 2 is the result of digestion of gastric proteins by proteases active above pH 7.0 (see G5).

1.5. Starch Block Electrophoresis

Gräsbeck in 1955 (G26, G27) collected normal human gastric juice on ice, after i.v. stimulation with insulin, neutralized it *in vitro*, removed mucus by centrifugation, and dialyzed and lyophilized the supernatant.

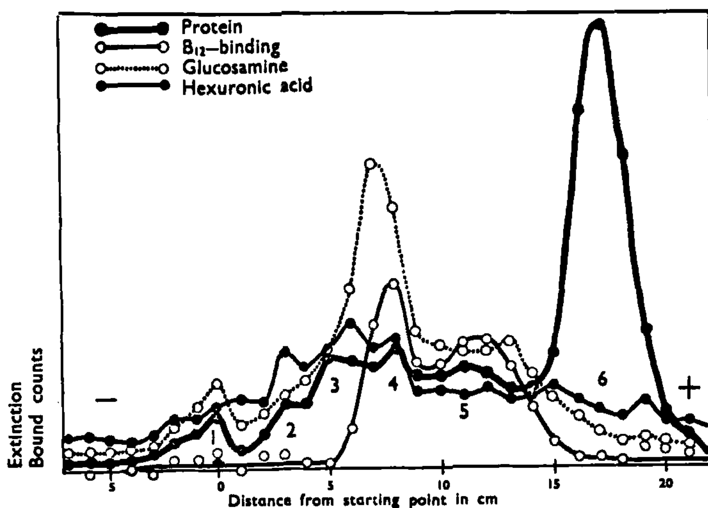

Fig. 17. Electrophoretic pattern of neutralized gastric juice on starch block, pH 6.1, 30 hours, 400 volts. The figures indicate regularly occurring protein peaks. From Gräsbeck (G27).

Nondialyzable solids from 50 ml gastric juice could then be fractionated in one electrophoretic run. Following this, the block was cut into 35 pieces, about 1 cm wide. Every segment was extracted with 10 ml buffer, and protein, glucosamine, and hexuronic acid were determined in each of the eluates. The pattern obtained included 6 protein peaks, of which the most anodic was the highest (Fig. 17). The slower electrophoretic components were rich in hexosamine, while the hexuronic acid was distributed throughout the partition showing a peak value coinciding with the peak of hexosamine. To the reviewer, the high uronic acid content in these studies appears to be due to the method used, which gives high uronic acid readings in the presence of other sugars. The same applies to Wada's (W4) and Grossberg *et al.* (G32) results as well as our

own early uronic acid determinations in "glandular mucoprotein" (see G5). Most of the pepsin traveled with the leading anodic peak, being distributed only in traces in other areas of the partition. Slight proteolytic activity at pH 4.0 was found in the same area as pepsin.

Katzka (K7) followed Gräsbeck in starch block electrophoretic fractionation of gastric juice, but used the Kunkel and Slater procedure and the Reco electrophoretic chamber, cooled by running tap water. Runs were performed in 0.1 *M* veronal buffer of pH 8.6 at 300 volts and 40–50 mA for 16 hours. At the end of the run, the starch was cut into segments which were eluted with physiological saline. Fasting gastric juice showed a protein pattern consisting of 4 major peaks marked A, B, C, D and various subpeaks, A1, A2, C1, C2, and C3. The latter were not consistently present in the gastric juices studied. Peak A was localized slightly toward the cathode from the application point, the largest one, peak B, was located closed to the application point, while peaks C and D were anodic. After histamine stimulation, peak C was higher than in the fasting juice, as was peak D. In gastric and marginal ulcer, patterns were similar to those of normals. In 4 patients with pernicious anemia in remission, peak B was present at a high concentration and practically represented the protein total, peaks A and C being very low. Peak D was absent from all pernicious anemia juices. Electropherograms of 2 patients with histamine anacidity, without pernicious anemia, were similar to those elicited in pernicious anemia. Since peak B was usually found in electropherograms of saliva as well, Katzka considered the possibility that it might have originated from swallowed saliva, but that it is normally destroyed by the proteolytic enzymes present in normal gastric juice. However, in juices devoid of proteolytic activity (pernicious anemia), this protein would remain unchanged. The findings of Uchino and Schwartz (G23, U1, U2), who used vertical paper electrophoresis in our laboratory, corroborate this point of view to some extent. The "intermediate B_{12} binder," related perhaps to a mucosubstance of salivary origin in gastric juice, disappears from the acid-pepsin-containing gastric juice due to proteolysis. Similarly, Brummer and Seppälä (B13) noted disappearance of the central band on paper electrophoresis after acid-pepsin digestion. Thus, it is probable that saliva contributes, in part, to the formation of Katzka's peak B, Mack *et al.* (M1) peak G2, our tertiary intermediary B_{12} binder (G4), and β_2-like precipitation lines of Hirsch-Marie and Burtin (H7a) on immunoelectrophoresis; the salivary contribution does not account for the entire material present in this electrophoretic area, however.

The nature of protein peaks on starch block electrophoresis and their

relation to those obtained on paper electrophoresis, however, cannot be definitively ascertained (G2a). It seems probable that Katzka's fastest peak, D, on starch block electrophoresis corresponds to pepsin. Peak C (and additional small peaks in this area) probably correspond to M1–M2 materials, as well as the albumin-containing area of the paper electropherogram. Peak B probably corresponds to M3–M4 materials on paper electrophoresis and may also include γ-globulin, while peak A is perhaps the starch block equivalent of the cathodic component of Gräsbeck (G27) and cathodic peptide peaks X2, Y1, Y2, and Z of vertical paper electrophoresis (G19).

Horowitz and Hollander (H13, H18) used starch block electrophoresis in studying acetylcholine-stimulated anacid mucinous secretions from unoperated dog stomach after injection of methacholine and i.v. injection of I^{131}-labeled albumin. A protein band with superimposed radioactivity was found on starch block and paper electrophoresis, demonstrating serum albumin mobility. This suggested that serum albumin contributed to the protein content of the normal gastric mucinous secretion in the dog. However, when acid gastric juice was collected after i.v. injection of I^{131}-labeled albumin, no peak was found; instead, several peaks were observed on the cathodic side of the electrophoretic partition. Incubation of the anacid methacholine-stimulated gastric mucus with acid gastric juice or acidification of this material to pH 1.2 resulted in the formation of cathodic bands; these were analogous to those observed in human gastric juice as a result of peptic degradation of serum albumin (B13, G3, G9, G34).

1.6. Starch Gel Electrophoresis

Smithies' technique, modified by Pert *et al.* (P2) and found applicable to fractionating gastric juice, was further developed and perfected by Jeffries *et al.* (J4). Soluble starch in 0.027 *M* borate buffer of pH 9.05 was used, which after cooling had a pH of 8.6 ± 0.2. Gastric juice collected after histamine was centrifuged, dialyzed, and lyophilized. Nondialyzable gastric solids (5–10 mg) were made into a paste with prepared starch and applied to the origin. Electrophoresis was carried out at 10°C for 5 hours, at a constant voltage of 6 volts/cm. On completion of the electrophoretic run, gel strips were cut in half along the horizontal plane. One half was stained with saturated solution of amido black 10B in methanol-water-acetic acid solvent (5:5:1 by volume) for 12–16 hours. The other half was sectioned and eluted with buffer for chemical assay of proteins and sugars, or determination of radioactivity if radioactive

vitamin B_{12} was added to gastric juice prior to electrophoresis (J3, J3a) (see Section 1.9.3).

Gastric juice, after starch gel electrophoresis, clearly separated into 5 zones of anodal staining, the slowest being 1 cm from the origin (Fig. 18). A wide zone located 2–4 cm from the origin toward the anode followed. This was preceded by 3 bands of more anodic mobility appearing at the

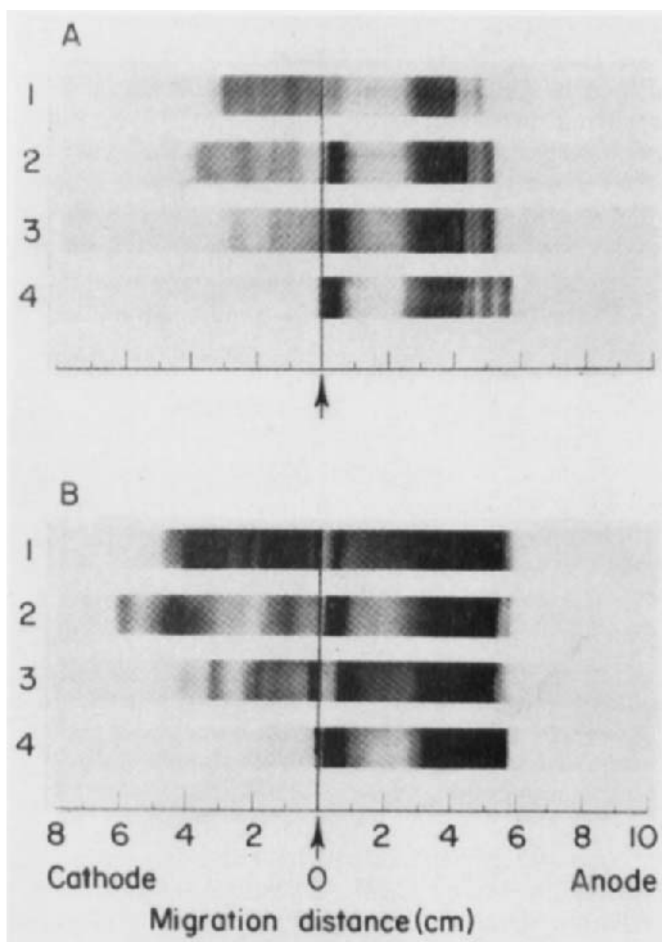

FIG. 18. Starch gel electrophoretic patterns of lyophilized gastric juice from 4 normal subjects. A, 5 mg of lyophilate; B, 20 mg of lyophilate. Numerals refer to individual subjects. Electrophoresis in borate buffer at pH 8.6 for 5 hours. These electrophoretic patterns were selected to illustrate the variation in cathodal staining (amido black 10B stain). From Jeffries *et al.* (J4).

migration front. When a larger amount of gastric juice was utilized, the wide zone migrated 3–6 cm from the origin and merged with the more rapidly migrating zones. The protein distribution in the eluate corresponded to protein localization, as determined by amido black staining. Cathodic zones staining with amido black contained no protein on chemical assay, similarly to cathodic peptide peaks X2, Y1, Y2, and Z on paper electrophoresis (G16, G17). Proteolytic activity in eluates from starch gel was localized in the single anodic area, corresponding to the broad anodic band preceded by a faster narrow anodic area. Both proteolytic activities (at pH 1.5 and 3.5) were confined to this area (J4).

Another change in the electrophoretic pattern of gastric juice, as a result of peptic digestion, was the marked decrease in size of the protein component localized about 1 cm toward the anode from the origin. This parallels findings obtained under similar conditions with other techniques, i.e., decrease or disappearance of component B on starch block electrophoresis (K7), of component M4 on horizontal paper electrophoresis (B13), and of the "intermediate tertiary B_{12} binder" on vertical paper electrophoresis (G23, U1, U2).

The most comprehensive studies of normal and pathological gastric juices with the use of starch gel electrophoresis have been most recently published by Hartmann, Cornet, and their associates Bignon, Ollier, and de Traverse (H1). The data obtained were compared with those on paper- and immunoelectrophoresis, and are of great interest to all those interested in comparative value of various techniques for the resolution of macromolecules of the gastric juice. They should be consulted in the original (H1, H1a).

Castro-Curel in our laboratory used Smithies' vertical starch gel electrophoresis in fractionating human gastric juice. Although several fractions were obtained, similar in appearance to those of Jeffries, the resolution was not as consistent as that obtained by horizontal starch gel electrophoresis (unpublished work).

1.7. Agar Gel Electrophoresis

This procedure was performed by several authors as a preliminary step to immunoelectrophoresis (F1, G24, G25, H20). It was more thoroughly studied by Hirsch-Marie and Burtin (H7), who used gastric juices neutralized *in situ* before electrophoresis. They found 5 anodic and 2–3 cathodic components stainable with amido black (Fig. 19). Anodic bands were labeled 1 to 5, and cathodic ones 6 to 9. The first most rapid component contained no carbohydrates, but exhibited marked proteolytic activity with an optimum of pH 2–3.5. The second band, close to the

first, also showed marked proteolytic activity at an acid pH, but stained slightly with glycoprotein stains. Band 3 in *in situ* neutralized gastric juice had the mobility of serum albumin, did not contain proteolytic activity, and did not stain with carbohydrate-staining material. Anodic band 4, localized close to the application point, had α-globulin electrophoretic mobility, was very rich in glycoprotein, and showed slight proteolytic activity at pH 8 (trypsin?). The fifth component, almost at

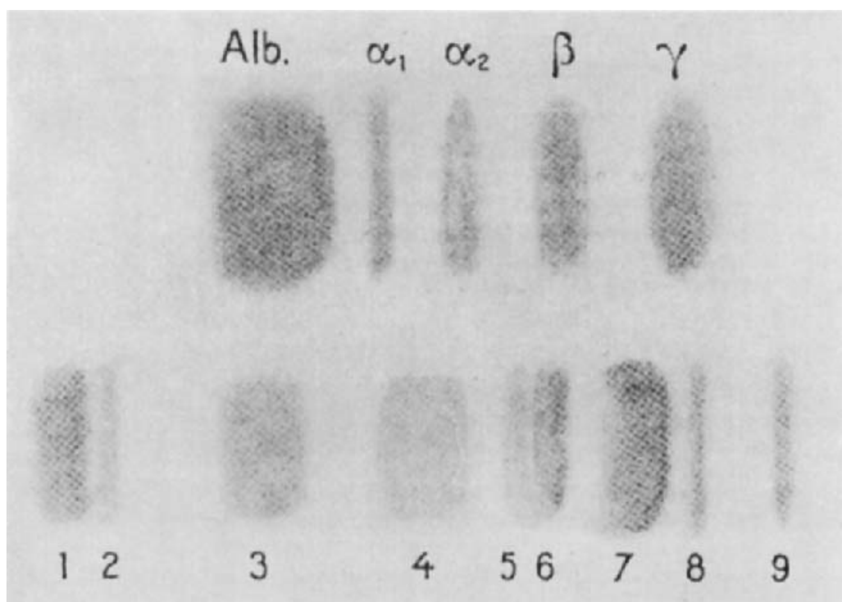

FIG. 19. Agar gel electrophoresis of proteins of concentrated gastric juice after *in vivo* alkalinization. Top row: Normal human serum. Bottom row: Concentrated gastric juice. From Hirsch-Marie and Burtin (H7).

the application point, stained strongly with glycoprotein stains and again showed proteolytic activity at pH 8. Of cathodic materials, band 6, almost at the application point, stained only with a protein stain and did not demonstrate proteolytic activity. Component 7, which was of greatest importance quantitatively, had electrophoretic mobility intermediate between that of β- and γ-globulins and contained about one half of total protein-staining material. It stained very little for glycoproteins and exhibited no proteolytic activity. Finally, the most cathodic components, 8 and 9, did not stain for glycoproteins and did not demonstrate proteolytic activity on agar.

Additional studies with agar gel techniques were reported by the same authors on a large number of gastric juices neutralized *in vitro* after collection.

If one attempts to correlate the agar gel electrophoretic components in gastric juice with those obtained on vertical paper electrophoresis (G19),

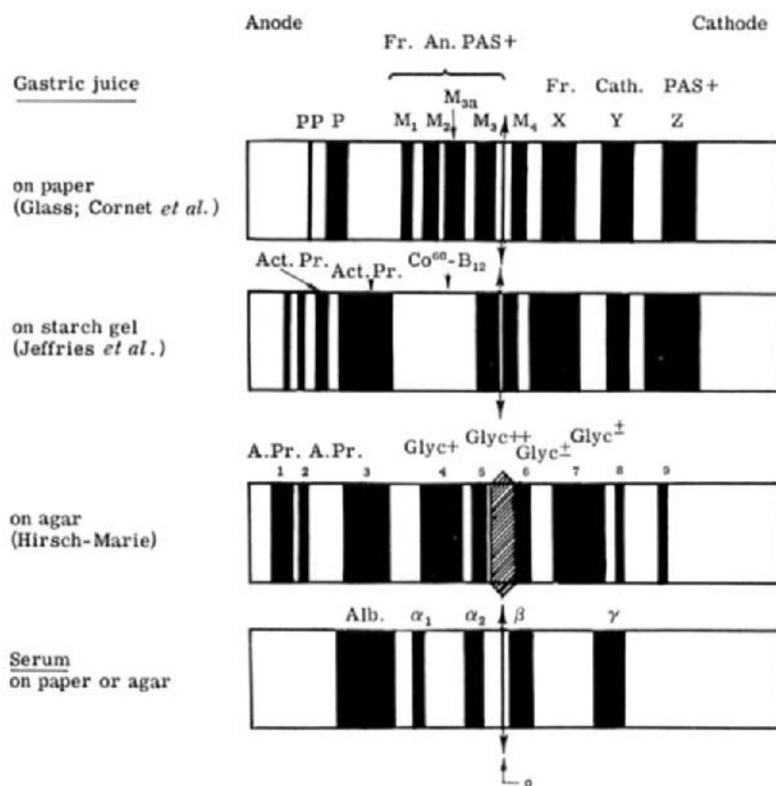

FIG. 20. Comparative results of different electrophoretic methods applied to gastric juice. From Cornet *et al.* (C5a).

the results would be as follows: band 1, on agar, corresponds to component P (pepsin) on paper; band 2 is most probably identical with component M1; band 3 corresponds to serum albumin. Bands 4 and 5 on agar correspond to materials M2 and M3 on paper, mucosubstances rich in neutral carbohydrates, and bands 6 and 7 correspond to components M4 and X1 on paper, which include γ-globulin. Finally, bands 8 and 9 probably correspond to peptides X2, Y, and Z on paper, pro-

teolytic degradation products of serum albumin and perhaps of some mucosubstances.

