ADVANCES IN CLINICAL CHEMISTRY

Volume 8

Harry Sobotka &
C. P. Stewart

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Advances in CLINICAL CHEMISTRY

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FOREWORD

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 a subject such as clinical chemistry. Media already existing furnish a comprehensive list of publications and an encyclopedic summarization of their contents; the present serial publication *Advances in Clinical Chemistry*—like other *Advances*—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

This volume presents a cross section of clinical chemistry, and deals with mineral, gaseous, and protein constituents of blood and tissues. As in previous volumes, some articles emphasize analytical procedures whereas others deal with their topic from a general physiological and clinical viewpoint without neglecting the pertinent clinical chemistry laboratory methods.

The editors' concept of clinical chemistry, as stated in previous volumes of this serial publication, encompasses a wide field of subjects—from the mechanism of blood coagulation to the microbiological assay of vitamins, and from the significance of trace metals in health and disease to the therapeutic application of increased gas pressure. The natural history of clinical conditions and their comparison with normal controls share one common feature, namely, the central position of some species of molecules, be they small or large, all of them the natural subject of chemical research. Hence, practical methods develop, either manual or mechanized, the results of which become increasingly more useful for the clinician in diagnosis, therapy, and prognosis.

As in the past, the editors wish to extend their thanks to the contributors and publishers.

October 1965

HARRY SOBOTKA
C. P. STEWART

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by DEREK WATSON

COPPER METABOLISM[1]

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1. Introduction and Historical Review

Copper was one of the first metals known to man, first used probably between 8000 and 7000 B.C. Copper utensils were found in Egyptian tombs dating back as far as 5000 B.C. The word copper derives from the Latin *cuprum*, an altered version of *cyprium*. *Cyprium* is an abbreviation of *aes cyprium*, the original name for copper in Latin, and refers to the island of Cyprus where the ancient copper mines were located (S11).

In spite of the long history of association between copper and man, the fact that copper is present in living organisms did not become known until 150 years ago, when copper was found in plants (B31, M18) and soon after

[1] During the preparation of this review the author was supported by a grant from the John A. Hartford Foundation of New York.

in animal tissues (S6). Nevertheless for some time thereafter it was considered that copper in living organisms, as well as some other trace elements, was a mere contaminant introduced from the environment either *in vivo* or during the process of sampling.

The first indication that copper is an essential body constituent, at least in some species, came with the recognition of the copper-containing respiratory pigment hemocyanin in cold-blooded animals (F8), and the discovery of turacin, a copper-containing pigment in the feathers of certain birds (C10).

Actual nutritional dependence of animals on copper was not conclusively proven until 1928, when Hart *et al.* (H4) showed copper to be an essential nutrient for the rat and other animal species (E7).

The recognition of naturally occurring copper-deficiency states in a number of domestic animals was a further important step toward the establishment of copper as an essential nutrient. Cattle, sheep, and pigs feeding on plants grown on copper-deficient soil were reported to be affected in North America (N2), in Europe (S29, S30), and in Australia (B14).

It had long been suspected that copper in the living organism is concerned with "catalytic" functions (G15). With the advent of modern biochemistry it was possible to prove that this is indeed the case. Copper was recognized to form an essential constituent of the molecule in a number of enzymes. In many of these, copper is a part of or essential for the function of the active site of the enzyme. Some of these enzymes, like cytochrome c oxidase, are widespread in the living tissues of a many animal species and indispensably involved in basic life processes. This establishes copper as an essential element for a wide variety of living organisms more forcefully than the evidence from nutritional studies.

While trace amounts of copper are essential for life, an excess of copper is toxic. There are many reports on the toxic effects of copper in a wide variety of animals and in man; these are well summarized in Underwood's monograph on trace metals (U2). Copper is well known as an inhibitor of many enzymes in extremely low concentration, around 10^{-6} M. The mechanism of such inhibition may be through an antagonistic effect on other essential metals, by the binding of the copper to the active site of the enzyme and thus rendering it inactive, and by a tendency of copper ions to combine with essential cofactors such as glutathione, etc. (R7).

It seems evident from all this that in living organisms, including man, elaborate physiological mechanisms must function to ensure that copper is made available in sufficient amount and in the right form whenever and wherever it is needed and, at the same time, that accumulation of toxic amounts is prevented.

These mechanisms, all part of the field of copper metabolism, will be the subject of this review. An attempt will be made to summarize present-day knowledge in the field from the literature and from the author's experience. Preference will be given to the observations made in man. The results of experimental work in animals and of studies *in vitro* will be used to fill in the gaps. Emphasis will be placed on recent contributions and on the few human pathological conditions related to disturbances of copper metabolism. Because of space limitations and the plethora of recent developments in the field, some important lines of information must be omitted. The reader is referred to a number of relatively recent, excellent monographs and review articles on copper metabolism (A1, A2, C2, S13, S17, U2). Much useful information is also contained in recent reviews and symposia on Wilson's disease, the most important aberration of copper metabolism in man (B5, S24, W8).

2. Methods of Investigation

Some of the methods of investigation essential for the study of copper metabolism will be surveyed in this chapter. In addition to the analytical methods for the detection and quantitative determination of copper in biological materials, the techniques for determination and detection of one of the biologically important copper proteins (ceruloplasmin) will also be discussed. Finally, the general principles and techniques involved in the use of radiocopper will be surveyed.

2.1. Determination of Copper in Biological Materials

Only trace amounts of copper are present in biological material, in the general range 0.1–10 μg/g (0.1–10 ppm). These trace amounts are in complex mixture with innumerable other metals and elements, some of which are present in much larger quantities. In addition the sample size is often limited, as in blood samples from infants and small animals or needle biopsy specimens from tissues. Methods for copper therefore must be highly sensitive as well as highly specific, since the analyst faces the problem of trace analysis on a micro- or ultramicroscale.