The comparison of electrophoretic patterns of gastric juice as obtained on paper, starch gel, and agar has been studied by Cornet *et al.*, and is illustrated in Fig. 20.

1.8. Immunoelectrophoresis

The first immunoelectrophoretic study of gastric juice under normal and pathological conditions was performed by Fasel and Scheidegger (F1), using the latter's microimmunoelectrophoretic method. Rabbit antiserum to gastric juice was obtained by bimonthly intramuscular injections of 0.1–0.3 mg normal lyophilized human gastric juice, suspended in 0.1–0.3 ml Freund's adjuvant and administered over a period of 5 months. Of 6 rabbits used, only one provided adequate titer of the antibody. Of 9 lines the second was attributed to pepsinogen, the third was probably due to albumin, and the seventh appeared only occasionally. In gastric ulcer, line 7 was frequently present and often disappeared after treatment. Line 5 was less pronounced in *in vivo* neutralized gastric juices, indicating that this line is the product of peptic degradation of some gastric juice components. The immunoelectrophoretic pattern of intragastrically neutralized gastric juice was about the same as that of *in vitro* neutralized juice, but fewer lines were elicited. Absorption with normal human serum of the antiserum to gastric juice did not abolish gastric juice antibodies. Thus, according to Fasel and Scheidegger (F1), normal human serum did not contain antibodies to proteins of normal acid gastric juice. In addition, since the pattern obtained with rabbit antiserum to gastric juice was not obtained with horse antiserum to human serum, the authors concluded that materials contained in acid gastric juice were different from those present in normal serum.

Tenorova *et al.* (T7) found 3–10 precipitation lines on immunoelectrophoresis of anacid human gastric juices run against rabbit antiserum to human serum. No precipitation lines were found when immunoelectrophoresis was performed against acid gastric juice. Others (B3), using immunoelectrophoresis against antiserum to human serum, analyzed three neutralized *in vivo* gastric juices of patients with gastric cancer, Menetrier's disease, and atrophic gastritis. In each case, immunoelectrophoresis demonstrated presence of albumin and γ-globulin, and in some instances α- and β-globulins as well. Similarly Witschi *et al.* (W16), using microimmunoelectrophoresis, identified albumin, γ-globulin, α_1-macroglobulin, β_{2a}-globulin, and siderophilin in human gastric juice. In our laboratory Ohara as well as Kubo and Dolinski, using immunoelec-

trophoresis and Ouchterlony plates demonstrated the presence of serum albumin (G3), β_{2a}, 7 S γ- and β_{2m}-globulins, as well as ceruloplasmin, transferrin, and some haptoglobins in gastric juices from patients with gastric anacidity (unpublished work). Götz and Scheiffarth (G24, G25, S4) studied human gastric juice, gastric mucosa, and extracts from the gastric wall on immunoelectrophoresis against horse antiserum to human serum. With gastric juice, one precipitation line formed in the area of the albumin, two in that of β-globulins, and one in the γ-globulin zone. Immunoelectrophoretic analysis of gastric mucosal extracts yielded 11 protein components: 2 pre-albumins, albumin, $2\alpha_1$-, $2\alpha_2$-, 1 β-, 1 $\beta\gamma$-, 1 β_{2a}-, and 1 γ-globulin. A cross-relationship existed, with some differences, between proteins of gastric juice, mucous membrane, and gastric wall.

One of the most comprehensive immunoelectrophoretic works in this field was carried out by Hirsch-Marie and Burtin (H7) in Grabar's laboratory. They collected fasting gastric juice after intragastric instillation of borate buffer of pH 9. Gastric juice was then concentrated by dialysis against 30% solution of PVP and dissolved in borate buffer to final concentration of 20–40 mg proteins/ml. Viscous gastric juices were liquefied either by ultrasonics or by 0.5–2-hour incubation at 37°C with α-chymotrypsin. Immunoelectrophoresis was performed on agar in the Grabar-Williams cell in veronal buffer of pH 8.2, ionic strength 0.024–0.05, 5 volts/cm, or by the micromethod of Scheidegger. Materials tested were exposed to horse or rabbit antiserum to human serum, as well as antiserum to human gastric juice obtained by immunizing rabbits with human gastric juice. Several antisera absorbed with human serum were also used.

Immunoelectrophoresis against antiserum to human serum showed arcs of γ-globulin, β_{2a}-globulin, α-globulin, and 1 or 2 albumins; antiserum to gastric juice yielded 1 γ arc, 2–3 β_2 lines (of which one was localized close to the central trough and a less dense one further inside), 1–2 β_1, 3–4 α (of which one was localized as far as β_2), and finally 1 arc with pre-albumin mobility (Fig. 21).

In addition to several proteins, identical with those found in human serum, the authors demonstrated the presence of 6 proteins in the fasting gastric juice of normal subjects apparently not present in serum, the main fraction having a mobility similar to β_2-globulin. Several fractions have also been found common to those present in human saliva. The authors consider the possibility of extragastric (salivary) origin of the β_2-like protein in gastric juice, although they do not rule out the possi-

bility that this protein is a common component in several digestive secretions of man (saliva, gastric juice, duodenal content).

Hirsch-Marie and Burtin (H7a) then studied 143 normal and pathological gastric juices by immunoelectrophoresis against antiserum to human serum, gastric juice, and gastric mucosa serum. Gastric juices were collected under fasting conditions and after histamine and were neutralized *in vitro* with a borate buffer and concentrated against PVP;

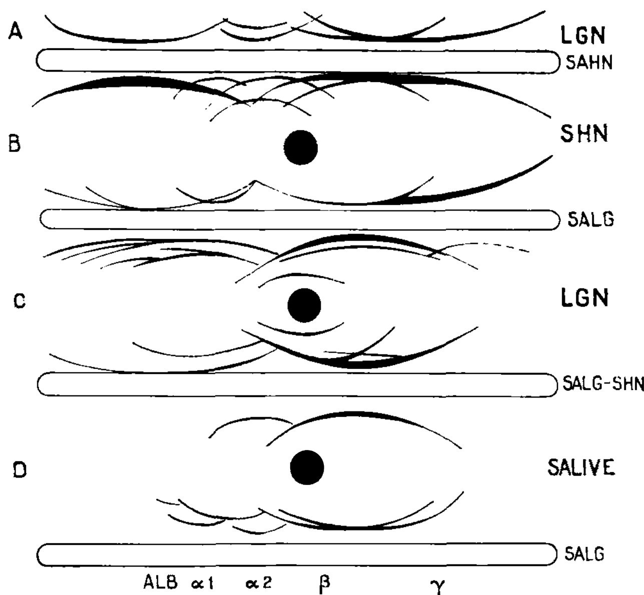

FIG. 21. Immunoelectrophoretic study of proteins. Normal concentrated gastric juice (LGN), concentrated saliva (Salive), normal human serum (SHN) in the presence of antiserum to normal human gastric juice (SALG), and same antiserum absorbed by normal human serum (SALG-SHN). From Hirsch-Marie and Burtin (H7).

the residue was liquefied with α-chymotrypsin or ultrasonics. Agar gel electrophoresis revealed 7 zones of mobility, while immunoelectrophoresis against the anti-normal human serum demonstrated the presence of albumin, α_2-globulin, β_{2a}- and γ-globulins and 11 other precipitation lines (against anti-gastric juice serum as well). Five of these were similar to those obtained in saliva. Gastric juices contaminated with blood, as well as those of patients with hypertrophic gastritis, certain ulcer cases, cancer and exudative gastroenteropathy showed an increase in albumin, β_{2a}- and γ-globulin. α-Globulins and siderophilin were found

only occasionally in gastric juice not contaminated with blood. An increase in the other components not related to the serum proteins was also observed in exudative gastroenteropathy while in gastric and duodenal ulcer, no characteristic patterns were observed, except for some increase in the 3rd and 4th precipitation lines. In gastric cancer, an increase in lines 4 and 5 was observed.

Another very extensive study was performed by Hurlimann (H20) on 121 gastric juices, of which 32 were collected after *in vivo* neutralization of gastric juice by phosphate buffer of pH 8. Dialyzed and lyophilized gastric juice, collected after histamine, was subjected to microimmunoelectrophoresis at 4–8% concentration in veronal buffer for 40 minutes against horse antiserum to human serum or rabbit antiserum to gastric juice.

The immunoelectrophoretic pattern of human gastric juice, when exposed to antiserum to human serum showed up to 12 precipitation lines, some perhaps due to proteolysis and different from those seen on immunoelectrophoresis of serum. Gastric juices, on immunoelectrophoresis against the rabbit antiserum to gastric juice, showed 4 lines corresponding in localization to serum albumin, α_2-globulin, β-globulin, and γ-globulin. When rabbit antisera to human gastric juice were absorbed with human serum, serum albumin, or γ-globulin, no precipitation arcs resembling these two proteins were found. All *in situ* neutralized gastric juices showed the presence of serum proteins on immunoelectrophoresis. However, of 89 nonneutralized gastric juices, only 5 demonstrated definite protein arcs, 22 protein traces, and the others no protein lines. This was certainly due to the proteolysis of serum protein in gastric juice by pepsin at low pH. In the 32 *in vivo* buffered gastric juices, albumin, and α_1-, β_1-, β_2-, and γ-globulin were found. The only serum protein which was usually absent (with the exception of 4 cases) was the α_2-globulin. The absence or very small concentration of α_2-globulin in gastric juice was due to its extreme proteolytic sensitivity, but perhaps also to translocation difficulty through the mucous membrane. An attempt to quantitate the amount of serum proteins in gastric juices by absorbing horse antisera with increasing amounts of serum failed. The quantity and composition of serum proteins varied from case to case. However, respective concentrations of different serum proteins did not correspond to their ratio in the serum. Immunoelectrophoretic patterns of various gastric juices neutralized *in situ* were practically the same, without regard to clinical diagnosis. On the other hand, when gastric juices were nonneutralized *in vivo*, an immunoelectrophoretic pattern similar to that of serum indicated the presence of hypochlorhydria, anachlorhydria,

anacid gastritis, or cancer of the stomach. In four gastric juices of patients with multiple myeloma, some abnormal serum proteins were found in the gastric juice (H20).

Some of the precipitation arcs found on immunoelectrophoresis of gastric juice were considered to be specific products of the gastrointestinal tract. These materials did not represent proteins or their degradation products, since pepsin-digested albumin or γ-globulin yielded no precipitation arcs to the antisera. Some of the precipitation lines (3, 5, 7, and 9) were similar to those found in saliva or bile.

Simons and Gräsbeck (S9) have submitted 15 gastric juices to immunoelectrophoresis. In order to identify the precipitation arcs obtained, the authors compared them with those produced by less complex gastric materials, such as human pepsin, gastricsin, albumin, and glandular mucoprotein and mucoproteose fractions. Using Scheidegger's technique and rabbit antisera to human gastric juice and serum, Simons and Gräsbeck detected 9 precipitation lines in the *in vitro* neutralized gastric juice, where neutralization was carried on up to pH10 to inactivate pepsin and then down to pH 7.0. Precipitation arcs were stained with protein stains and, in cases where Co^{57}-vitamin B_{12} was added to gastric juice before fractionation, they were also studied by autoradiography (see Section 1.9.3).

Pepsin and gastricsin mobilities corresponded to line 2, and albumin to line 3. There was little difference in patterns of the *in situ* and *in vitro* neutralized gastric juice, except for somewhat fewer precipitation lines in the former. About half the gastric juices submitted to immunoelectrophoresis against the antiserum to human serum gave precipitation lines with serum albumin and γ-globulin. When 3 gastric juices were neutralized intragastrically, the immunoelectrophoretic patterns obtained with the antiserum to human serum were similar to those obtained by Tenorova *et al.* (T7). They showed, in addition to albumin and γ-globulin, precipitation lines of α-globulins. Five pernicious anemia gastric juices yielded fewer lines with antiserum to gastric juice than the native, acid *in situ* neutralized gastric juice; only lines 4, 5, and 6 were detected, but not line 7.

Pernicious anemia juices formed many precipitation lines against the antiserum to human serum, and several precipitation lines with the antiserum to saliva (S9). Immunoelectrophoresis of 12 gastric juices against rabbit antiserum to human saliva resulted in the formation of a few precipitation lines. The immunoelectrophoretic patterns of intragastrically neutralized and *in vitro* depepsinized gastric juices, when subjected to immunoelectrophoresis against the antiserum to saliva, differed.

The intragastrically neutralized juices resembled those of pernicious anemia and showed many more lines than gastric juices neutralized *in vitro*. Since salivary contamination was avoided, both saliva and gastric juice apparently contained immunologically common components, which undergo rapid destruction in the presence of acid and pepsin, even if only a few minutes have elapsed between the secretion of the acid and its neutralization *in vitro*.

Gräsbeck *et al.* (G28a) compared the immunoelectrophoretic cross reactivity of gastric juice with saliva and other body fluids, and studied the occurrence of the vitamin B_{12}-binding components in other body fluids. They found that saliva, gall bladder, bile, and cerebrospinal fluid gave a large number of common precipitation lines with antiserum to gastric juice. Since most precipitation lines disappeared after absorption of the antisera by blood serum, the obvious explanation was the presence of serum proteins in these body fluids. In addition, however, some other common antigens were found in saliva, gastric juices, and other body fluids.

Kaminsky *et al.* (K6) studied the products of albumin proteolytic degradation by these immunoelectrophoretic techniques.

1.9. Application of Electrophoresis to Special Problems

1.9.1. *Localization, Fractionation, and Quantitation of Gastric Proteases*

Two protease fractions were observed by Herriott (see G5) on free boundary electrophoresis of crystalline swine pepsin, and two proteases were separated by Merten *et al.* from hog gastric extracts (M4), as well as by Taylor from human gastric juice (T6). Like pepsin, both moved to the anode at pH 2.5.

Grossberg *et al.* (G32), using free boundary electrophoresis, found peptic activity distributed through practically all 5 electrophoretic components of acid canine gastric juice. However, this is not so with zone electrophoresis of human gastric juice (see below).

Merten *et al.* (M4) precipitated HCl extract of hog gastric mucosa by 60% saturation with ammonium sulfate below pH 6.0 and, after dissolving, submitted it to paper electrophoresis at pH 4.6. The most anodic protein fractions contained proteases with a pepsin to cathepsin ratio of 10.9:1, while the second peak contained both these enzymes at a ratio of 1.6:1. After re-electrophoresis, the first peak demostrated peptic activity only at pH 1.4–2.4, with no activity at pH 3.5. Prolonged electrophoresis in the Tiselius apparatus of crystalline hog pepsin showed heterogeneity of this relatively pure material in acetate and phosphate buffers of pH 3.9, 5.9, and 8.0 (H10).

Norpoth *et al.* determined pepsin and cathepsin, by formol titration using human serum as a substrate, in the eluates from electrophoretic strips (N2). Peptic activity was concentrated in the most anodic segment of the partition, corresponding to the first and beginning of the second peak, while cathepsin was present in the area corresponding to the fourth peak. Of the total number of 6 anodic peaks, 2, 5, and 6 were free of enzymes. In gastric juices with histamine-fast anacidity, the first anodic peak was absent and no pepsin could be eluted. Heinrich (H3), could not, however, separate by paper electrophoresis, the proteases acting on edestin at pH 1.8 and 3.2.

Presence of proteolytic enzymes active at pH 1.5 and 3.5 in the anodic bands of the paper electropherograms was also directly demonstrated with elution techniques in our laboratory (G3, G21). Proteolytic activities, at both pH 1.5 and 3.5, were localized in the area of the first, most rapid band of the electropherograms of acid-pepsin-containing gastric juice run in pH 4.5 buffer. In addition, catheptic activity (at pH 3.5, without activity at pH 1.5) was detected at the application point of gastric juice, when subjected to paper electrophoresis in acetate buffer of pH 4.5 (Fig. 22). This corroborated and extended Norpoth's findings (N2), as well as the observations of Tang *et al.* (T2–T5a) regarding gastricsin.

Using vertical paper electrophoresis with acetate buffer of pH 4.5, we found peptic activity at pH 1.8 exclusively in the eluates from the most anodic band of the electropherograms of human gastric juice (G3, G21). We were also able to correlate grossly the results of electrophoretic quantitation of pepsin in borate buffer of pH 9.0 by integration of the leading anodic peak (G3, G21) with the units of the proteolytic activity of the same native juices at pH 1.5, as determined by the hemoglobin digestion method.