Because of the ubiquitous presence of copper in nature and especially in the modern environment, contamination of samples before or during analysis is an ever present danger. For example, ordinary distilled water from copper or tinned copper stills contains quite regularly 10–200 μg/liter of copper. This of course precludes its use for the rinsing of glassware or as a diluent of samples and reagents. Glass-distilled water or distilled water passed through suitable cation-exchange resins must be used, and its quality frequently checked. Glassware must be of highest quality glass, free

from extensive etching, meticulously cleaned and washed in dilute acid followed by extensive rinsing in copper-free distilled water. It is well to remember that filter paper, dialyzing membranes, etc., may contain copper and at the same time may retain copper from the samples. Reagents must be of highest analytical grade, carefully screened for copper contamination. Solvents and concentrated acids may have to be redistilled. As is customary in trace analysis, reagent blanks and standards carried through the whole procedure must be included with practically each batch of determinations. Much useful information concerning the dangers of contamination in trace analysis and its elimination is given by Thiers (T1).

Copper has a marked tendency to form complexes with organic substances, especially proteins, polypeptides, and amino acids. In complexed form, copper may not react with reagents or may not be extracted quantitatively. Special care must therefore be taken to free copper from these bonds quantitatively before its determination. This often cannot be achieved without dry or wet ashing.

Numerous methods are available for the determination of trace amounts of copper. The statement has been made, "The methods now available are in the main very satisfactory and leave little to be desired" (S3). While this may be true in general, there is still room and need for improvement. Simplification of existing methods would reduce the danger of contamination, and increase in sensitivity would allow the use of smaller samples.

2.1.1. *Colorimetric Methods*

These methods have the great advantage of not requiring expensive apparatus or highly specialized technical knowledge for operation of the apparatus. The sensitivity range of present-day colorimetric methods is adequate for most purposes; their specificity and accuracy are also considerable.

As a rule, preliminary ashing to destroy organic material is necessary. Dry ashing, even at temperatures not higher than 500°C, is less satisfactory than wet ashing. This is not so much because of the danger of volatilization of copper, but because of its partial conversion to acid-insoluble forms by reaction with constituents of ash or the vessel wall (S3). Wet ashing with sulfuric and nitric acids or perchloric acid is preferred because there is less danger of loss of material; however, there is danger of contamination unless the acids used are of the highest degree of purity. The use of perchloric acid carries the danger of explosion in inexperienced hands.

There are a great number of color reagents for copper, some of which are listed in Table 1. Their relative sensitivity can be compared on the basis of their molar absorbancy indices. The references given in the last column of Table 1 pertain to the sensitivity, specificity, and general charac-

TABLE 1
COLOR REAGENTS FOR COPPER

Reagent	Solvent	Wavelength (mμ)	Molar absorbancy index (1 cm)	Reference
Diethyldithiocarbamate	Water	440	8,000	(G14, P5)
	Isoamyl alcohol	440	12,700	(E3, M4, P5)
	CCl_4	435	14,700[a]	(B17)
Dibenzyldithiocarbamate	CCl_4	435	16,200[a]	(B17, G5, M13, M17, V2)
Dithizone	CCl_4	508	24,600	(L8)
2,2-Biquinoline (cuproine)	Isoamyl alcohol	540	5,900[a]	(B17, H18, P5)
2,9-Dimethyl-1,10-phenanthroline (neocuproine)	CCl_4-EtOH	454	8,000	(J5, P5, S32)
4,7-Diphenyl-1,10-phenanthroline		420	12,000	(P5, S34)
2,9-Dimethyl-4,7-diphenyl-1,10-phenanthroline (bathocuproine)	*n*-Hexanol	480	14,200	(S32, S33, Z1)
Biscyclohexanoneoxalyldihydrazone (cuprizone)	Water	600	16,000	(P5)
Oxalyldihydrazide + acetaldehyde	Water	542	29,500	(G10, R11, S37)
1,5-Diphenylcarbohydrazide	Water	540	158,000	(T8)
	Benzene	540	55,000	(S46)

[a] Calculated from data available in reference (B17).

teristics of these reagents and describe applications of copper determination in biological materials. Each reagent has its proponents, and the relative merits are difficult to judge from the rather voluminous literature.

The most time-honored and certainly dependable method for serum, urine, and tissue copper determination is that described by Eden and Green in 1940 (E3). Wet ashing with a combination of sulfuric, perchloric, and nitric acids is followed by the deionization of iron with citrate and/or pyrophosphate in strongly alkaline medium. Diethyldithiocarbamate is the color reagent and the complex is extracted into amyl alcohol. Provided blanks can be kept low, which can be achieved only by applying extreme care to avoid contamination of the many reagents, copper can be determined reliably in 0.5–1.0 ml of serum. While this method undoubtedly represents a great improvement over previous methods (M4, T5), it is somewhat cumbersome; nevertheless it has proved its worth in many hands and has been the basis of a number of modifications and applications.

Gubler *et al.* (G14) have described another method for the determination of copper in blood by dithiocarbamate. They found ashing unnecessary, and recommend liberation of copper from its organic complexes by incubation with hydrochloric acid preliminary to precipitation of the proteins by trichloroacetic acid. Citrate and pyrophosphate are added, followed by alkalinization with ammonia, as in the previous method. Diethyldithiocarbamate is used as color reagent. The color is read in the aqueous solution, extraction into organic solvent being omitted. The method can be applied to whole blood as well as plasma. This method has not been used as widely as the former. Recovery of protein-bound copper without digestion may not be complete. As can be seen from Table 1, dithiocarbamate produces less color in aqueous solution than in organic solvents. Also, in water the copper-dithiocarbamate complex is in colloidal suspension, which makes colorimetric readings uncertain (P5, S3).