We determined pepsin, comparing the results of these 2 methods in 165 lyophilized human gastric juices (Fig. 23). The data show reasonably adequate correlation, although occasionally significant discrepancies were noted in some gastric juices with histamine-fast anacidity. This discrepancy was interpreted as evidence of the irreversible inactivation of pepsin in the gastric juice at pH 7.0, which therefore escaped enzymatic assay but still showed as a pepsin band on paper electrophoresis. It is possible that, in these cases, pepsinogen was activated to pepsin at low pH but later, owing to secretion of alkaline mucus or the reflux of duodenal content, the pH of gastric content was raised to 7. Further cause of discrepancy may be the presence of proteolytic enzyme in gastric juice having electrophoretic mobility identical with that of pepsin,

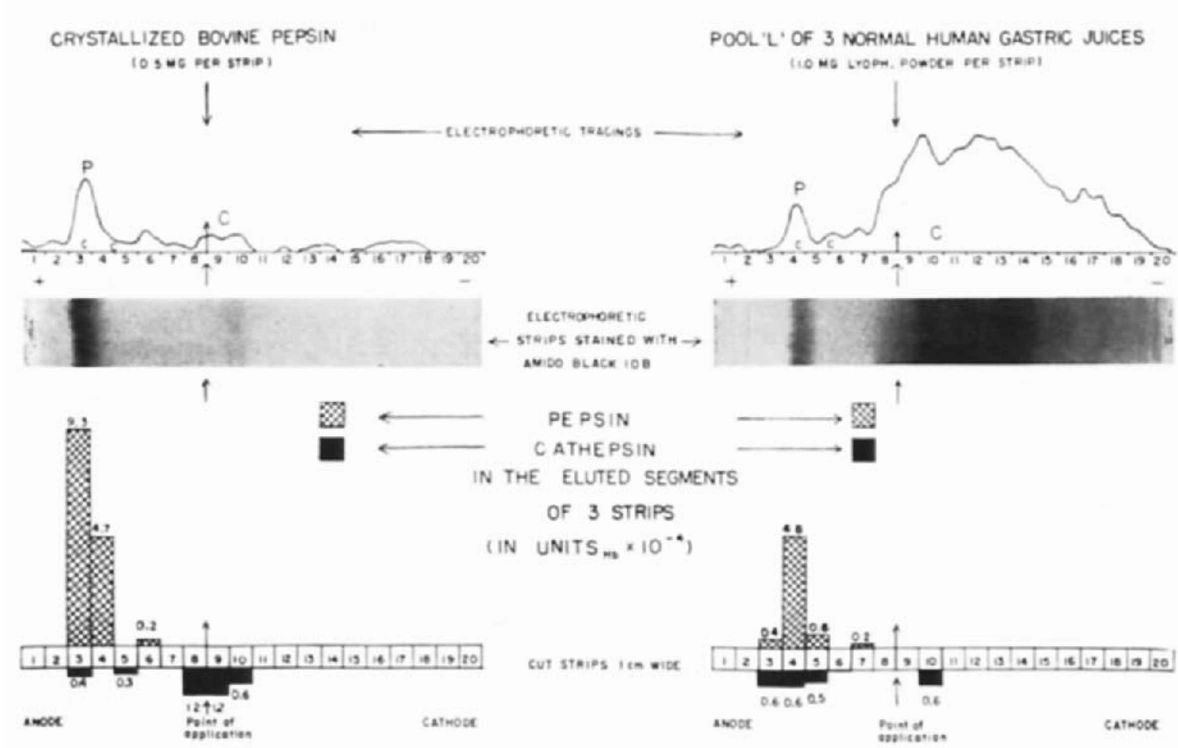

FIG. 22. Elution of proteases from paper electropherograms of gastric juices and crystalline pepsin. Acetate buffer, pH 4.5, Γ/0.1, 0.4 mA/cm, 120 V, 5½ hours. From Glass (G3).

but having different optimum of proteolytic activity. Since some protease, active at pH 3.5, has the same electrophoretic mobility as pepsin, and may be eluted from the leading pepsin peak (G3, G21, T6), this may add to the difference in the results of both assays.

The localization of the pepsin peak in paper electropherograms in acid gastric juice is not difficult. With our technique (G3) the pepsin band in native or dialyzed and lyophilized acid gastric juice travels on paper 5–5.5 cm from the application point within 5½ hours, whereas the

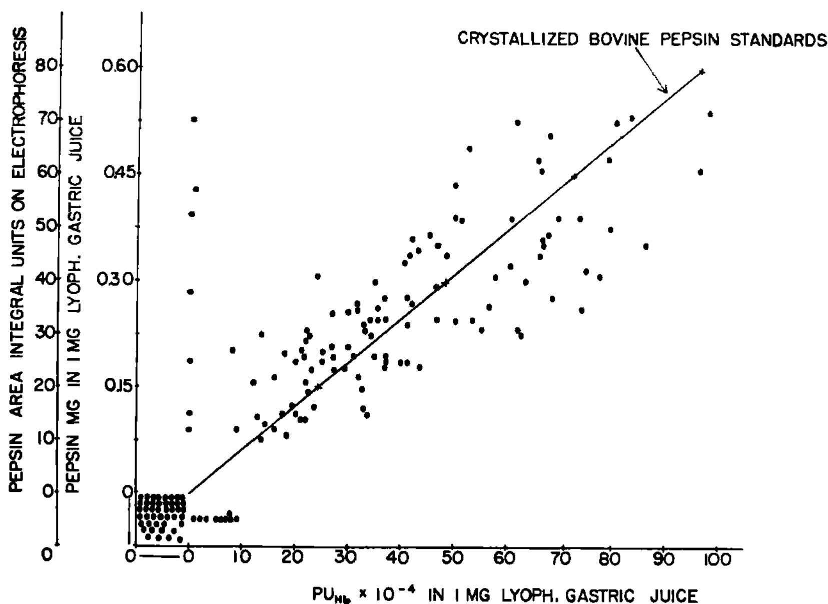

FIG. 23. Quantitation of pepsin by paper electrophoresis and hemoglobin digestion in 165 lyophilized gastric juices of humans. From Glass (G3).

next peak, M1, has an electrophoretic mobility of 4. The albumin peak in the anacid gastric juice is localized only 3–3.5 cm from the application point toward the anode, while the degradation products of albumin, subject to peptic digestion at a low pH, have lower electrophoretic mobility than the native albumin, as shown by Ohara in our laboratory (G3). This permits their differentiation. However, when albumin is digested by gastric proteases at pH 3.0–3.5, its first degradation product has electrophoretic mobility somewhat higher than that of native albumin; this results in its spreading to the area localized about 4 cm from the origin.

In the anacid gastric juice, however, which contains nondigested serum albumin but may still contain some inactivated pepsin, the latter forms an aggregate with albumin and differs from it only very slightly in electrophoretic mobility (G3). When pepsin is added to crystalline serum albumin in alkaline buffer, which prevents the albumin degradation, and both are subjected to electrophoresis, pepsin forms a complex with albumin, showing only as a spearhead of the albumin band and travels about 4 cm from the origin. This may be a cause of error in quantitation of pepsin by electrophoresis in anacid gastric juice containing serum albumin (see Fig. 6).

Piper *et al.* (P3) neutralized gastric juice *in vivo* with bicarbonate, and reported their most rapid and prominent band on cellulose acetate strips to have the electrophoretic mobility of serum albumin. These authors considered, therefore, that the most rapid band in the electropherogram of gastric juice is formed by albumin, and not pepsin. In this they followed the view of Henning *et al.* (H5a), who applied the serum protein nomenclature to the electropherogram of *in vitro* neutralized gastric juice and referred to the most rapid band as serum albumin. It is certainly correct that the first anodic peak in anacid gastric juice represents albumin. However, this holds true only for the anacid gastric juice or the acid gastric juice neutralized intragastrically to a pH above 7.0. In the acid gastric juice containing pepsin, as well as in the *in vitro* neutralized gastric juice (to pH 7.0), the first peak having higher negative charge than albumin corresponds to pepsin. This is demonstrated by the enzymatic assay of the eluates from the first anodic band of the strip (G3). Furthermore, in some *in vivo* neutralized gastric juice, a prealbumin peak has been noted by Gullberg and Olhagen (G34), which probably corresponds to the "pepsin spearhead."

The presence of the highest concentration of pepsin on continuous electrophoresis was demonstrated by Wada *et al.* (W8) in the most anodic area of the electropherogram, while the minor peak of proteolytic activity had a less negative anodic charge (Fig. 10). In the anacid gastric juice, the proteolytic activity was localized more toward the middle of the partition, probably due to the presence of nonactivated pepsinogen in this area, having slower anodic mobility.

Using starch block electrophoresis, Gräsbeck (G27) and Horowitz and Hollander (H18) found, on elution, activity in the most anodic segment of the electropherogram of the gastric juice. Similarly Jeffries *et al.* (J4), using starch gel electrophoresis, found pepsin in the most anodic segment of the partition.

Hurlimann (H20) detected proteolytic activity in the agar gel electro-

pherograms of 26 gastric juices by the Uriel method. In addition, another zone of proteolysis was found to have less rapid anodic migration. No peptic activity could be demonstrated on immunoelectrophoresis in the precipitation lines themselves.

Similarly, Hirsch-Marie and Burtin (H7) eluted proteolytic enzymes active at pH 2 and 3.5 from the first two bands of agar gel electropherograms of normal gastric juice, which were collected after intragastric neutralization with borate buffer. The two bands from which proteolytic activity at pH 7.0 and 3.5 was eluted preceded the third band, which was identified with serum albumin by immunoelectrophoresis.

Further proof that the first main anodic band in acid juice corresponds to pepsin was supplied by Simons and Gräsbeck (S9). Human pepsin, purified by fractionation on DEAE-cellulose, gave a precipitation arc with rabbit antiserum to human gastric juice, which corresponds exactly to the localization of the major fast precipitation arc observed in normal acid gastric juice, immediately ahead of the albumin precipitation arc (Fig. 24). This is at variance with the earlier findings of Hirsch-Marie and Burtin (H7) and Hurlimann (H20), who were unable to find precipitation arcs against pepsin on immunoelectrophoresis of gastric juice that would correspond to the area where the proteolytic activity was detected on agar gel electrophoresis. The probable explanation of these differences is that the titer of the antibodies, in the work of the latter authors, must have been relatively low as compared to that of Gräsbeck. This may be substantiated by a far larger dose of immunizing injections in the latter's schedule. Since the antibodies to pepsin are produced at a low titer only (see G5), a very potent antiserum to pepsin is necessary to obtain arcs of precipitation with pepsin antigen.

The recent work of Grabar's group (H7a, R1a) has proven the correctness of this interpretation:

Rapp and Burtin (R1a) studied the antigenic constituents and enzymatic activity of gastric mucosal extracts by immunoelectrophoresis at pH 8 on agar gel. They found 9 gastric antigens and 4 zones of proteolytic activity. After activation at low pH, these formed active enzymes, of which 3 had antigenic characteristics. Two of these proteases were also discovered in the human plasma.

Hirsch-Marie and Burtin (H7a) determined proteolytic activity of human gastric juice after histamine by electrophoresis on agar, using, as a substrate, human or bovine serum albumin at 0.2% concentration in a glycine-HCl buffer of 0.2 molarity and pH 2. Acid gastric juices with pH below 5.8 showed 4 proteolytic constituents on agar gel electrophoresis. These corresponded in their pH optimum of activity and in immuno-

chemical characteristics to that of the proteases obtained by acidification of the proenzymes from the human gastric mucosa. Alkalinization to pH 8 only decreased, but did not abolish the proteolytic activity of gastric juice. The second, third, and fourth proteases formed precipitation lines with the antiserum to gastric juice and rabbit antiserum to gastric mucosa.

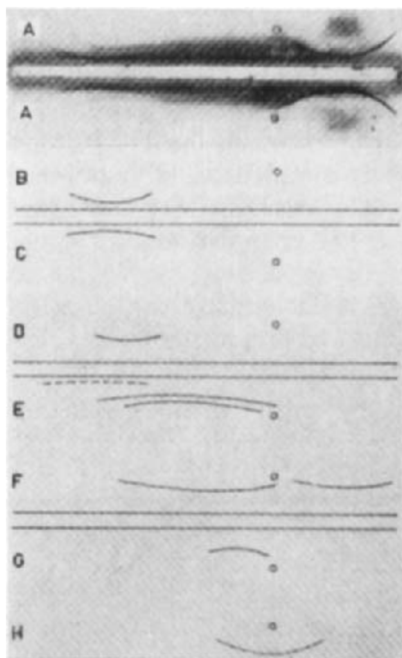

FIG. 24. Immunoelectrophoresis of human gastric juice. Immunoelectrophoretic patterns: A, *in vitro* depepsinized normal gastric juice; B, crystalline human pepsin; C, crystalline human gastricsin; D, human serum albumin; E, glandular mucoprotein; F, mucoproteose; G, rapid vitamin B_{12} binder; H, slow vitamin B_{12} binder. Anti-gastric juice immune serum was used throughout. From Simons and Gräsbeck (S9).

In subsequent work, Rapp and Burtin (R1a) studied the gastric mucosa extract from 30 controls and 7 cancerous stomachs by immunoelectrophoresis. In the extracts of cancerous stomachs, the first 2 anodic fractions disappeared, fractions 3, 6, and 7 decreased, while the most centrally located fraction (4) was increased. Three out of five zones of carboxylic esterase activity disappeared in cancer juices, while all of the 4 zones of proteolytic activity were either absent or markedly decreased. The adjacent gastric mucosa, not revealing the carcinomatous infiltration, showed similar abnormalities.

The difficulties and controversies encountered in the localization of pepsin on electrophoresis are due to its forming aggregates with adjacent proteins of the gastric juice, which obviously changes their electrophoretic mobility. This was manifested in results of the chemical fractionation of gastric juice in the laboratory of Babkin and Komarov (see G5) and our own. It was best demonstrated by the polydispersity of pepsin on free boundary electrophoresis (G32). This has been corroborated also by chemical analysis of glandular mucoprotein in our laboratory [see companion review (G5)]. Also DeGraef (D1) found that his mucoprotein fraction (MPV), which was identical with glandular mucoprotein, contained both pepsin and carbohydrate. Furthermore, the *in vivo* neutralization of gastric juice to pH 9–10 may cause alteration of the pepsin-protein and result in change of its electrophoretic mobility. With pH 7.0–8.0, the inactivation of pepsin is probably not complete (G27). Some part of the proteolytic activity can be reactivated, and agar (H7) and paper curtain electrophoresis (W4, W8), at an alkaline pH, would still detect proteolytic activity in some of the fast anodic fractions. However, the intragastric alkalinization of gastric juice with bicarbonate (P3) may raise the pH so high that its mobility is altered and the enzymatic activity lost as well.

1.9.2. *Fractionation of "Dissolved Mucin"*

Pugh *et al.* (P7) and Mack *et al.* (M1a, M2), using free boundary electrophoresis, showed that "mucoprotein" and "mucoproteose" fractions of the "dissolved mucin" (G5) had different electrophoretic mobilities: mucoprotein fast anodic mobility ($5.5–6.9 \times 10^{-5}$ cm^2 sec^{-1} volts^{-1}), mucoproteose slow anodic mobility ($0.5–1.0 \times 10^{-5}$ cm^2 sec^{-1} volts^{-1}) in veronal buffer of pH 9.2 (Fig. 1). Soluble mucus had intermediate mobility of -3.5×10^{-5}. It was also noted by Mack *et al.* (M1) that the mucoprotein fraction processed from the acid human gastric juice, when run by itself on Tiselius electrophoresis or when added to acid gastric juice, did not have as fast mobility as the fastest anodic component of the gastric juice, which had a mobility of $7.4–7.5 \times 10^{-5}$ cm^2 sec^{-1} volts^{-1} and which probably, as we know now, corresponded to the complex of pepsin and mucoprotein (see G5).

Norpoth *et al.* (N3) compared electrophoretic patterns of mucoprotein and mucoproteose with those of various other fractions precipitated from the gastric juice. When gastric juice was precipitated by Martin's method (see G5) the "gastroglobulin," which is similar to dissolved mucin, showed two peaks which had electrophoretic localization of the glandular mucoprotein and mucoproteose fractions, respectively. Collodion filtration of

gastric juice by Kutzim's technique gave 2 similar components. Two similar fractions were obtained by precipitation of gastric juice with the use of acetic acid, methyl alcohol or absolute ethanol (N2). Norpoth's results, obtained with horizontal paper electrophoresis, were similar to those recorded with the Tiselius method (M2, P7), i.e., the fast component of dissolved mucin was the glandular mucoprotein fraction and the slow component the mucoproteose.

Bélanger and Crevier (B4, C7, C8) injected rats with sulfate-S^{35} and submitted the gastric juice to electrophoresis. On autoradiography of the gastric juice electropherograms, radioactivity was found in the area corresponding to that of the glandular mucoprotein fraction. They concluded that this material contains sulfates and belongs therefore to the sulfated aminopolysaccharides. In light of more recent information available on the heterogeneity of glandular mucoprotein, these sulfated aminopolysaccharides are probably derived from the germinative (transient) region of the surface epithelium in the depth of the crypts or from the pyloric glands [see references B12, G3a, M54 in companion review (G5) in this volume].

The protein components of the glandular mucoprotein fraction, precipitated from acid gastric juice and submitted to vertical paper electrophoresis in borate buffer of pH 9.0 in our laboratory (G3, G19), had the electrophoretic mobility of bands M1 and M2 when stained with amido black 10B. On PAS staining, carbohydrate material was detected in variable concentration and was localized in the area of bands M1, M2, and also slightly more centrally. On the other hand, the mucoproteose fraction consisted of 2 major protein components having the electrophoretic mobility of bands M3 and M4, as well as of a large carbohydrate moiety which occupied the area of component M3b extending from there slightly more toward the anode into the area of M3a. Furthermore, a small carbohydrate moiety was detected in the cathodic area of M4 (Fig. 25). The as yet unpublished work of De Graef (D1a) shows similar differences in electrophoretic pattern of proteins and carbohydrates in these fractions.

Wada *et al.* (W7) studied mucoprotein and mucoproteose fractions by horizontal paper electrophoresis in veronal buffer of pH 8.6 and in acetate buffer of pH 4.5. They processed these materials from gastric juices of normals, patients with histamine-fast anacidity, and those with gastric cancer, which were collected after insulin stimulation. They also subjected the trichloroacetic acid precipitate of gastric juice to electrophoresis, as well as the supernatant fraction remaining after acetone precipitation of the trichloroacetic filtrate of the gastric juice.