The diethyldithiocarbamate copper complex is unstable in acid medium and is light-sensitive. Dibenzyldithiocarbamate (available as zinc salt) lacks these disadvantages; moreover, it is more sensitive (Table 1) and more specific (M13, S3). Maytum *et al.* (M17) and most recently Giorgio *et al.* (G5) have described methods for urinary copper determination with this reagent. The latter method does not require preliminary ashing. The only disadvantage of these methods is the use of carbon tetrachloride, which is potentially toxic.

Dithizone has been used widely in the past for the determination of copper; however, there is little to recommend its use now. It is less sensitive (Table 1) and far less specific than other reagents.

2,2-Biquinoline and 2,9-dimethyl-1,10-phenanthroline are very specific reagents for copper but lack sensitivity (B17); nevertheless the latter has been used successfully for the determination of copper in certain foodstuffs (J5). 4,7-Diphenyl-1,10-phenanthroline and bathocuproine are more sensitive; the latter is specific for copper to such an extent that it can be used for the simultaneous determination of both copper and iron in serum (Z1).

Rice (R11) and Welshman (W9) have described methods for the determination of serum copper using oxalyldihydrazide (see Table 1) as the color reagent, based on Stark and Dawson's (S37) recommendations for the use of oxalyldihydrazide and the Gubler *et al.* (G14) method of liberating protein-bound copper without ashing. These methods are simple, claimed to be accurate, and more sensitive than previous methods.

Morell *et al.* use another method for determining microgram quantities of copper in biological materials.[2] The sample is wet ashed by a method similar to that of Eden and Green (E3), and dicyclohexanoneoxalyldihydrazide is used as the color reagent (see Table 1), recommended by Peterson and Bollier (P5). Morell's method takes few short cuts and seems to combine relative simplicity and ease of operation with high sensitivity. An ultramicro modification of this method is used for the determination of copper in needle biopsy specimens of the liver.

Finally, Stoner and Dasler's method utilizes the most sensitive color reagent for copper, 1,5-diphenylcarbohydrazide (S46). Wet ashing is included in this method, followed by performance of the color reaction and extraction of the colored complex into specially treated benzene. This method seems most promising and may prove to be more sensitive than the others.

The above methods have all been, or could easily be, adapted for the chemical determination of copper in tissues.

Histochemical demonstration of copper is possible with the aid of rubeanic acid (U3). This method, applied to a needle biopsy specimen, has been suggested as a useful diagnostic aid in Wilson's disease (C8). Unfortunately, the method is rather erratic and does not function in all hands. Improvements in this technique or new, more sensitive methods with high optical resolution for histochemical demonstration of copper are necessary. Of special importance would be the demonstration of copper at the electron microscopic level; unfortunately, such methods are not yet available.

[2] The latest version of this method has not been published, but can be obtained from the authors on request (A. G. Morell, Albert Einstein College of Medicine, Yeshiva University, New York 61, New York).

2.1.2. *Emission Spectrography*

Emission spectrography has been applied fairly extensively for trace metal analysis in biological material. Early methods were semiquantitative. More exact quantitation has become feasible lately by determining the relative intensity of the photographed spectrum lines by densitometry. The method is particularly suitable for parallel determination of large numbers of elements in the same sample. Butt *et al.* (B34), Koch *et al.* (K13), and Tipton *et al.* (T3, T4) have reported human tissue analysis for copper and a great number of other elements by this method. Long (L9) has reported the use of emission spectroscopy for the detection of metals in serum protein fractions separated by starch block electrophoresis. The technique of emission spectrometry has recently been improved by the use of a direct reading attachment which replaces the more cumbersome and less accurate photographic recording methods. Nusbaum *et al.* (N8) reported the use of this latter method for tissue analyses, and Valberg *et al.* (V1) for red blood cells. The spectroscopic method for the determination of copper is both sensitive and specific; its only disadvantage is the need for complicated and expensive apparatus, which will preclude for some time its widespread use.

Another form of emission spectrometry is flame spectrophotometry. Newman and Ryan (N5) have adapted the method for the determination of copper in urine and serum. This method is less sensitive and requires wet ashing as well as the extraction of copper as a dithizone complex into carbon tetrachloride followed by oxidation of dithizone and re-extraction of the copper into water.

2.1.3. *Atomic Absorption Spectrometry*

Willis (W12) has recently summarized the principles and applications of this method. A short note appeared recently regarding the use of atomic absorption spectrometry for serum and urine copper analysis (B15). The sensitivity of this method for copper is rather less than for such other biologically important trace metals as magnesium, zinc, and sodium. The sensitivity can be improved by extracting the copper as dithiocarbamate or pyrollidinedithiocarbamate complex (A7) into methyl isobutyl ketone. While this method is less sensitive than some others, it is nevertheless very specific and the apparatus is only moderately expensive.

2.1.4. *X-Ray Spectroscopy*

Alexander (A6) determined copper, iron, and zinc in dry ashed tissues or plasma by an X-ray fluorescence method. As it stands now, 20–250 mg

of tissue or plasma ash is necessary as a single sample. Perhaps this amount can be reduced, but until this occurs the method cannot be considered a microtechnique. The high degree of specificity and the need for only dry ashing and plating to prepare a sample for measurement are great advantages of this method. The apparatus is expensive and not yet easily available.

2.1.5. *Neutron Activation Analysis*

This is presently the most promising method for the determination of trace amounts of copper on an ultramicroscale. The radiochemical properties of copper are such that one theoretically should be able to detect 10^{-10} g of this element without much difficulty (S31). There are two naturally occurring nonradioactive copper isotopes, Cu^{63} and Cu^{65}. Exposure to a high flux of thermal neutrons converts these to radioactive Cu^{64} and Cu^{66}, respectively.