The mucoprotein fraction from the normal acidic gastric juice on horizontal electrophoresis had the mobility of the first anodic peak, B1, of the gastric juice (W7). It contained very little carbohydrate. Whereas only one large band was observed in veronal buffer of pH 8.6, three small bands were detected when mucoprotein was submitted to electrophoresis

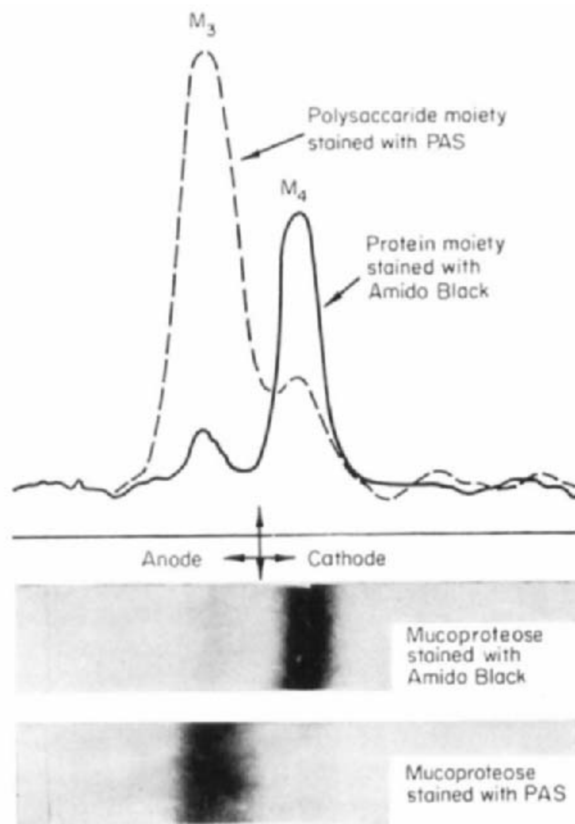

FIG. 25. Paper electropherograms of gastric mucoproteose fraction. From Glass (G3).

in acetate buffer of pH 4.5. The mucoprotein fraction from patients with histamine-fast anacidity differed in electrophoretic pattern from that obtained from acid gastric juice, in that it demonstrated two peaks of B1 and B2 mobility in both veronal and acetate buffers. In patients with gastric cancer, some low, poorly differentiated, and rather wide bands were observed instead.

The mucoproteose fraction, processed from gastric juice of normal

controls, consisted of one slow, single band of B4 mobility in pH 8.6 veronal (W7). It remained at the origin in acetate buffer of pH 4.5. In gastric juice from patients with histamine-fast anacidity, two peaks with mobilities of B3 and B4 were noted in pH 8.6 veronal buffer, and a wide band remaining at the origin in acetate buffer. In gastric carcinoma, a similar pattern was observed, but band B4 was higher than in gastric juice from patients with histamine-fast anacidity. Mucoproteose fractions upon staining for carbohydrates showed a very high content of carbohydrates, especially in patients with gastric cancer (W7). Similar observations with the use of horizontal electrophoresis were reported by Kakei (K2).

The acetone supernatant of the trichloroacetic acid filtrate of acid gastric juices from controls, when run in veronal buffer of pH 8.6, showed bands B3, B4, and some additional small cathodic bands, B5 (W7). In acetate buffer at pH 4.5, at least 3 components moved to the cathode, and peak B4 was especially high. The patterns of this fraction from gastric juice of patients with histamine-fast anacidity and gastric cancer were not distinct. Wada concluded (W1) that the composition of the mucoprotein and mucoproteose fractions processed from acid and anacid gastric juice, as well as that from gastric cancer patients, varies and suggested adjusting of pH prior to precipitation in order to make results more uniform. The reasons for these variations are discussed in the section on mucosubstances (G5).

Immunoelectrophoretic studies of the mucoprotein and mucoproteose fractions from human gastric juice were performed by Simons and Gräsbeck (S9). Neither mucoprotein nor mucoproteose was homogeneous on immunoelectrophoresis against rabbit antiserum to human gastric juice, and each gave several faint precipitation lines. The excessive extension of some of these lines suggested to the authors molecular alterations during preparation of the fractions. Mucoproteose contained in its immunoelectrophoretic pattern a precipitation line with mobility corresponding to the most cathodic precipitation line, 9, which stained strongly with amido black (peptide?). In addition, mucoproteose gave another prolonged precipitation line which, like γ-globulin, extended from the origin to 2/3 of the anodic portion of the pattern. Mucoprotein, on the other hand, gave 3 anodic precipitation lines, of which one was shared with the mucoproteose. The two other precipitation lines of mucoprotein were parallel to each other and localized at the most anodic portion of the partition in the area where the proteolytic enzymes and serum albumin were found. These results corroborate the previously discussed heterogeneity of both these fractions; they possibly share some

common component, but also are markedly different in immunoelectrophoretic behavior.

1.9.3. *Study of Vitamin B_{12} Binders and Intrinsic Factor*

Latner *et al.* were the first to apply paper electrophoresis to the study of vitamin B_{12} binders in the gastric juice (L2). They concentrated pooled and neutralized gastric juice by ultrafiltration and lyophilization and reconstituted the ultrafiltrate residue with buffer for 150–200-fold concentration. This material was submitted to paper strip electrophoresis in veronal buffer of pH 8.6 or phosphate buffer of pH 6.35, and strips were stained with naphthalene black and traced with the densitometer. Five to 6 components were obtained of which 3 were anodic and 2 or 3 cathodic. The largest component was close to the application point and moved slightly to the cathode. The protein-staining peaks, after elution, were assayed microbiologically for vitamin B_{12}. All protein peaks showed B_{12}-binding capacity, which was probably due to overloading of strips with the material applied. Two components exhibited intrinsic factor activity when tested, upon elution, in patients with pernicious anemia in relapse. Of these, one was localized on the cathodic side and the other on the anodic side of the partition, the latter being the more active of the two and containing either a mucoprotein or mucopolysaccharide. The presence of bound vitamin B_{12} at the anodic side of the electrophoretic partition was corroborated by others with the use of microbiological as well as isotope techniques (G2, G23, S5, U1, U2).

Schilling and Deiss first performed paper electrophoresis of gastric juice to which radioactive vitamin B_{12} had been added (S5). This was done by the conventional technique used for serum, in veronal buffer of pH 8.6 and 0.075 molarity at 4°C for 16 hours. After electrophoresis, papers were cut into segments, and the radioactivity of each was determined and correlated with distribution of proteins, as stained by bromphenol blue. The main B_{12} binder was located on the anodic side of the partition, relatively close to the application point. In 8 experiments performed with 6 different gastric juices, the major anodic B_{12} binder did not coincide with any of the major protein bands in the gastric juice, but was localized between them. The technique used, however, did not permit differentiation between various B_{12} binders present in the gastric juice.

Continuous electrophoresis on paper curtain was applied in our laboratory in fractionating intrinsic factor activity of human gastric juice (G20). In each run, 30–100 mg of dialyzed and lyophilized gastric juice dissolved in borate buffer of pH 9.2 or acetate buffer of pH 4.0 (0.15 and 0.1 ionic

strength, respectively) was fed for 24 hours from a container to a Whatman No. 3MM or No. 4 serrated paper curtain, at 25 mA and 150–250 volts. Thirty-eight fractions derived from 12 electrophoretic fractionations of 7 lyophilized samples of gastric juice were tested for intrinsic factor activity in 45 tests, using the method of hepatic uptake and labeled vitamin B_{12}. Intrinsic factor-active materials were found in two areas of the partition: one having fast anodic mobility and the other localized more centrally. These two areas were usually divided by a zone containing an inert material. The most potent intrinsic factor activity was contained in one human gastric juice fraction active at a dose of 2.2 mg of dry weight. The existence of two areas of intrinsic factor activity in each of the electrophoretic partitions of normal gastric juice was caused, as we presently know, by proteolytic degradation of intrinsic factor.

We repeated these studies a few years later using another paper curtain unit (Spinco), as well as a modified fractionation technique (G3a). The intrinsic factor activity of the acid gastric juice was again distributed in two areas of the electrophoretic partition. The more centrally located material corresponded to native intrinsic factor (IF) and "primary B_{12} binder," whereas the more anodic material represented pepsin-digested IF and the "secondary B_{12} binder."

Our studies in association with Uchino and Schwartz (G2, G23, U1) and Kakei (G13, G14, K4) have shown that there are at least three types of vitamin B_{12} binder in the gastric juice. These can be separated by various physicochemical methods, including paper electrophoresis (G23, K4) and gel filtration (G13, G14). According to our classification (Table 3), there are two IF-related B_{12} binders: the primary B_{12} binder, which is related to native IF, and the secondary B_{12} binder, which is the product of proteolytic degradation of the primary binder (G23, U2) (Fig. 26). Furthermore, a "tertiary" IF-unrelated binder is present in anacid gastric juices (G23, U1, U2), which has electrophoretic mobility intermediate between the primary and secondary binders, and is related to some mucosubstances endowed with B_{12}-binding capacity (Fig. 27). Acid-pepsin digestion destroys the tertiary binder. The primary binder has slower electrophoretic mobility than the secondary one, which has fastest anodic mobility of all B_{12} binders. It is absent or present in only traces in pernicious anemia gastric juice. The secondary binder is less thermolabile, less sensitive to peptic digestion, and has less B_{12} affinity than the primary binder. It may represent that form of IF-related B_{12} binder which carries B_{12} through the gastrointestinal tract, since IF is normally exposed to peptic digestion in the stomach. This binder is present in any normal acid gastric juice and in high concentration in the

hypersecretory stomach (G23, U1, U2). The quantitation of the radioactivity bound by primary B_{12} binder on the electropherogram of the human gastric juice to which labeled vitamin B_{12} was added was suggested by our laboratory (G7) as an *in vitro* test for pernicious anemia.

Gullberg used the intragastric *in situ* neutralization of gastric juice technique to study B_{12} binders of the gastric juice from normals and patients with pernicious anemia by paper electrophoresis (G34). Phos-

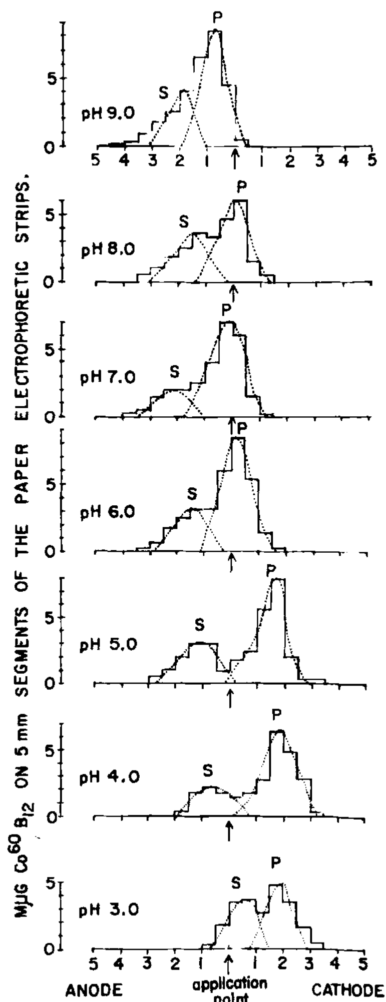

FIG. 26. Primary (P) and secondary (S) B_{12} binders on electrophoresis of normal human gastric juice. From Kakei and Glass (K4).

TABLE 3[a]

SOME CHARACTERISTICS OF B_{12} BINDERS IN HUMAN GASTRIC JUICE

Features	Primary Binder (1)	Secondary Binder (2)	Tertiary Intermediate Binder (3)
1. Nature and relation to intrinsic factor	Native, intrinsic factor-related binder	Proteolytic enzymes digested, intrinsic factor-related binder	Nonrelated to intrinsic factor
2. Derivation	Glandular secretion of the fundus and body of the stomach	Glandular secretion of the fundus and body of the stomach	Saliva? Mucus secretion of surface epithelium and/or pyloric glands?
3. Occurrence	In acid gastric juices and in anacid gastric juices, except for pernicious anemia, where it is absent or in traces	In acid gastric juices only	In anacid gastric juices, including pernicious anemia
4. B_{12}-binding capacity	High	Moderate	Moderate or low
5. Thermolability	Destroyed after 10 minutes of boiling at pH 1.5, and 30 minutes of boiling at pH 7.0 in the absence or presence of B_{12}	Destroyed after 30 minutes of boiling at pH 1.5 in the presence or absence of B_{12}, partly destroyed by 30 minutes of boiling at pH 7.0 in the absence of B_{12}, but little affected by the same in the presence of B_{12}	Only partly destroyed by 30 minutes of boiling at pH 1.5 or 7.0 in the absence or presence of B_{12}
6. Peptic digestion at pH 1.5	Partly destroyed in the absence of B_{12}, but little affected in the presence of B_{12}	Little affected in the absence or presence of B_{12}	Mostly destroyed in the absence of B_{12}, but little affected in the presence of B_{12}
7. Electrophoretic mobility on paper electrophoresis	Slow anodic, in borate buffer of pH 9.0	Fast anodic, in borate buffer of pH 9.0	Intermediate between (1) and (2)

TABLE 3 (*Continued*)

Features	Primary Binder (1)	Secondary Binder (2)	Tertiary Intermediate Binder (3)
8. Correlation with underlying protein and carbohydrate material on electrophoretic strips	None	None	Present
9. Probable correspondence to B_{12} binders detected by other authors with other techniques	"Slow anodic" binders of Gräsbeck—on starch block electrophoresis, Gullberg—on paper electrophoresis, Jeffries *et al.*—on starch gel electrophoresis, Okuda and Gräsbeck, and Simons and Gräsbeck—on agar gel electrophoresis (binder S).	"Fast anodic" binders of Gräsbeck on starch block electrophoresis, and Jeffries *et al.* on starch gel electrophoresis as well as intermediate binder of Simons and Gräsbeck on agar gel electrophoresis (binder I).	"Fast anodic" binder of Gullberg on paper electrophoresis of gastric juice neutralized *in situ*, and "rapid anodic binder" of Okuda and Gräsbeck, and Simons and Gräsbeck on agar gel electrophoresis (binder R).

[a] From Uchino *et al.* (U2).

phate buffer of pH 7.2 was introduced into the stomach before collection. The *in situ* neutralized gastric juice was then concentrated by ultrafiltration through collodion membrane for 24 hours, and subjected to paper electrophoresis in borate buffer of pH 9. Electropherograms were exposed to Kodirex autoradiographic films for 4–7 days. Two vitamin B_{12} binders were found in normal gastric juice, of which the slower one represented the native, IF-related B_{12} binder. Increase of B_{12} binding by the slower

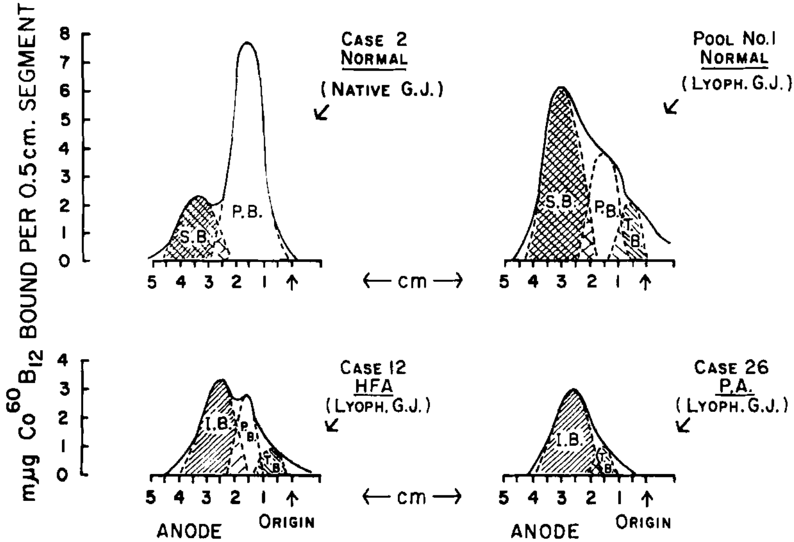

FIG. 27. Vitamin B_{12} binding in gastric juice electropherograms. 50 mμg Co^{60}-vitamin B_{12} added per 1 mg or ml gastric juice; 1 mg of dialyzed and lyophilized gastric juice was applied to each electrophoretic strip. P.B., primary binder; S.B., secondary binder; I.B., intermediate tertiary binder. From Glass *et al.* (G23) and Uchino *et al.* (U1).

binder was noted following cholinergic stimulation with Carbachol (G34), which is in line with the stimulation of IF secretion by vagal stimuli (see G5). The second, fast B_{12} binder of Gullberg bound only relatively small amounts of radioactive B_{12} and, according to our interpretation, corresponded to the IF-unrelated tertiary intermediate binder (G4) (Fig. 28). In gastric juice from patients with pernicious anemia, the slow, IF-related B_{12} binder was invariably absent, while the faster component was always present. Immune rabbit sera containing anti-intrinsic factor antibodies block electrophoretic mobility of intrinsic factor-related B_{12} binder on paper electrophoresis (L3).

On starch block electrophoresis of gastric juice (see Section 5), Gräsbeck (G26, G27) found that the two major protein peaks showed marked

B_{12}-binding ability (Fig. 17). A third minor B_{12}-binding fraction of intermediate electrophoretic mobility was also noted. Of the two B_{12}-binding peaks, the more anodic one was considered by Gräsbeck to be the product of proteolytic autodigestion of gastric juice. Its increase was noted after digestion of gastric juice by pepsin at an acid pH.

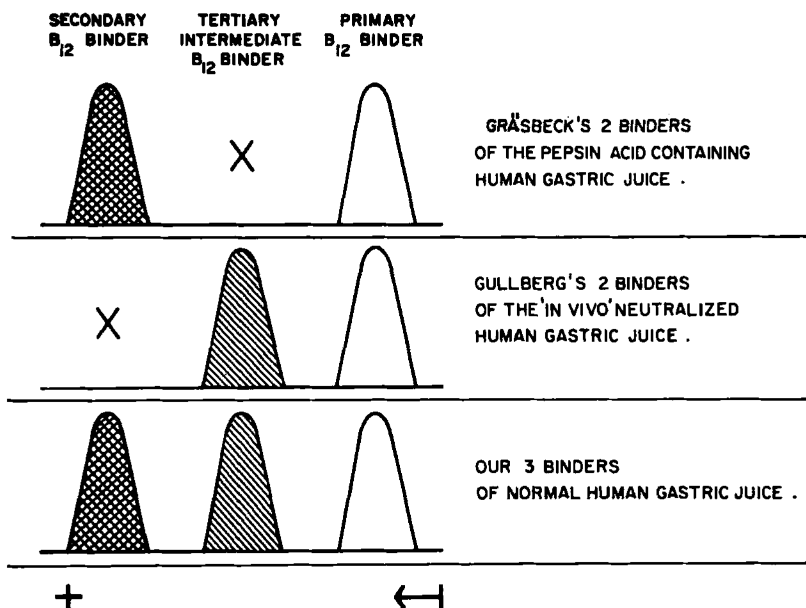

FIG. 28. Electrophoretic localization of three vitamin B_{12} binders in human gastric juice. From Glass, unpublished data.

On starch gel electrophoresis of human gastric juice to which Co^{60}-vitamin B_{12} was added prior to fractionation, Jeffries (J4) found Co^{60}-vitamin B_{12} binders contained in a single broad peak localized 1–2 cm toward the anode from the origin, where the amido black staining was light and protein content relatively low (Fig. 18). When gastric juice was submitted to peptic digestion prior to electrophoresis, the B_{12}-binding material was located more toward the anode, namely, about 3.5 cm from the origin. Intrinsic factor activity was found in both areas of B_{12} binding. Some pernicious anemia sera containing anti-intrinsic factor globulin block the anodic mobility of intrinsic factor-related B_{12} on starch gel (J3, J3a), as they do on paper electrophoresis (L3).

Rapp and Burtin (R1) applied electrophoretic and immunoelectrophoretic techniques to the study of the normal and carcinomatous gastric

mucosa in humans. At least two immunoelectrophoretically distinct vitamin B_{12} binders were found in gastric juice, but were also in the normal gastric mucosa. Electrophoretic localization of the slow binder

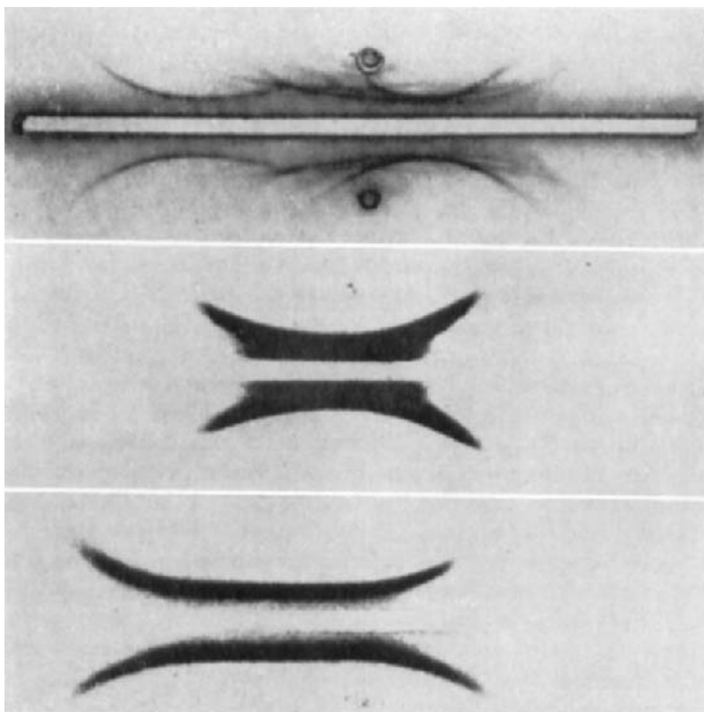

FIG. 29. Autoradiographs of the immunoelectrophoresis of gastric juice. *Top:* Immunoelectrophoresis with rabbit antiserum to neutralized gastric juice. *Middle:* Autoradiography of immunoelectrophoresis with the same serum. *Bottom:* Autoradiography of immunoelectrophoresis with rabbit antiserum to neutralized gastric juice immune serum. From Hurlimann (H20).

coincided with the location of one of the carboxyl exterases and was decreased in the gastric juice from patients with carcinomatous gastric mucosa.

Simons and Gräsbeck (S9), using immunoelectrophoresis, did not detect line 7 in pernicious anemia gastric juice. Since line 7, according to the authors, corresponds to the native intrinsic factor-related B_{12} binder, the absence of this line in pernicious anemia may have significance for the diagnosis of this disease. The "rapid" vitamin B_{12} binder was tentatively identified as line 4, whereas the "slow" (native, intrinsic factor-related) B_{12} binder was localized in the area corresponding to line 7.

Electrophoretic analysis of hog intrinsic factor concentrates has been extensively used for characterization of their composition (C6, E1–E3, G2, G11, G29, G30, H11, J1, W14, W15). The discussion of these findings would exceed the limits of this review and the reader is referred to the author's review on "intrinsic factor" (G4). Hurlimann (H20) studied by autoradiography vitamin B_{12} binders in the gastric juice and confirmed the presence of two binders of different electrophoretic mobility (see Fig. 29).

2. Immunodiffusion

The double immunodiffusion method was first applied by Holman *et al.* (H16) and Gullberg and Olhagen (G34) to detect albumin and γ-globulin in gastric juice. By this method, serum proteins were found in gastric juice from normal individuals and patients with various disease. Stimulation of gastric secretion with histamine diminished the serum protein concentration in gastric juice, which Holman *et al.* (H16) believed to be due to either proteolysis or dilution.

In 3 cases of Barta and Tichy (B3) with protein leakage into the gastric juice, serum proteins were identified by the Ouchterlony method. Hollander and Horowitz (H13), using this diffusion technique in canine gastric juice, demonstrated presence of serum albumin in canine gastric juice.

Heiskell *et al.* (H4) used this method as well as microimmunoelectrophoresis on 175 specimens of lyophilized gastric juices from 58 adult male patients with various gastric disease. Gastric juices, collected under fasting conditions and after histamine and insulin stimulation, were lyophilized and subjected to immunodiffusion against antisera to human albumin, human γ-globulin, and normal whole human serum. Gastric juice of patients with gastric cancer or histamine-fast anacidity with atrophic gastritis gave precipitation reactions with antiserum to normal human serum. Albumin was identified by means of antiserum to albumin in 6 of 9 patients with atrophic gastritis and in 7 of 9 with gastric carcinoma. This technique detected as little as 0.3 mg albumin and as little as 0.1 mg globulin per 100 ml. In many instances, precipitation lines obtained with antisera to normal whole human serum indicated possible presence of α- and β-globulins in gastric juice. However, these could not be identified conclusively by their immunoelectrophoretic characteristics. In duodenal ulcer, only 5 of 17 gastric juices gave precipitation reactions and the albumin and globulin concentration was obviously lower than in anacid specimens. It was evident that serum proteins were altered here by peptic digestion. Similarly, immunological activity of normal

whole serum, after addition of pepsin and HCl, was significantly decreased following as little as 5-minute incubation at 37°C. γ-Globulin was somewhat more resistant to proteolysis, but after 2 hours of digestion faint traces of ceruloplasmin and β-globulins only could still be demonstrated.

In an extension of these studies, Wada *et al.* (W1) reported the incidence of positive precipitation reactions for serum proteins by the Ouchterlony technique to be 77.0% in gastric cancer (26 cases) and 64.5% in chronic gastritis (31 cases). However, this held true in only 15–20% of peptic ulcer cases. In atrophic gastritis, 6–8 precipitation lines were obtained with antisera to human serum and these included intensive lines of albumin and γ-globulin and also faint lines of orosomucoid, α_2-globulin, ceruloplasmin, transferrin, and β-globulin.

Kubo and Dolinski, in the unpublished work from our laboratory, used Ouchterlony immunodiffusion technique to identify the immunoglobulins present in human gastric juice. Using this method, they identified 7 S γ-globulin, and large amounts of β_{2a}-globulin and some β_{2m}-globulin in gastric juices from patients with histamine-fast anacidity.

3. Column Chromatography on Exchange Resins

3.1. Amberlite IRC-50

Fractionation of human gastric juice on ion-exchange columns was pioneered by Caputto (C2) and Richmond *et al.* (R3b) in Wolf's laboratory (W17). Initially, a large preparative Amberlite IRC-50 column (4.5 cm wide and 25 cm tall) was used in chromatographing a large pool of dialyzed and lyophilized gastric juices from 100 healthy human subjects. The column was eluted with citrate and phosphate buffers of increasing pH (3.5–8.0) and fractions collected were analyzed (Fig. 30). The first effluent peak (at pH 3.5) contained most (about 60%) of the hexosamine and hexoses of gastric juice, as well as blood group substance A. This was followed by a large protein peak. Peptic activity appeared only later, in the effluent of pH 3.8–4.3. This was preceded and followed by other larger and smaller protein peaks, a total of about 15. In subsequent work, Richmond and Caputto (R3–R3b) eluted 2 peaks of proteolytic activity from the column in the effluent of pH 3.9 and 4.3. Of these, the first had the characteristics of pepsin, the second those of gastricsin.

In further work of these authors, chromatography on Amberlite IRC-50 XE-64 columns yielded two sharply delineated fractions differing in proteolytic activity curves (R4, R5, T3–T5a). Pepsin activity was eluted at effluent pH of 4.0, gastricsin at effluent pH of 4.4. Proteases eluted

in these two peaks also differed in other properties, which are discussed in the author's companion review in this volume (G5).

Faulkner (personal communication) in the same laboratory used column chromatography for clinical purposes and for fractionation of individual gastric juices. Nondialyzable solids from 50 ml gastric juice were applied to a 1 × 10 cm column and eluted with citrate and phosphate buffers of increasing pH (3.2–8.0). Welsh *et al.* (W12, W12a)

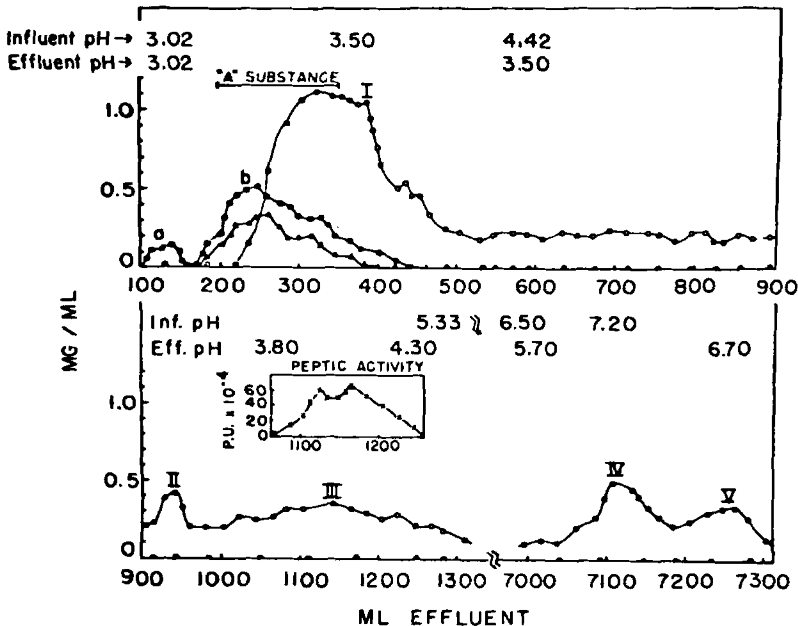

FIG. 30. The pattern of the effluent from the Amberlite IRC-50 resin column of pooled human gastric juice. Ninhydrin material, -○-○-; hexose, -●-●-; hexosamine, -▲-▲-. From Caputto *et al.* (C2).

extended this work and found that, in normal gastric juice, proteins were contained in 6 peaks appearing at well-duplicated elution areas (Fig. 31). Of these, peak 1 was rich in carbohydrates and contained blood group substances A and B; peak 2 contained pepsin, peak 3 gastricsin, and peaks 4 through 7 were of unidentified significance. Hexoses were eluted in peaks 1, 4, and 5. Protein concentration in peak 4 correlated inversely with the acid concentration in the original juice. Chromatograms from normals and patients with duodenal ulcer were similar, while those of pernicious anemia and gastric cancer patients contained low peaks 2 and 3, i.e., low content of pepsin and gastricsin and very high peaks 4,

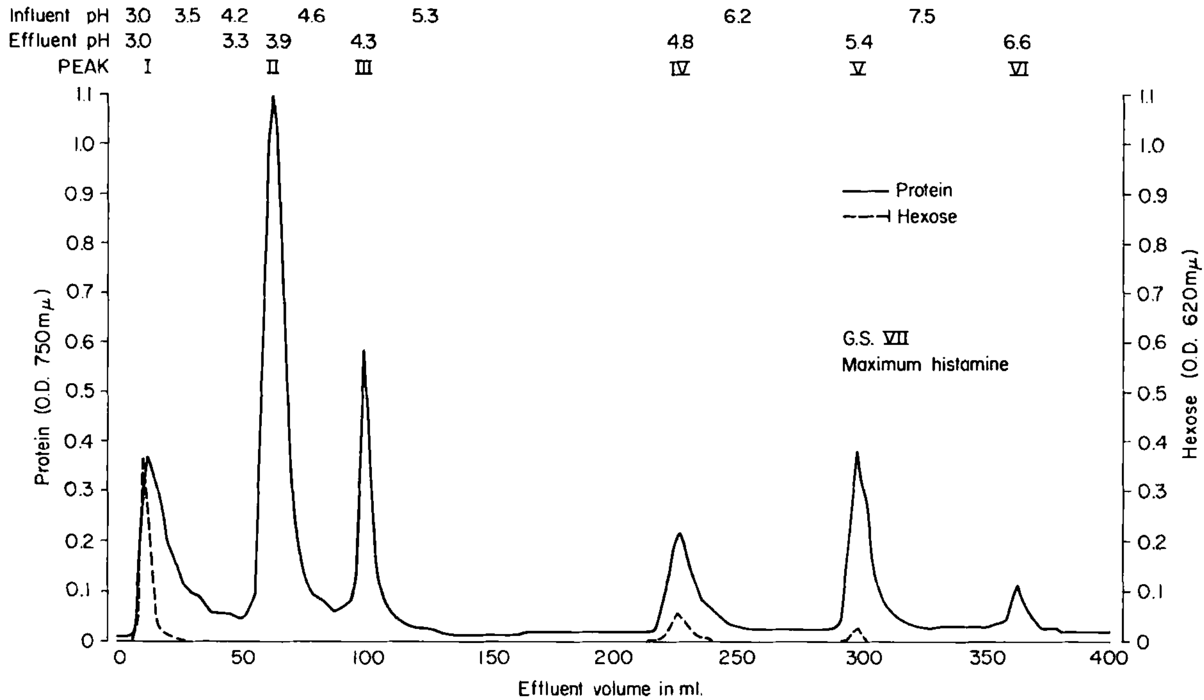

FIG. 31. Chromatography of normal acid gastric juice on Amberlite IRC-50 column. The chromatogram of a post-histamine stimulated acid-containing gastric juice specimen from a normal individual. There are 6 protein peaks; peak II contains pepsin and peak III, gastricsin. Hexoses are always eluted with peak I, and almost always with peaks IV and V. Blood group substances A and B, when present, are eluted in peak I. From Welsh *et al.* (W11).

5, and 6. These authors also chromatographed normal and abnormal gastric juices to which vitamin B_{12} was added prior to chromatography. The first B_{12}-binding peak, which appeared at pH 3.1 of the effluent, was associated with mucopolysaccharides and blood group substance activity. The second B_{12}-binding peak appeared at pH 4.2 and was associated with the second of 2 peaks having proteolytic activity; the third B_{12} binding peak appeared at pH 4.8.

Subsequently, Welsh *et al.* (W11) extended this work and found B_{12} binding in 3 of the protein peaks: 1, 4, and 5 (Fig. 32). Paper electro-

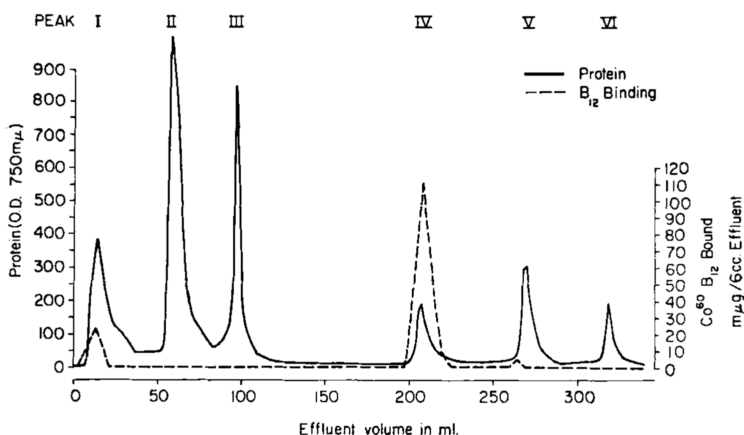

FIG. 32. Chromatography of normal acid gastric juice on Amberlite IRC-50 column. Example of Co^{60}-vitamin B_{12} binding of effluent obtained from column chromatographic separation of an individual gastric juice specimen. The vitamin B_{12} binding is expressed as mμg Co^{60}-vitamin B_{12} bound/6 ml effluent. From Welsh *et al.* (W11).

phoresis of the B_{12}-binding material in peak 1 showed a single, anodic band which corresponded to the tertiary, intrinsic factor-nonrelated B_{12} binder, in the nomenclature of our laboratory (G23). From 55 to 91% of B_{12}-binding material was eluted, however, in peak 4 (at pH 5.2 of the effluent). It contained intrinsic factor, as shown by the urinary excretion test. On paper electrophoresis it yielded two B_{12}-binding bands: a slow one, which corresponded to our native, intrinsic factor-related primary binder, and a fast one which corresponded to the intrinsic factor-related secondary binder (G23, U1), degraded by proteolysis. Thus, the Amberlite IRC-50 chromatography resulted in separation of the IF-related and IF-unrelated B_{12} binders in human gastric juice.