Most authors use Cu^{64} for detection; Kaiser and Meinke (K1) see advantages in the use of Cu^{66} with a half-life of 5.1 minutes. Their recovery, however, using rat tissues for analysis, is not more than 80%, which seriously limits the value of the method. When using Cu^{64} it is best to measure the 0.511-MeV annihilation radiation. The main problem in the case of biological samples is the production of 15-hr half-life Na^{24} during activation. The back-scattered radiation from the large 1.37-MeV photopeak of Na^{24} completely obscures the relatively small photopeak of Cu^{64}. For this reason the copper must be separated from the sodium, which may precede the neutron activation or, more conveniently, be carried out following the activation step. In the latter case, carrier copper can be added, which means that the separation can be carried out on a macroscale and of course contamination with inactive copper is of no importance whatsoever. Kanabrocki *et al.* (K2) described a neutron activation technique for the determination of copper in biological fluids in which sodium is separated by dialysis, before activation. Bowen (B20) uses a chemical precipitation technique to separate the copper following activation. Kjellin (K8) and Fritze *et al.* (F10) use ion-exchange chromatography as the method of separation following neutron activation to determine copper in cerebrospinal fluid and in small samples of animal tissues. The great advantage of the chromatographic method of separation is a 100% yield in terms of copper, which cannot be said of the chemical separation techniques.

At present activation analysis is the most sensitive method for copper determination. However, it requires the analyst to have access to a nearby source of thermal neutron radiation, as well as a special laboratory and personnel for radiochemical work.

2.1.6. *Electrometric Titration*

Johnson (J3) has reported a relatively simple method for determining copper in serum by electrometric titration.

2.2. Determination of Ceruloplasmin

Colorimetric, enzymatic, and immunochemical methods are available for the determination of ceruloplasmin.

2.2.1. *Colorimetric Method*

The colorimetric method is based on the intense blue color of this protein, which disappears under the influence of high concentrations of ascorbic acid or cyanide. The absorption maximum at 610 mμ and the absorbancy index were first established by Holmberg and Laurell (H14). Scheinberg and Morell (S16) described a technique by which ceruloplasmin can be determined in ceruloplasmin-rich purified plasma fractions. It is said that this method with slight modifications can also be used for the determination of ceruloplasmin in citrated plasma. Due to the relatively low concentration of ceruloplasmin in plasma, however, this method is impractical since it requires large amounts of serum and is disturbed by the slightest turbidity change. It has nevertheless been used to standardize the enzymatic methods and has great value in this respect.

2.2.2. *Enzymatic Method*

This method of ceruloplasmin determination is based on the weak polyphenol oxidase activity of ceruloplasmin, the only oxidase of this kind in human and animal plasma. The best known substrate (*in vitro*) is *p*-phenylenediamine or *N*,*N*-dimethyl-*p*-phenylenediamine. The rate of oxidation can be followed by measuring the oxygen uptake manometrically or, more conveniently, by measuring colorimetrically the rate of appearance of the purple reaction product. The exact nature of this reaction is not known. Rice suggested that Bandrowski's base is formed through the condensation of 3 molecules of *p*-phenylenediamine (R12). Colombo and Richterich (C11), however, were unable to confirm this. The recent work of Peisach and Levine (P4) suggests that Bandrowski's base is at best only an intermediary product. The end product of the reaction is unknown.

The enzyme reaction is influenced by many factors and therefore rigidly controlled conditions must be observed in order to obtain results which reflect only the variation in enzyme concentration. Several well described methods are available (C11, R4, S16).

The pH optimum of the reaction is somewhere between 5.0 and 6.0,

depending on such factors as the choice of buffer, molarity, etc. An interesting initial lag period of the enzyme reaction has been noted by several authors (H16, H20, P4); the reason for this lag is at present unknown. Humoller *et al.* (H20) have suggested that the ascorbic acid present in the serum will reduce the *p*-phenylenediamine oxidized by the enzyme until it is used up in this reaction. This is disputed by Colombo and Richterich (C11). The lag period is more likely explained on the basis of the complex nature of the oxidation of *p*-phenylenediamine which proceeds in several steps, according to the recent work of Peisach and Levine (P4). Whatever the explanation, the lag period must be taken into consideration when measuring ceruloplasmin activity by colorimetry. It may not suffice to determine the optical density of the reaction mixture at the start of the reaction and once later. Because of the lag period, especially if there is a short time-interval between the two observations, the straight line between the two points will not represent the rate of the reaction for obvious reasons. A recording photometer will measure the true rate of the reaction.

Certain metals have a profound influence on the activity of the enzyme. Copper in concentrations as low as those resulting from contamination of glassware and reagents will catalyze nonenzymatic oxidation of *p*-phenylenediamine. For this reason it was recommended that 10^{-3} *M* EDTA (ethylenediaminetetraacetic acid) be incorporated in the reaction mixture (B28). However, higher concentrations of EDTA inhibit the enzyme, and it has been suggested that EDTA may form a complex with copper in ceruloplasmin and thereby inactivate the enzyme (H20, K3). The addition of EDTA to the reaction mixture therefore may not be desirable. We found that by simply avoiding copper contamination, by the use of precautions necessary in copper determinations, the nonenzymatic oxidation of *p*-phenylenediamine slows to an imperceptibly low rate, and in this situation there is of course no need to add EDTA.

Levine and Peisach called attention to the effect of Fe^{2+} ions on the reaction (L7). Fe^{2+} ions in low concentration stimulate and in higher concentration inhibit the reaction. Their *p*-phenylenediamine preparation was the source of ferrous ion contamination. Micromolar concentrations of EDTA abolish this effect by chelating the iron. We feel that *p*-phenylenediamine free of iron contamination should be used. This will obviate the need for EDTA, since the iron present in serum normally does not disturb the reaction (C11), probably because it is present in the ferric state (Fe^{3+}) and bound to transferrin as well.