Heatley *et al.* (H2) have used the Amberlite XE-64 column for the

purification of the IF principle from human gastric juice. The elutions were performed with 0.2 M phosphate of pH 4.9–5.5 or Latner's phosphate buffers A and B (L1). Paralleling Latner's results with hog gastric mucosal extracts, they obtained IF-active material from 100 ml human gastric juice in peak 2 of their elutions. Results of other authors concerning the chromatography of hog gastric mucosa on Amberlite resins for the purpose of IF purification are described elsewhere (G4).

In the joint research project with C. F. Code's group, we attempted to fractionate gastric inhibitory activity ("gastrone") on Amberlite IRC-50 column (K8). Chromatography on these columns of 16 gastric juice pools by pH and molarity gradient elution showed the following: (1) no gastrone activity in the effluent of pH 3.2 containing fucomucins and

	POOLS	1	2	3	4	PA
	BLOOD GROUPS	NON-SECR. (A)	NON-SECR. (O)	SECR. (A)	SECR. (AB)	MIXED
HEXOSE		48.5	48.8	37.9	50.4	42.4
HEXOSAMINE		32.3	31.3	47.6	27.5	38.2
FUCOSE		7.4	13.2	7.8	18.7	13.7
URONIC ACID		10.0	5.2	5.4	3.4	4.2
SIALIC ACID		1.8	1.5	1.3	0	1.5
		100.0 →				

FIG. 33. Distribution of carbohydrates in % in peak "A" eluted from Amberlite XE-64 column. Uronic acid readings are not corrected for the hexose color contribution. From Glass (G3b).

blood group substances, (2) inhibitory activity associated with pyrogenicity in pH 4.6 effluent fractions, and (3) definite but inconsistent activity, depending on the gastric juice pool, in effluents of pH 5.2.

In as yet unpublished work from our laboratory, Ibanez (G3b) demonstrated differences in the chemical composition of fractions eluted from the Amberlite IRC-50 column from gastric juice pools of individuals with differing blood group status: Large gastric juice pools having similar secretory status and blood group type were collected, in association with J. A. Buckwalter, and eluted on Amberlite IRC-50 columns. In line with the observations of Richmond *et al.* (R3–R3b), we found that the bulk of the blood group substances is eluted in the first peak from the Amberlite IRC-50 column, with citrate buffers of pH 3.2–3.5. The first peaks containing the bulk of blood group substances were then assayed for fucose, hexoses, hexosamine, uronic acid, sialic acid, and protein content (Fig. 33).

In secretors, the fucose to hexosamine ratio ranged from 0.50 to 0.89 and was highest in the AB secretors, next in A secretors, then in B secretors, and lowest in O secretors. The mean for this ratio, from all secretor pools, was 0.65, while in the ABO(H) nonsecretor groups who had blood group type O, A, or AB, the ratio of fucose to hexosamine was 0.27–0.58, with a mean of only 0.46. This difference was not statistically significant, however, with P value above 0.1. Sialic acid content of the first effluent in nonsecretors was somewhat higher, on the average, than in the secretor group. The significance of these findings is not yet sufficiently documented. The hexuronic acid content of the first gastric juice peak from the Amberlite column in nonsecretors with the blood group type A and AB in the blood was also higher than in the ABO(H) secretors, but the values were not corrected for the hexose color contribution to the reaction.

3.2. Dowex

Ibayashi (I1) analyzed the high molecular constituents of gastric juice by cation-exchange column chromatography on Dowex-50 X8. Elution chromatograms of acid gastric juice from normals and patients with gastric cancer and histamine-fast anacidity were obtained with citrate, phosphate, and borate buffers (0.2 *M*) of pH from 2 to 10. Seven peaks assayed by tyrosine content were obtained from a pool of normal acid gastric juice. Highest peaks were formed by fractions 6 and 7 and then by 1 and 5. Protein-related polarographic activity (double-wave peaks) was high in fraction 1, and low in fractions 3 and 6. Carbohydrates, including hexoses, hexosamine, sialic acid, and hexuronic acid, were eluted in fractions 1, 3, and 5, with highest carbohydrate content in fraction 1; this was eluted at pH about 2. Similar amounts of hexoses were present in fraction 3 (effluent at pH 4.5–5.1), while less hexoses, hexosamine, and hexuronic acid were found in fraction 5.

In patients with gastric cancer and histamine-refractory anacidity there was marked decrease of tyrosine peaks throughout the whole range of effluents, and a marked increase in carbohydrate components, especially hexoses, in fraction 1 (I1). The ratio of hexoses to tyrosine was much greater here than in controls, and hexosamine and hexuronic acid, normally found in peak 5 in chromatograms of patients with gastric cancer, were contained in peak 3 instead. Polarographic activity of the first fraction was higher in these juices than in normal juice. Gastric juices from patients with histamine-fast anacidity had features intermediary between those of controls and those with gastric cancer.

Wada *et al.* (W2) extended this work and studied by this method

other biologically active materials in gastric juice: pepsin, blood group substances, KIK factor, and toxohormone. Using the method of column chromatography on Dowex-50, they found pepsin in the first peak. In patients with gastric carcinoma, pepsin concentration was low, while in patients with noncarcinomatous histamine-fast anacidity, after insulin stimulation, pepsin traces were found. Blood group substance A demonstrated highest activity also in fraction 1, with smaller amounts or traces in fractions 2, 3, and 4. Blood group substance B was found in fraction 5, with lower concentrations in other fractions. In gastric cancer patients there was high activity of both blood group substances A and B in fraction 5. KIK factor was found almost exclusively in fraction 7, while toxohormone activity was present in fraction 1. Thus, complete separation of the two activities was achieved by column chromatography, similar to the aforementioned separation obtained by Masamune *et al.* on continuous paper electrophoresis (see G5).

Vita *et al.* (V2), using highly basic Dowex-1 X2 resin in Cl^- and OH^- form, separated acidic aminopolysaccharide (hexuronic acid content of 35–37%) and neutral mucopolysaccharide (hexosamine content 25–30%) from an aqueous extract of hog gastric mucosa.

3.3. DEAE-Cellulose

Chosy and Schilling (C4) used chromatography on Amberlite CG-50 and DEAE-cellulose to purify intrinsic factor from normal human gastric juice. Pepsin was inactivated in gastric juice by neutralization, radioactive vitamin B_{12} was added, and the dialyzed material was concentrated by ultrafiltration and chromatographed on Amberlite CG-50 resin, with acetate buffer of pH 5.4 and 0.58 molarity. Two peaks containing bound B_{12} were obtained, which were further purified by ultrafiltration and gel filtration on Sephadex G-75 column. The excluded fraction from Sephadex G-75 was concentrated by ultrafiltration and rechromatographed on DEAE-cellulose column with a phosphate buffer of pH 7.5, and a gradient elution of 0.02–0.2 molarity. Two major peaks of optical density were obtained, of which the first contained all bound B_{12} in a single, sharp peak and intrinsic factor (IF) activity.

While the native gastric juice, before fractionation, bound 87 mμg B_{12}/100 mg N, one of their best fractions had a B_{12}-binding capacity of 4100 mμg B_{12}/100 mg N, i.e., about 6.6 mμg/mg protein, assuming that the concentration of N in IF is similar to that in the serum proteins. Their most active fractions exhibited IF activity on urinary excretion test at a dose of about 10 μg N, i.e., at an approximate dose of 60–100 μg protein, which makes them some of the most potent IF materials processed from

human gastric juice, similar in potency to the materials of Gräsbeck *et al.* (G28).

The latter group concentrated gastric juice by ultrafiltration and chromatographed it on CM- and DEAE-cellulose and DEAE-Sephadex. Gräsbeck *et al.* (G28) found B_{12}-binding material in two of their four protein peaks eluted by phosphate-NaCl buffer at pH 6.7–6.2. This B_{12}-binding material showed IF activity in the Schilling test in a pernicious anemia patient at a dose of 1.7 mg protein. (Since these materials probably also contained the carbohydrate moiety, the active dose must have been somewhat higher.) The IF-active B_{12}-binding material was rechromatographed on DEAE-Sephadex and G-200 Sephadex. It split into two peaks, one of which was eluted at 0.07 *M* NaCl concentration and was very active at a dose as low as 10 mg N. This may correspond approximately to doses of 60–100 mg protein, which classifies it as one of the two highest potency IF materials obtained from human sources.

Column chromatography of hog gastric mucosa on DEAE-and CM-cellulose was used by various authors for the purpose of purifying intrinsic factor (E2, E3, H11). They are reviewed elsewhere (G4).

Castro-Curel, in our laboratory (C3a), fractionated intrinsic factor and proteolytic enzymes of human gastric mucosa on DEAE-cellulose column. Extracts from 5 *in situ* neutralized and resected stomachs from duodenal ulcer patients were sequentially precipitated by 20–80% saturation with ammonium sulfate, and the 40–50% saturation fractions, with and without addition of tracer dose Co^{57}-vitamin B_{12}, were eluted with pH 7.0–7.3 phosphate and NaCl of increasing molarity. Fractions were pooled according to ultraviolet absorbance peaks at 280 mμ. The IF activity, assayed on guinea pig intestinal mucosa homogenates (C3), coincided with the main B_{12} binders, determined by staining and radioactivity counting of electropherograms of effluents. On 12 columns, IF emerged in effluents of 0.04–0.14 (mean 0.10) total molarity, showing little ultraviolet absorbance and devoid of proteolytic activity (Fig. 34). Precipitates at 45–50%ammonium sulfate saturation were richer in IF than those at 40% saturation. Proteases active at pH 2.0 and 3.5 emerged only in later effluents at total molarities 0.16–0.36 (mean 0.31) (Fig. 35). The ratio of pepsinogen to activated pepsin was highest in late effluents, of which the latest one contained the most protease active at pH 3.5. Thus elution of extracts from human fundic mucosa, at neutral pH with molarity gradient, permits separation of IF from proteases active at pH 2.0–3.5, and pepsinogen, all devoid of IF activity.

Using Ecteola ion-exchange resin columns with acetate buffer and sodium chloride gradient from 0.06 to 0.22 *M*, Seijffers *et al.* eluted two

pepsin fractions from dialyzed human gastric juice (S7). The two peaks showed differences in pH optima of activity. While peak 1 showed 2 optima at pH 1.6 and 3.0–3.2, the other peak showed only one optimum at the wide range 1.5–2.8. These two peaks also showed differences in the resistance to heat and alkaline inactivation; pepsin from peak 1 was more heat- and alkaline-resistant than that from peak 2.

In further studies, the authors (S7a, S7b) were able to concentrate and identify three distinct pepsinogens from human gastric mucosa by column

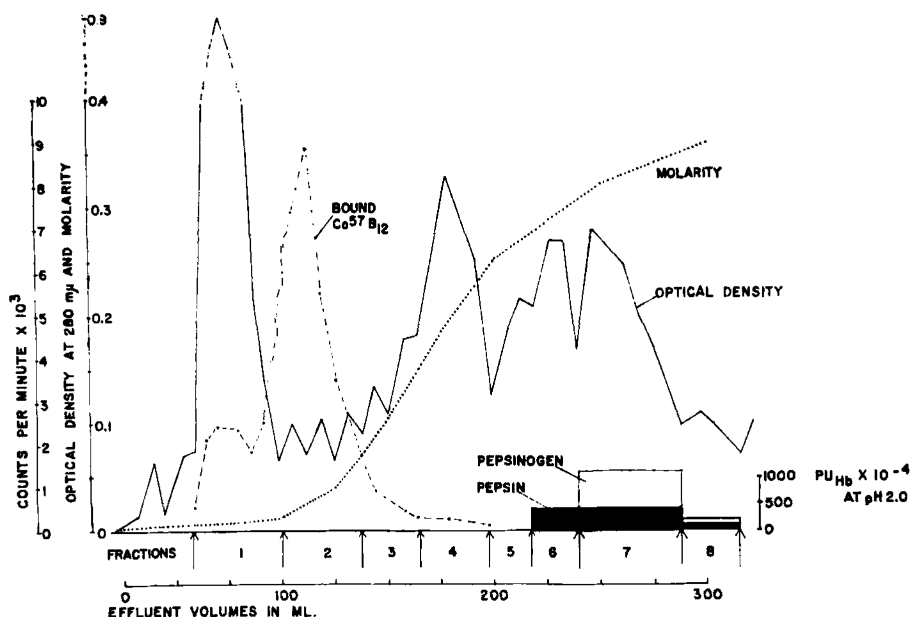

FIG. 34. Chromatography on DEAE-cellulose of human gastric mucosa extract (with Co^{57}-vitamin B_{12} added). From Castro-Curel and Glass, unpublished data.

chromatography on DEAE-cellulose, which they designated as pepsinogens 1, 2, and 3. They inferred that pepsinogens 2 and 3 are secreted only by the fundic glands of the gastric mucosa, while pepsinogen 1 is secreted by the pyloric as well as the fundic mucosa.

In subsequent observations (S7c), the authors noted that acidification of all 3 pepsinogens to pH 2 for 8 minutes, followed by incubation at various pH's, from 1.1 to 7.2, and then alkalinization at pH 7.8, leads to formation of intermediate compounds between pepsinogens and pepsins. These were designated as pepsin-pepsin-inhibitor complexes (HPPI). These complexes have no milk-clotting activity at pH 5.5 and

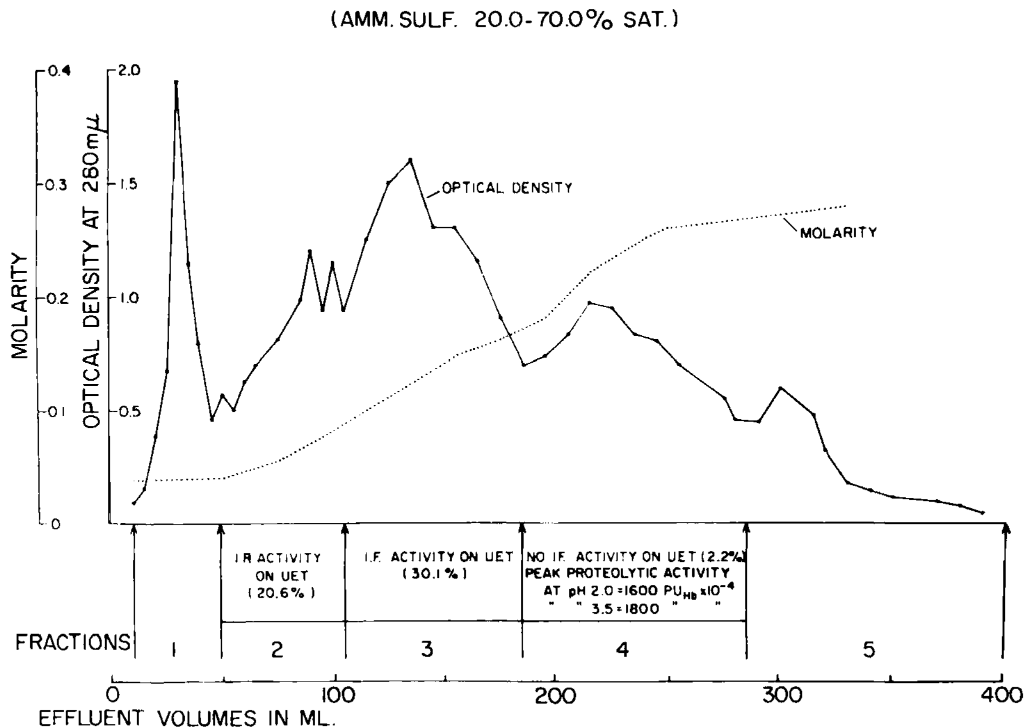

FIG. 35. Chromatography on DEAE-cellulose of human gastric mucosa. From Castro-Curel and Glass, unpublished data.

are stable to alkali at pH 7.8. They were unstable at or above pH 9.2, in which they differed from pepsinogens. Thus, during the conversion of pepsinogen to pepsin, probably some polypeptide is split off, which is then slowly digested by pepsin with a pH optimum of about 4. This digestion is stopped, if the reaction mixture is placed in the ice-cold buffer of pH 7.8, resulting in formation of a combination product of pepsin with the remaining undigested polypeptides.

Kushner *et al.* (K9) reported four electrophoretically distinct constituents of human gastric mucosal extracts displaying proteolytic activity at pH 2. These four constituents were partially or completely separated by an exchange chromatography and each of them demonstrated an increase in electrophoretic mobility following acidification. Three of these pepsinogens were antigenic, and there was an immunological relationship between the unacidified and acidified antigens. One human pepsinogen was immunologically related to purified hog pepsinogen. The human gastric mucosa, according to these authors, contains four physicochemically and immunochemically distinct pepsinogens.