Finally, the effect of a number of anions on the oxidase activity of ceruloplasmin should be mentioned. Holmberg and Laurell (H16) and later Curzon (C18) showed that chloride, acetate, nitrate, bromide, thiocyanate,

sulfate, and fluoride all have inhibitory effects. Serum albumin was claimed to be inhibitory, but was found later to inhibit only the nonenzymatic oxidation of *p*-phenylenediamine and not to inhibit or enhance the oxidase activity of ceruloplasmin (R4).

These observations underline the need for meticulous standardization of enzymatic assay techniques for determining ceruloplasmin. With proper standardization, the enzymatic methods are reliable. However, one must always be alert to the possibility that factors other than the concentration of ceruloplasmin may influence the *p*-phenylenediamine oxidase activity.

The results of enzymatic determinations of ceruloplasmin are often expressed in arbitrary units, and the values judged in the light of a series of results obtained in normal subjects by the same method. Expression of the enzyme activity in milligrams of ceruloplasmin per unit volume of serum is also possible. The relation between oxidase activity and the amount of ceruloplasmin in serum can be determined by measuring in parallel samples of sera both the oxidase activity and the change of optical density at 610 mμ before and after the addition of ascorbic acid or cyanide. On the basis of the known absorbancy index, the ceruloplasmin concentration can be calculated (see Section 2.2.1) and the relation between it and the enzyme activity determined. Alternatively, purified human ceruloplasmin can be used for standardization of the enzymatic method. The ceruloplasmin content of the purified preparation can be determined colorimetrically or, in the case of a highly purified preparation, by nitrogen analysis. Predetermined increments of ceruloplasmin can then be added to aliquots of a selected serum. It is convenient to select a serum with relatively low ceruloplasmin level to start with. Serum of a patient with Wilson's disease, some of whom have no measurable amount of enzyme activity, would be ideal for the purpose; however, Walshe (W5) has recently found an inhibitor in these sera.

Mention should be made of a simple enzymatic screening method whereby individuals with very low ceruloplasmin blood levels can be detected (A4). The method employs a filter paper disc (Whatman No. 3). A drop of 0.4% *p*-phenylenediamine in acetate buffer of pH 5.2, 1.0 *M*, and a drop of plasma are placed on the filter paper in succession. The discs are then wrapped in a polyethylene sheet, placed in a tray, and incubated at 37–40°C. A control sample with a borderline low ceruloplasmin level is included among the unknowns. The intensity of the blue color, which develops as the result of ceruloplasmin-catalyzed oxidation of *p*-phenylenediamine, is noted and compared to that produced by the standard. Convenient kits for the performance of this screening test can be obtained commercially.[3]

[3] Dade Reagents Inc., Miami, Florida.

2.2.3. *Immunochemical Method*

Immunochemical methods are also available for quantitative determination of ceruloplasmin (H11, M11, S15). These require a highly purified preparation of human ceruloplasmin, which serves as antigen and is given parenterally to another species, usually rabbits, to incite the production of specific antibody against the human ceruloplasmin. The specificity of the antibody depends entirely on the purity of the antigen. Further purification of the antibody can be achieved by absorption on sera devoid of ceruloplasmin, e.g., sera of certain patients with Wilson's disease. The immunochemical method of determination of ceruloplasmin yields results which correlate well with serum copper levels and with the results of the enzymatic method.

2.3. Determination of Non-Ceruloplasmin-Bound Copper in Serum

A small but important fraction of serum copper is not bound to ceruloplasmin. Most of this "non-ceruloplasmin-bound copper" is attached to serum albumin. The amount of this fraction of serum copper varies in physiological and especially in pathological conditions. It can be measured since it will react with diethyldithiocarbamate directly, whereas ceruloplasmin-bound copper will react only after acidification. A method for the determination of "direct reacting" copper in serum has been published by Gubler *et al.* (G12). Unfortunately, this method is not very accurate, especially in the case of normal subjects where the figures are very low. More exact measurements can be obtained when the values are higher as in certain pathological states.

2.4. Use of Radioactive Copper

There are two useful radioactive isotopes of copper. Certain properties of these are shown in Table 2.

TABLE 2
Properties of Useful Radioactive Copper Isotopes[a]

Property	Cu^{64}	Cu^{67}
Half-life	12.8 hr	58.5 hr
Energy of principal γ emission	0.511 MeV (from β^+ decay)	0.184 MeV 0.093 MeV
Maximum energy of β emission	β^- 0.57 MeV β^+ 0.66 MeV	β^- 0.40 MeV 0.48 MeV 0.58 MeV

[a] Data were taken from reference (R1).

Copper-64 is readily available commercially, and so far has been more widely used than Cu^{67}. Its half-life is short and it therefore can be used only for relatively short-term experiments. By virtue of positron emission, it yields two rather energetic γ-rays through an annihilation reaction which can be detected and measured with relatively high efficiency.

Copper-67 (not available commercially) has been produced and used for some experiments (S44). Its half-life is longer than that of Cu^{64} and it is also readily measured by its γ-emission. The energy peaks of Cu^{64} and Cu^{67} are conveniently different, which permit their use in double-labeling experiments (S44).

Both Cu^{64} and Cu^{67} emit β-radiation; thus they can be measured by β-counting techniques and detected by autoradiography. Unfortunately, their maximum β-energy is rather high and the autoradiographic resolution therefore is rather poor, which impairs their suitability for microautoradiography.

Copper-64 of a medium degree of specific activity can be produced by bombarding Cu^{63}, one of the stable copper isotopes, with thermal neutrons to yield Cu^{64} by the following reaction.

$$Cu^{63}\ (n,\gamma)\ Cu^{64}$$

Very high specific activity, in fact almost carrier-free Cu^{64}, can be produced by fast neutron bombardment of Zn^{64}.

$$Zn^{64}\ (n,p)\ Cu^{64}$$

This is followed by chemical separation of the copper from zinc (F9). Lots containing 20–25 mC of activity in less than 1 μg of copper have been produced by this method.

Copper-67 can be produced from Ni^{64} (an enriched preparation of which is necessary) by a beam of accelerated α particles from a cyclotron.

$$Ni^{64}\ (\alpha,p)\ Cu^{67}$$

The resulting Cu^{67} is contaminated to some extent with Cu^{64}.

Copper-67 can also be produced in a reactor by fast neutron bombardment of Zn^{67}. An enriched preparation of Zn^{67} is necessary. The reaction is

$$Zn^{67}\ (n,p)\ Cu^{67}$$

and is analogous to the production of Cu^{64} from Zn^{64}. Here again chemical separation of the Cu^{67} from Zn^{67} is necessary. The specific activity of the resulting preparation is expected to be high.

In vitro incorporation of radioactive copper into ceruloplasmin is possible

(S44). Ceruloplasmin tagged with I^{131} has also been used for experimental purposes (K1).

3. Copper in Tissues and Biological Fluids

3.1. Copper Content

The copper content of the normal human adult is widely quoted as 100–150 mg. This figure, as far as we could determine, is taken from a paper published in 1935 by Chou and Adolph (C9). These authors measured the copper content of 9 organs taken at autopsy from 2 adults, one of whom died of opium poisoning, the other of a ruptured aortic aneurysm. The total copper content of these organs was calculated with figures for water content and weight of the organs from standard tables. The approximate total body copper of 115.9 mg was obtained by adding the total copper content of the 9 organs. The authors state that their figure is only a rough estimate since brain, skin, and other tissues were not included; nevertheless they suggested, "Probably the total copper content (of the human body) is between 100 and 150 mg."

Tipton and Cook (T3) recently have given the results of a quantitative emission spectrographic analysis of 24 trace elements in 29 human tissues. Tissues were obtained from 150 adults from various parts of the United States, who were free of disease and died suddenly, usually as a result of accident. Copper was one of the elements studied, and, since these data constitute the most complete material so far obtained by the most carefully standardized methods of collection and analysis, the results are shown in Table 3. In the original paper, the copper content is given as μg/g ash. For easy reference and since tissue copper levels in the literature are usually given on a dry or wet weight basis, we have calculated these with the aid of ash as per cent of the dry and wet weights given by Tipton and Cook in their paper. The median and the 80% range are shown in the table for each organ, expressed as μg/g ash, dry weight, and wet weight. Using the organ mass values established for the "standard man" (R9), the median and 80% range of total copper content were calculated in each organ and are shown in the last column of Table 3.

The data in Table 3 speak for themselves. It is interesting to note that liver, brain, heart muscle, and kidney have the highest concentration of copper, in decreasing order. There is a higher concentration of copper in the upper parts of the gut than in the lower parts. Skeletal muscle has a relatively low copper level; nevertheless, because of its large mass, muscle as a compartment contains more copper than any other tissue. One-third of the total body copper is contained in muscle. The copper content of

TABLE 3
COPPER CONTENT OF HUMAN ORGANS AND TISSUES

Organs and tissues	(1) μg Cu/g ash[a]		(2) μg Cu/g dry weight		(3) μg Cu/g wet weight[b]		(4) Mass[c]	(5) mg Cu in whole organ[d]	
	Median	80% range	Median	80% range	Median	80% range	(g)	Median	80% range
Brain	360	240–500	21.9	14.6–30.5	5.40	3.60–7.50	1.500	8.10	5.40–11.25
Liver	510	320–1300	18.8	11.8–48.0	6.63	4.16–16.9	1.700	11.28	7.07–28.70
Kidney	260	190–340	11.9	8.75–15.6	2.86	2.09–3.74	0.300	0.86	0.83–1.11
Heart	350	250–460	14.0	10.0–18.4	3.85	2.75–5.06	0.300	1.16	0.83–1.52
Diaphragm	140	110–200	4.76	3.74–6.80	1.34	1.06–1.92	—	—	—
Muscle	74	50–120	3.11	2.10–5.05	0.89	0.60–1.44	30.000	26.70	18.00–43.20
Skin	100	80–150	1.70	1.36–2.55	0.70	0.56–1.05	2.000	1.40	1.12–2.10
Omentum	190	110–280	0.38	0.22–0.56	0.38	0.22–0.56	10.000[e]	3.80[e]	2.20–5.60[e]
Spleen	89	67–120	4.45	3.35–6.00	1.25	0.94–1.68	0.150	0.19	0.14–0.25
Pancreas	140	92–210	4.90	3.22–7.35	1.68	1.12–2.52	0.070	0.12	0.08–0.18
Lung	120	92–180	5.75	4.41–8.65	1.32	1.02–1.98	1.000	1.32	1.02–1.98
Larynx	33	14–150	3.07	1.30–13.9	0.99	0.42–4.50	—	—	—
Trachea	56	25–96	3.02	1.35–5.19	0.90	0.40–1.54	—	—	—
Esophagus	120	84–200	4.56	3.19–7.60	1.08	0.76–1.80	0.365	0.39	0.28–0.66
Stomach	230	150–320	7.36	4.80–10.2	1.84	1.20–2.56			
Gut									
Duodenum	260	160–420	10.5	6.25–16.4	2.08	1.28–3.36			
Jejunum	230	160–330	8.97	6.25–12.9	2.07	1.44–2.95	2.000	3.34[f]	1.13–2.55[f]
Ileum	240	170–400	8.16	5.78–13.6	1.75	1.29–3.04			
Cecum	220	150–280	4.40	3.00–5.60	1.32	0.90–1.68			
Sigmoid	210	150–340	5.25	3.75–7.00	1.47	1.05–2.38			
Rectum	170	110–270	5.27	3.41–8.38	1.19	0.77–1.89			
Adrenal	200	160–250	2.20	1.76–2.75	0.92	0.74–1.15	0.020	0.02	0.015–0.023
Thyroid	94	43–240	3.48	1.59–8.88	1.03	0.47–2.64	0.020	0.02	0.009–0.053
Testis	80	58–110	4.55	3.30–6.27	0.88	0.64–1.21	0.040	0.035	0.026–0.048
Prostate	100	57–140	4.50	2.56–6.30	1.10	0.63–1.54	0.020	0.022	0.013–0.031
Ovary	120	85–180	5.28	3.74–7.91	1.16	0.82–1.75	0.008	0.009	0.007–0.014
Uterus	98	73–130	4.21	3.14–5.59	0.96	0.72–1.27	—	—	—
Urinary bladder	110	76–180	2.53	1.75–4.15	0.88	0.61–1.44	0.150	0.132	0.091–0.216
Aorta	90	50–140	3.42	1.90–5.32	1.26	0.70–1.96	—	—	