The details of this work are as follows: Kushner *et al.* (K9a) attempted to separate and characterize the immunologically active constituents of normal, human gastric mucosa, precipitated with ammonium sulfate and dialyzed and lyophilized, by chromatography on DEAE cellulose. They used phosphate buffers ranging from 0.005 M at pH 7.0 to 0.4 M at pH 5.3, and obtained 6 chromatographic peaks which, on immunoelectrophoresis, were tested for proteolytic and carboxylic esterase activity. Several antigens were found in the same peak. The most cathodically located pool 6 was rich in nucleic acid, and contained proteases 1, 2, and 3, while pools 4 and 5 contained proteases 2 and 3, and pool 3 only protease 4 and albumin and globulin. The last pool, No. 6, also contained a protease active at pH 7.6, which migrated in the cathodic electrophoretic zone. Protease 2 gave an activity curve characteristic for pepsin, with peak activity at pH 1.8. The material precipitated at 33% ammonium sulfate saturation contained more protease 3 and less protease 2, while that precipitated at 50% ammonium sulfate saturation contained all 4 proteases. These results are, to some extent, related to those reported by Castro-Curel in our laboratory (C3a). The precipitate obtained at 33–40% ammonium sulfate saturation, after fractionation on DEAE cellulose column at a neutral pH, has resulted mostly in the elution of the nonactivated zymogen. Activated zymogen was eluted from the column in larger concentration when the ammonium sulfate precipitate at higher concentration was used (C3a).

4. Paper Chromatography

Caputto *et al.* (C1) applied chromatographic analysis in studying the large molecular components of nonfractionated fasting human gastric juice. They used the ascending technique with the following solvents: 15 and 20% acetone in water, 15–30% alcohol in water, or 20–30% alcohol in 0.5 *M* phosphate buffer of pH 7.2. Chromatograms were developed at 4° or —15°C for 15–68 hours and spots were stained with bromphenol blue. Four to 6 spots were identified in most specimens with R_f values in 35% diluted·alcohol of 0.05, 0.23, 0.70, 0.80, and 0.95, respectively. No spots appeared which would stain with benzidinetrichloroacetic acid for polysaccharides. After chromatography, papers were eluted and peptic activity was localized at the point of origin and in the spot having R_f 0.80. Blood group substance A was found in a wide zone which extended for about 5 cm in width from the point of origin, and also in the spot having R_f 0.80, where protein and peptic activity was found. This method was not promising and was abandoned shortly thereafter.

Brummer and Kulonen (B12) submitted 23 gastric juices (13 acid and 10 anacid) collected before and after histamine stimulation, to amino acid chromatography on paper, after precipitating with 80% alcohol and dissolving the residue in 1% HCl containing acetone. Descending chromatograms were run in butanol-acetic acid or butanol-ammonia as solvent and stained with 0.2% ninhydrin solution in acetone. A purple ninhydrin-staining streak was found, which migrated more rapidly than leucine and which was seen in all gastric juice samples having pH below 3.0. It disappeared after acid hydrolysis and did not react with sulfanilic acid, biuret, and Ehrlich reagents. The compound producing this spot was retained on Dowex-50 columns.

This spot was also found in canine acid gastric juice, but was absent from the juices of patients with achlorhydria. It was likewise found in anacid gastric juice of patients with and without pernicious anemia after acidification (B14).

It was initially assumed that this spot was due to the presence of two or more peptides split from pepsinogen during the process of activation to pepsin. However, when gastric juices were studied by means of paper electrophoresis, Brummer and Seppälä (B13) changed their initial interpretation and accepted the concept that this peptide originated from proteolytic degradation of albumin and mucoproteins. Methionine spots were found principally in anacid juices and declined with increase in acidity. They were not present in chromatograms of canine gastric juice.

The amino acid pattern of the gastric juice was first studied by Gilligan *et al.* (G1), using two-dimensional paper chromatography, then by Kansky [cited in (B10)] and Oh-uti and Awataguchi (O1). Gilligan *et al.* (G1) noted the presence of peptides in dialyzates and ethanol filtrates of gastric juice. They found leucine, isoleucine, alanine, valine, methionine, proline, tyrosine, phenylalanine, glycine, lysine, glutamic and aspartic acids, glutamine, arginine in gastric juice. This group also found large concentrations of aminobutyric acid, while Kansky demonstrated serine and threonine in gastric juice. Higher amounts of amino acids were reported by the latter in gastric juice of patients with gastric cancer. Furthermore, Kiyotat and Sun-ichi (see B10) found proline and threonine in gastric juice of patients with gastric cancer, which were absent from peptic ulcer juices. They also found greater concentrations of valine, serine, alanine, leucine, and glycine in patients with gastric cancer, while peptic ulcer patients had closer to normal amounts. Warren *et al.* (W9) obtained similar results and found higher concentrations of free amino acids in gastric juice of patients with gastric cancer than in that of normals or subjects with peptic ulcer. They noted higher content of nonprotein nitrogen and proteins as well in these juices.

Hiller and Bischof (H6) determined amino acid concentration in 9 of 36 gastric juices studied, and found leucine in highest concentration, followed by glutamic acid, alanine, serine, lysine, threonine, serine, glycine, and valine, and in lowest concentration aspartic acid.

Bouda and Vesely (B10a) detected in the absence of HCl secretion large amounts of amino acids, which disappeared with increasing acid secretion. Neutral and acid amino acids were present in all juices. By means of electrochromatography, they found leucine, phenylalanine, methionine, valine, threonine, tyrosine, serine, glycine, lysine, arginine, and aspartic and glutamic acids. In addition, several other nonidentified spots and bands were obtained which were probably of peptidic character. In patients with gastric atrophy, the total amount of amino acids was high and chromatographic spots were rounded, sharply outlined, and corresponded to pure amino acids in R_f values. Conversely, peptic ulcer juices and those from individuals with high fundic gland activity had less amino acids, and formed, on chromatography, longer streaks of weakly staining peptides. The amino acid-peptide pattern of normal gastric juice did not differ from that of peptic ulcer patients. Makino *et al.* (M2) found an increased number and concentration of amino acids with increasing acidity of gastric juice.

Berg (B5) and Preisser *et al.* (P6a) hydrolyzed centrifuged human gastric juice in concentrated HCl at 100°C or in Permutit RS at 100°C

for 48 hours, removed acid by distillation, subjected the residue to ascending chromatography in butanol-acetic acid-water (4:1:1) and then in butanol-pyridine-water (6:4:3) and stained with aniline phthalate, ninhydrin and *p*-dimethylaminobenzeneacetyl acetone. Chromatograms were also read spectrophotometrically at 436 mμ and planimetrically quantitated with the use of various sugar standards. The authors found the presence of glucosamine, galactosamine, galactose, mannose, and fucose in human gastric juice, the mutual ratio of which varied in various cases, but remained rather unchanged under the effect of histamine stimulation. In gastritis, the concentration of galactose, mannose, and fucose was often increased, while the total content of sugars in gastric atrophy was rather decreased (see comment in Section 1.2.2d).

Schrager (S6) studied human gastric mucopolysaccharides and their secretory patterns under normal and pathological conditions, by means of paper chromatography. Chromatograms of 120 gastric secretions showed no spots of uronic acid on paper chromatograms, but all of them showed zones corresponding in their R_f to glucose and mannose, in addition to normally occurring galactose and fucose. However, in prior work, Werner did not find glucose in human gastric mucopolysaccharides [ref. (W9) in the companion review (G5)]. Since these materials were also found in saliva, the reviewer believes that glucose and mannose are not derived from the gastric secretion, but from the swallowed saliva and/or from the blood serum. The ratio of galactose to glucose was about 10:1–2. In patients with gastric carcinoma, Schrager found a marked decrease in galactose and a great increase in glucose.

5. Gel Filtration on Sephadex Columns

Gel filtration, introduced by Porath and Flodin (F3, P5, P6) to achieve a molecular sieving of materials of different molecular size, was first applied in our laboratory for fractionation of large molecular materials in gastric juice (G3a, G12, G12a) and gastric vitamin B_{12} binders (G13, G14). Kakei (K3) in our laboratory, using Sephadex G-25 columns, obtained good separation of free B_{12} from B_{12} bound to binders of normal human gastric juice. Free B_{12} and B_{12} bound to gastric juice, as separated by gel filtration, differed in dialyzability, electrophoretic mobility, and adsorption to activated charcoal (Fig. 36). Later other authors (C4, G28) also used Sephadex G-25 or G-50 columns for separation of bound from free B_{12} in purification of intrinsic factor.

In order to determine the approximate sequence and range of molecular sizes of the macromolecular materials contained in native gastric juice, as well as to dissociate some naturally forming large molecular

aggregates in the gastric juice, we combined paper electrophoretic analysis with prior gel filtration on Sephadex columns of various porosities (G12, G12a). We found that gel filtration of human gastric juice on Sephadex G-50, G-75, G-100, and G-200 columns, followed by paper electrophoresis, better fractionates nondialyzable components than electrophoresis alone. Additionally, it grades them according to respective molecular sizes. Gastric components excluded from the G-200 columns had the electrophoretic mobility of mucosubstances M3a and M3b (see Section 1.2.2). These demonstrated a large polysaccharide moiety stain-

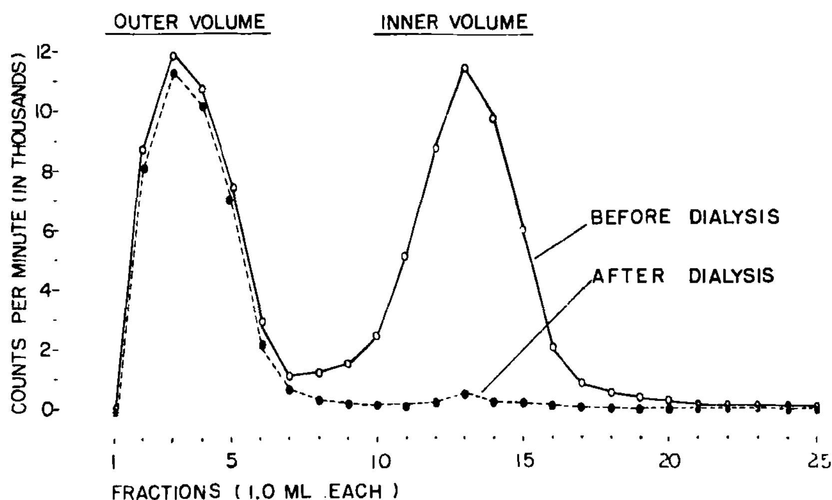

FIG. 36. Fractionation on Sephadex G-25 of Co^{60}-vitamin B_{12} (1.13 μg) + normal lyophilized human gastric juice (10 mg), followed by dialysis of the fractions. From Kakei and Glass (K3).

able with periodic-Schiff stain (Fig. 37). Succeeding in molecular size were materials high in protein and low in carbohydrate moiety and having electrophoretic mobility of γ-globulin, M4-X1, mucosubstance M2, and serum albumin. Their molecular weight was above 100,000. Following in succession were materials excluded from the G-75, but retarded on G-100 and G-200 columns. They had an approximate molecular weight of at least 30,000–40,000 and included carbohydrate-rich mucosubstance M4 from anacid gastric juice and pepsin. Next in size were materials excluded from G-25, but included into Sephadex G-50 (Fig. 38), which, in sequential order of appearance, consisted of components Y1, Y2, and PP, representing peptides of molecular weight above 4,000. Finally, the most retarded materials on Sephadex G-25 were components X2 and Z

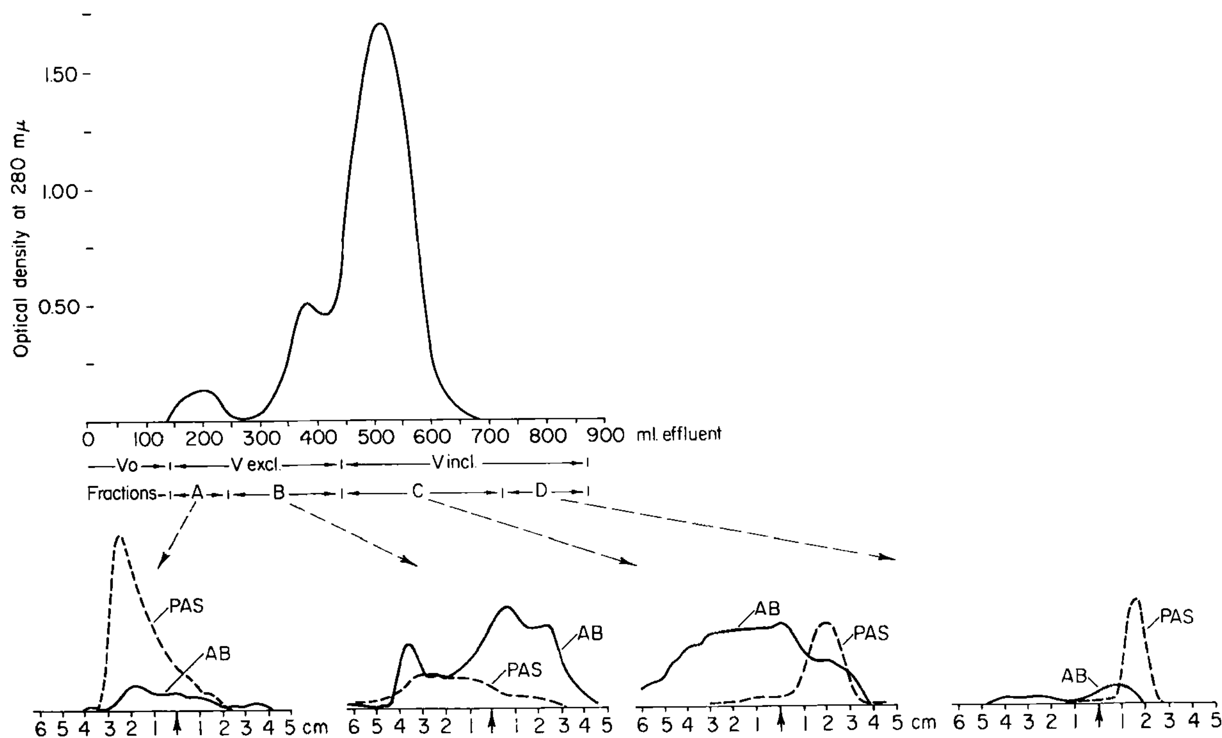

FIG. 37. Gel filtration diagram on Sephadex G-200 column is shown in the upper part of this figure. The lower part shows paper electrophoretic pattern stained with PAS and amido black (AB) of 4 main fractions A, B, C, and D eluted from the column. From Glass *et al.* (G12a).

(peptides of smallest molecular sizes?). In addition, gel filtration on Sephadex caused disaggregation of carbohydrate-protein complexes, such as detachment of uncharged (neutral) nondialyzable, carbohydrate-staining material from mucosubstance M3b (G12a).

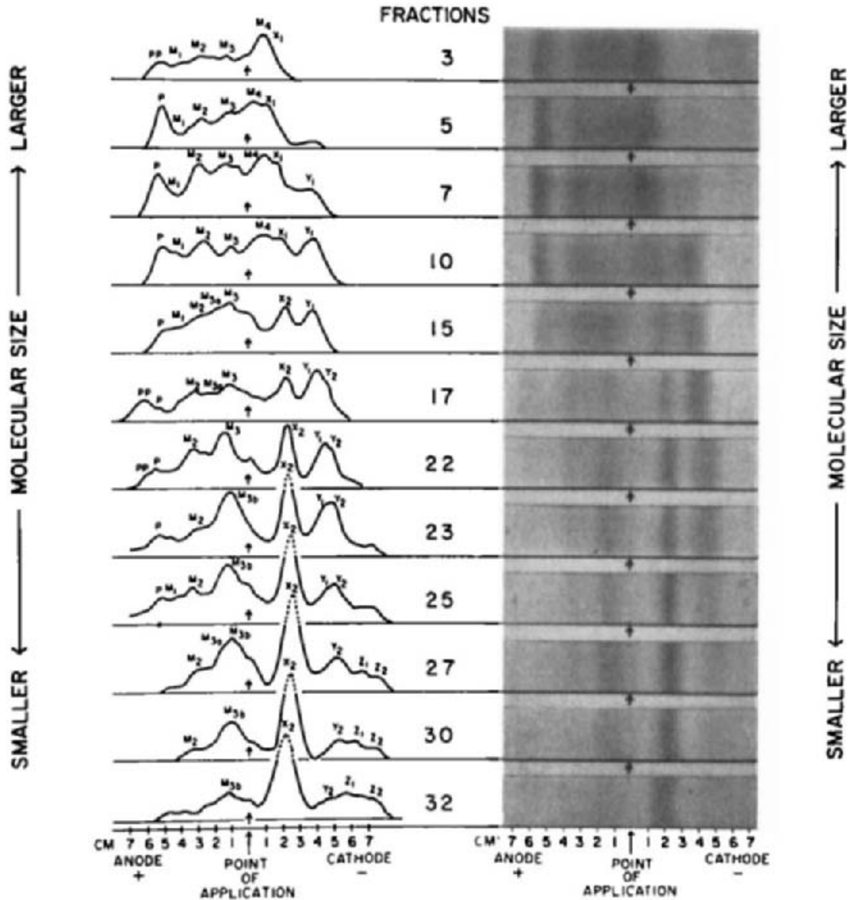

Fig. 38. Paper electrophoretic patterns of fractions obtained by gel filtration on Sephadex G-50 from a pool of normal human gastric juices. From Glass (G3a).

Kakei and Kubo (G13, G14) in our laboratory further demonstrated that vitamin B_{12} binders from the gastric juice were eluted into the excluded volume of Sephadex G-100 and G-200 columns. Bound radioactive B_{12} was excluded from the gels and formed one large symmetrical peak, appearing between the first and second peaks of proteins (Fig. 39).