Blood[g]	—	—	—	—	0.98	0.72–1.24	5.400	5.290	3.89–6.70
Bone (vertebra)[h]	5.0	2.0–5.0	1.45	0.58–1.45	0.75	0.30–0.75	7.000	5.250	2.1–5.25
Subcutaneous tissue, bone marrow, lymphoid tissue[i]	—	—	—	—	—	—	7.800	6.57	4.96–8.92
Estimated total body content:								76.0	50.0–120.0

[a] From Tipton and Cook (T3), except where indicated otherwise.

[b] Calculated from data given by Tipton and Cook (T3), except where indicated otherwise.

[c] From *Report of I.C.R.P. Committee II* (R9).

[d] Calculated from data in columns 3 and 4.

[e] Since omentum is mainly depot fat the copper content of depot fat was calculated here based on the total mass of fat in the body (shown in column 4) and the copper concentration in the omentum (column 3).

[f] Calculated on the basis of the arithmetic mean copper content of the various parts of the gastrointestinal tract.

[g] Concentration of copper in whole blood of man was taken from Wintrobe *et al.* (W15). Calculations were based on mean ± 2 standard deviations.

[h] Bone copper contents, personal communication from Dr. Isobel Tipton (1965).

[i] There are no data in the literature on the copper content of these tissues. The assumption was made therefore that the copper content of subcutaneous tissue is the same as that of skin, and that the copper content of bone marrow and lymphoid tissue is the same as that of blood.

human hair and nails (not shown in Table 3) is considerably higher than that of other organs, compared on a wet weight basis (M14, R13), no doubt due to the low water content of these tissues. Copper in hair and nail does not reflect the accumulation or depletion of copper in the body, thus the analysis of these easily accessible tissues is of little value for diagnostic purposes (M14, R13).

On the basis of the excellent data of Tipton and Cook (Table 3) with a few assumptions, which are detailed in the table, the total body copper content can be calculated. The mean of 75 mg with a range of 50–120 mg is lower than the previously quoted figures.

The average whole-body concentration of copper in adult vertebrates including man is given as 1.5–2.5 μg/g fat-free tissue (W10, W11). If 75 mg is taken as the total body content, calculated from Tipton's data (Table 3), and 60 kg as the fat-free average weight of a human subject (R9), the mean concentration of copper in the whole body of man is 1.25 μg/g fat-free tissue with a range of 0.83–2.0 μg/g. These figures are lower than those given by Widdowson *et al.* (W10, W11). With a few exceptions, the relative distribution of copper in the tissues of animals and of man, is not very different (U2). Sheep and cattle (ruminants in general) have a higher concentration of copper in the liver than other animals (A1).

The distribution of copper in organs and tissues has been studied by histochemical methods. In the liver most of the copper is in the parenchymal cells (H19, U3). Because of the low sensitivity of histochemical methods, this could be demonstrated only when the amount of copper in the liver was in excess of normal. There is wide variation in the copper content of various parts of the human brain. Gray matter in general has a much higher copper content than white matter. The locus coeruleus, a small blue-colored area in the brain, contains a surprisingly high amount of copper, 107–404 μg/g dry weight (E1). Copper can be demonstrated in nerve cells by histochemical methods only when their copper content is abnormally high (H19). By chemical methods, which are more sensitive, the presence of copper in white matter has been proven conclusively (E1).

The intracellular distribution of copper has been studied. A distinct pattern of distribution was found among the four subcellular fractions that can be separated by differential centrifugation. In rat liver, 64.3% of the total copper is found in the soluble fraction, 8.2% and 5.0% in the mitochondrial and microsomal fractions, respectively, and 20.3% in the fraction containing nuclei and cell residue. These results obtained by Thiers and Vallee (T2) were confirmed by Hermann and Kun (H10). In the livers obtained at autopsy of two adult men, Porter has found a similar subcellular distribution of copper (P11, P15).

The concentrations of copper in biological fluids of man are shown in Table 4. The blood levels of copper are around 100 μg/100 ml. Plasma and erythrocytes contain similar concentrations of copper.

The copper content of saliva is much lower than that of the blood. Bile contains highly variable amounts of copper, which is not surprising since it is the main excretory route for copper. The copper content of cerebrospinal fluid is controversial. According to the recent careful measurements of Kjellin (K8) (shown in Table 4), the copper concentration in the cerebrospinal fluid of normal adults is very low. Higher levels were reported by others (see literature quoted by Kjellin, K8).