Electrophoretic analysis of these fractions revealed that radioactivity corresponded to the area of the electropherogram in which the primary and secondary intrinsic factor-related B_{12} binders were localized (see Section 1.9.3). Administration of material containing bound radioactive vitamin B_{12} to patients with pernicious anemia in remission demonstrated its high intrinsic factor activity on urinary excretion test (Table 4). This indicates that intrinsic factor in gastric juice and intrinsic factor-related vitamin B_{12} binders either have a molecular weight above 200,000 or,

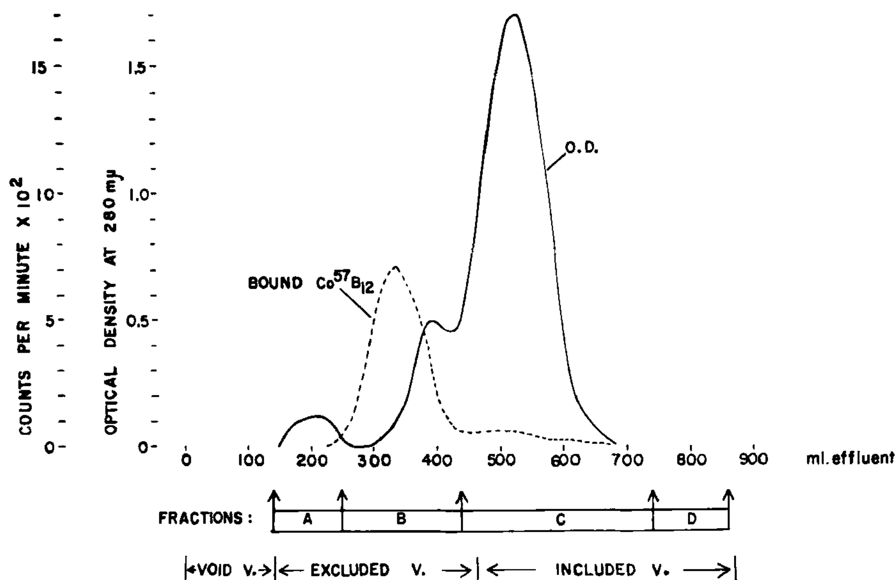

FIG. 39. Gel filtration on Sephadex G-200 of Co^{57}-vitamin B_{12} bound to normal human gastric juice. From Glass *et al.*, in preparation.

more probably, form a complex with large molecular gastric mucosubstances of this size.

Sephadex columns were also used by Chosy and Schilling (C4) and Gräsbeck *et al.* (G28) for purification of intrinsic factor from human gastric juice. The gel filtration raised the B_{12}-binding activity of the purified product several times over the activity of the starting material (see Section 3.3).

In a joint study with C. F. Code, we used gel filtration for fractionation of gastrone (K8, see G5). Gel filtration on Sephadex of various grades demonstrated highest inhibitory activity in fractions excluded from the G-75 column, indicating that the main inhibitor has a molecular weight

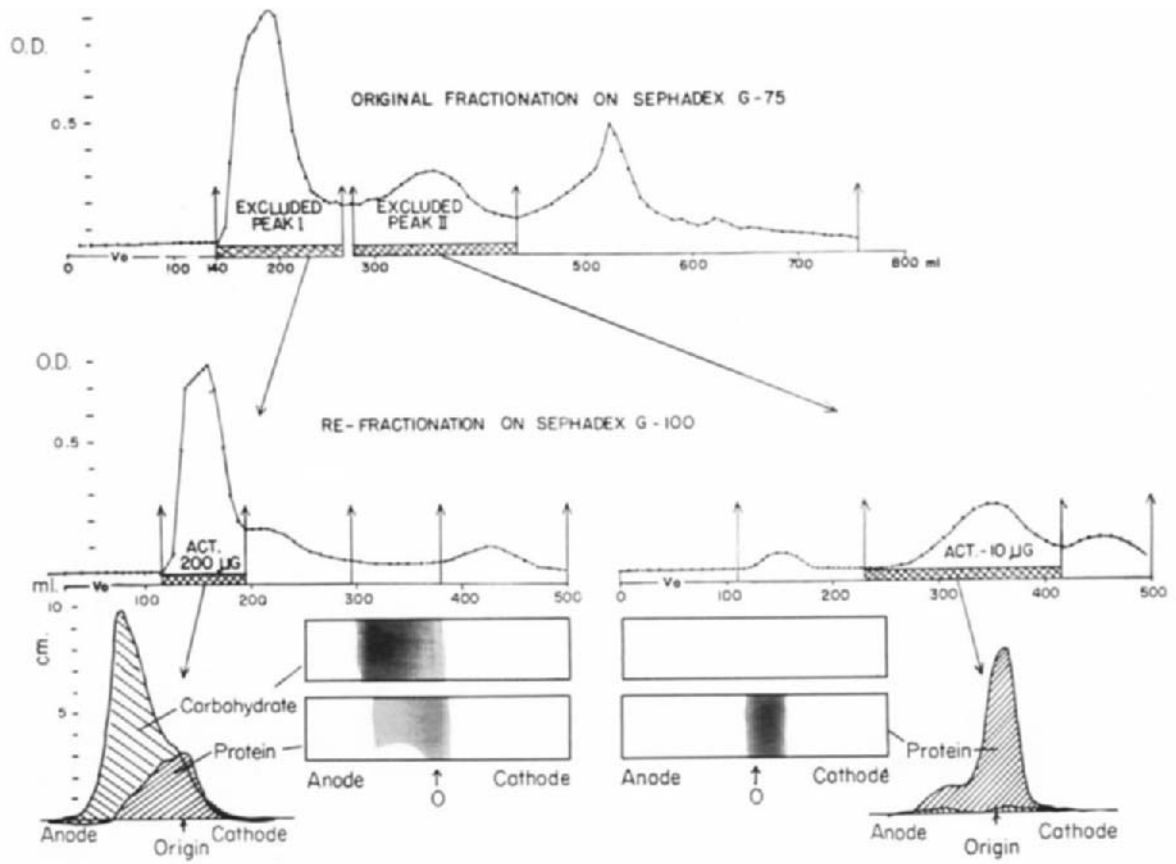

Fig. 40. Fractionation of a pool of anacid gastric juices by consecutive gel filtration on Sephadex G-75 and G-100. The "gastrone activity" (Act.) of fractions is expressed in micrograms of minimal dose, which causes 50% inhibition of gastric secretion on Shay rats. From Glass *et al.*. in preparation.

of over 40,000. Material eluted in this way from the anacid gastric juice pool, with the consecutive use of G-75 and G-100 columns, showed potent inhibitory activity on pylorus-ligated rats at a dose of 10–50 μg. On paper electrophoretic analysis it demonstrated mobility and staining properties of serum γ-globulin, which on immunodiffusion and immuno-

TABLE 4
INTRINSIC FACTOR ACTIVITY OF HUMAN GASTRIC JUICE FRACTIONS ELUTED ON SEPHADEX COLUMNS AND MEASURED BY THE URINARY EXCRETION TEST

Sephadex columns	Pooled normal human gastric juice in ml on the column	Urinary excretion of radioactivity in % of the oral dose[a]: Leading fractions excluded from the gel	Late fractions included in the gel
G-75	80	17.6	1.4
G-100	70	17.9	2.4
		16.3	
G-200	77[b]	2.1	1.7
	80	10.8	
	80	13.6	

[a] All these tests were performed with a 2 μg oral dose of Co^{57}-vitamin B_{12}, 48-hour urine collections and 2 injections of "cold" B_{12} at 24-hour intervals.
[b] Stored for 2 weeks in the refrigerator without neutralization.

electrophoresis was identified as β_{2a}- and 7 S γ-globulins (unpublished work with Kubo and Dolinski) (Fig. 40). Lower gastrone activity appeared in other fractions retarded on Sephadex columns.

6. Ultracentrifugation

Ultracentrifugation was used in the study of large molecular materials of gastric secretion for isolation and identification of intrinsic factor. It was here used in 1954 by Williams *et al.* (W14, W15) as one of the steps in fractionating hog gastric mucosa for processing intrinsic factor preparations. Ultracentrifugation, following fractional ammonium sulfate precipitation yielded several components. However, no pure materials could be obtained.

Jacob *et al.* (J1) used 4-step ammonium sulfate fractionation of hog gastric mucosal extracts, followed by ultracentrifugation, to obtain high-potency intrinsic factor-active materials (Table 5). The intrinsic factor-containing fraction precipitated between 39 and 47% ammonium sulfate saturation was active at a 1-mg level. About 90% sedimented with a constant of 1.78–1.82 S. Other heavier components formed only about

TABLE 5[a]
CHARACTERIZATION OF INTRINSIC FACTOR PREPARATIONS

				Carbohydrate content (w/w%)				
Material	Processing method	Sedimentation constant ($s_{20, w}$)	Paper electrophoretic pattern	Non-hexosamine hexoses	Hexosamine	Fucose	Sialic acid	Intrinsic factor activity
Precipitate, *D*	Fourth step of the ammonium sulfate precipitation at 47% saturation	1.82 (90%) 4.32 (10%)	6 components (4 anodic and 2 cathodic)	4.47	2.55	0.59	0.68	Very high at 1.0 mg level on isotope assay
Low molecular wt. fraction, *a*-2	Ultracentrifugation of 4 ammonium sulfate precipitates; *C*-*F* obtained at 39–69% saturation	0.78	6 components incl. 4 anodic (fast, intermediate, and slow) and 2 cathodic (in traces)	3.20	1.87	0.45	1.23	Questionable or weak at 2.0–4.0 mg level on isotope assay
Medium molecular wt. fraction, *c*-2	As above	1.73	6 components (4 anodic and 2 cathodic)	4.40	1.87	0.36	0.94	Very high at 1.0 mg level on isotope assay
High molecular wt. fraction, *e*-2	As above	3.76 (47%) 5.66 (53%)	6 components, incl. 2 anodic (slow and intermediate) and 2 cathodic (at high concentration)	8.88	7.99	2.36	0.94	Moderate at 1.0 mg high at 2.0 mg level on isotope assay
High molecular wt. fraction, *e*-*1*	Ultracentrifugation of precipitate *D*	3.26 (60%) 4.75 (40%)	6 components (4 anodic and 2 cathodic)					High or very high at 0.4–0.6 mg level on both isotope and hematological assays

[a] From Jacob *et al.* (J1).

10% of the material, and had sedimentation constants of 4.32–4.70 S. This starting material was then used for preparative ultracentrifugation. It yielded several fractions of various sedimentation constants. Some of them were homogeneous, while others were heterogeneous on ultracentrifugation, but all were highly heterogeneous in our laboratory (G11) on paper electrophoresis. Their sedimentation constants ranged from 1.73 to 5.66 S, and their estimated molecular weight from 15,000 to 100,000. One of the heaviest fractions exhibited high intrinsic factor activity at doses of 0.4–0.6 mg (G11).

Bromer and Davisson (B11) used ultracentrifugation in the last stage of processing one of the most active intrinsic factor preparations available from hog stomach. Hog gastric mucosa was submitted to digestion by pancreatin, followed by column chromatography on Amberlite XE-64 and calcium phosphate gel. Ultracentrifugation followed by electrophoresis constituted the last step. Highly potent material was obtained, active at a dose of less then 0.1 mg.

Several other authors used ultracentrifugation in identifying intrinsic factor from hog stomach, e.g., Andresen and Skouby (A1, A2), Wijmenga *et al.* (W13), and Holdsworth (H11). Taylor *et al.* (T6a) used differential ultracentrifugation and free boundary electrophoresis to separate material inhibitory to intrinsic factor from the intrinsic factor-active mitochondrial material in gastric human mucosal cells. In general, however, ultracentrifugation was of only limited value in preparatory fractionation of macromolecular materials of gastric juice. More recently, Berg *et al.* (B5c), applied the ultracentrifuge to the determination of the composition of glycoproteins in gastric juice and found that their composition is rather constant, while the concentration of these materials in gastric juice undergoes variations.

A large series of ultracentrifugation studies of normal and pathological gastric juices has been recently reported by Hartmann *et al.* (H1a). The presence of 4 components having different sedimentation constants was ascertained, as well as that of many other complexes of glycoproteins, enzymes, and polypeptides.

7. Polarography

The principle of polarography, developed by Heyrowský and Brdicka, was applied in studying gastric juice first by Sibata, Kanazawa and Sato (see K1), followed by Sasai (S1-S3) and Kakei (K1, K2, K5).

Sasai *et al.* (S1–S3), using polarography, found a dialyzable peptide in the filtrate of gastric juice after precipitation with methanol. It was perhaps related to that formed by peptic digestion of the protein

precipitated by sulfosalicylic acid from gastric juice (K1, K2, K5), and that which Gilligan detected in the filtrate of gastric juice precipitated with ethanol (G1).

Extending Sasai's work, Kakei (K1, K2, K5) found a high double-protein wave on polarography in the majority of anacid gastric juices

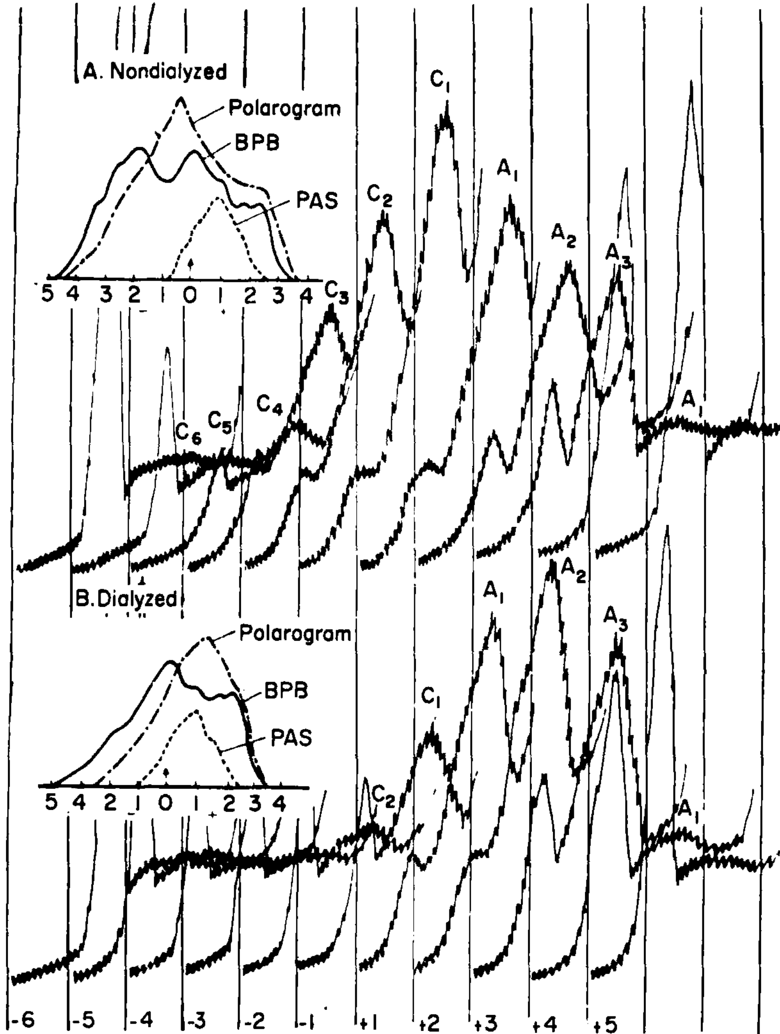

FIG. 41. The two insets on the left show the polarograms and electropherograms [stained with bromphenol blue (BPB) and PAS] of the nondialyzed and dialyzed acid gastric juice obtained from normal person. The polarographic waves shown here were obtained on the elutes of 1 cm wide segments cut off the paper electropherogram. From Sasai *et al.* (S2).

from gastric cancer patients. The protein content was increased and when very high, gastric cancer was often present. In cases of gastric cancer with preserved HCl in gastric juice, a double-protein wave was not found. Instead, a high single polarographic peptide wave appeared in the supernatant of the trichloroacetic acid filtrate of the gastric juice after precipitation with methanol. Whenever the increase in this peptide was found, gastric cancer was present in Kakei's material. He also eluted segments of paper electropherograms of lyophilized gastric juice and submitted the eluates to polarographic analysis. Eluates from cathodic segments gave peptide-type polarographic waves and represented products of peptic digestion of gastric proteins (Fig. 41). Duodenal ulcer patients in whom peptides were highly elevated consistently showed histological features of glandular hypertrophy of gastric mucosa.

Kawarada (see B10) determined polarographic waves of mucus and dissolved mucin fractions, i.e., mucoprotein and mucoproteose. Direct correlation was established between HCl concentration and polarographic mucin waves. In gastric cancer, the polarographic wave of mucoprotein disappeared while that of mucoproteose increased. In peptic ulcer, no significant changes were found.

Also Umetani (U4) noted variations in the polarogram of human gastric juice, especially in gastric cancer. There was no correlation, however, between gastric juice viscosity, actual gastric juice pH, and polarogram.

Bouda and Vesely (B10a) measured the polarographic activity (in Brdicka solution) of eluates from various segments of gastric juice electropherograms. Highest activity was found in the middle of the partition, which did not coincide with the maximum concentration of glycoproteins or proteins. Similarly to Kakei's findings, a single (peptide-type) polarographic wave was localized in the cathodic area of the partition and a double (protein-type) polarographic wave appeared in the anodic area of the electropherogram. Anacid gastric juices always gave a double (protein-type) polarographic wave. Peptic digestion of anacid gastric juice at acid pH transformed the pattern to that found in normal acid gastric juice.

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