TABLE 4
COPPER CONTENT OF BIOLOGICAL FLUIDS IN MAN

Fluid	Copper (μg/100 ml ± S.D.)	Reference
Blood		
Whole	98 ± 13	(W15)
Plasma	109 ± 17	(W15)
Erythrocytes	115 ± 22	(W15)
Saliva	31.7 ± 15.1	(D1)
Bile	5.6–205.0	(D3, V4)
Cerebrospinal fluid	1.6 ± 0.4	(K8)
Urine	18.0 ± 7.2 μg/24 hr (4–30 μg/24 hr)	(B33)

Very little copper is excreted in urine. Here the concentration values have little meaning; the 24-hour excretion is shown in Table 4. The figures in this table were taken from the careful study of Butler and Newman (B33). Bearn and Kunkel (B7) and Porter (P9) found similarly low urinary excretions. According to our own experience, the 24-hour urinary copper excretion does not exceed 40 μg in normal individuals. Others accept figures up to 60 or even 100 μg per 24 hours as normal (see references quoted in reference B33).

3.2. The State of Copper in Tissues; Copper Proteins

The physicochemical properties of the element copper are such that free ionic copper probably does not exist in appreciable amounts in the living organism. An exception may be the stomach contents where a relatively high degree of acidity may allow the solution of copper ions. In the rest of the body, copper is in a complexed, more or less tightly bound form with proteins, peptides, amino acids, and probably other organic substances.

Many, perhaps all, proteins have some degree of copper-complexing property. Of the serum proteins, albumin and transferrin have known copper-binding properties. When, however, copper is added to serum in excess of a 1:1 molar ratio in respect to albumin, then, in addition to albumin and transferrin, several other globulins will also bind some copper.

While many proteins in tissues may thus easily form copper complexes, there are a few proteins of which copper is an integral part. Copper in these proteins is part of the molecular structure and not in dissociation equilibrium with ionic copper in the solution; there is a characteristic ratio between moles of protein and atoms of associated copper. These and a few other criteria, discussed in detail by Vallee (V3), make these proteins a special class of metal-protein complexes and establish them as metalloproteins.

An ever increasing number of copper metalloproteins is being recognized. Those regularly present in mammals are listed in Table 5 with some of their characteristics. Other important naturally occurring copper proteins, such as hemocyanin, laccase, and ascorbic acid oxidase, are not listed since they do not occur in mammals. The metalloprotein nature of some of the proteins listed in Table 5 has not been established fully as yet. The search for further copper proteins, copper-protein complexes, and other forms in which copper may be stored or transported in the body must continue.

Many of these copper proteins are enzymes, and the copper is a part of their active group (Nos. 1, 10–14 in Table 4), while others have no known enzyme activity (Nos. 2–9). As far as is known, none of these proteins functions as a respiratory carrier, as hemocyanin does in mollusks. It has been suggested, but not proven, that the "human liver copper protein" of Morell *et al.* (M32) and the "hepatic mitochondrocuprein" of Porter *et al.* (P13, P15) may function as copper storage proteins, similar perhaps to ferritin in the case of iron.

It may be questioned whether all these proteins are separate entities. There are many similarities between hemocuprein and erythrocuprein, and also between hepatocuprein, horse liver copper protein, and human hepatocuprein. Porter suggested that the erythrocuprein of Markowitz *et al.* (P14), cerebrocuprein I, and human hepatocuprein may be identical proteins, although he recognizes some differences between them.

The copper in some of these copper proteins accounts for a sizable portion or all of the copper present in the organ compartment where they are found. Copper locked in ceruloplasmin accounts for 90–95% of the copper in plasma (H14). Copper bound to "human liver copper protein," according to Morell, accounts for as much as 80% of the total copper in the subcellular soluble fraction of liver (M32), and another 7% is accounted for

TABLE 5

COPPER PROTEINS IN MAMMALS

No.	Protein	Source	Molecular weight (g)	Color	Cu content (%)	Cu (gram-atom per mole)	Enzyme activity	Physiological role	Reference
1	Ceruloplasmin	Human plasma	160,000	Blue	0.34	8	+	Unknown	(H14, K3, P3)
2	Hemocuprein	Bovine erythrocytes	35,000	Blue	0.34	2	Not found	Unknown	(M10)
3	Erythrocuprein	Human erythrocytes	28,000–33,200	Colorless	0.32–0.36	2	Not found	Unknown	(K7, M11, S26)
4	Hepatocuprein	Bovine liver	(not determined)	Colorless	0.34	—	Not found	Unknown	(M10)
5	Horse liver Cu protein	Equine liver	30,000–40,000	Bluish green	0.34–0.41	2	Not found	Unknown	(M27)
6	Human liver hepatocuprein	Human liver	(not determined)	Green	0.3	—	Not found	Unknown	(P14)
7	Human liver Cu protein	Human liver	~10,000	Colorless	0.1–2.7	(variable)	Not found	Cu storage?	(M32, S25)
8	Hepatic mitochondrocuprein	Human and bovine neonatal liver	(not determined)	(not known)	2.4	—	Not found	Cu storage?	(P13, P15)
9	Cerebrocuprein I	Human and bovine brain	30,000–40,000	Bluish green	0.29	2	Not found	Unknown	(P10, P12)
10	Cytochrome c oxidase	Bovine heart	93,000	—	0.07	1	+	Electron transport	(A9, S5, W1, Y2)
11	Tyrosinase	Melanoma, skin	33,000	Colorless	0.02	1	+	Phenol oxidase	(B18, F6, P8)
12	Uricase	Tissues of mammals except primates	100,000	—	0.056	1	+	Urate oxidation	(M5)
13	Monoamine oxidase	Vertebrate tissues	255,000	—	0.07	4	+	Oxidative deamination of catechol amines and others	(Y1)
14	β-Mercaptopyruvate transsulfurase	Rat liver	10,000	—	0.82	1	+	Transsulfuration of β-mercaptopyruvate	(F1)

by Porter's hepatocuprein (P14). In human red blood cells, 60% of the copper is in the form of erythrocuprein; the rest is bound to another protein which has not been characterized as yet (S26). The last five copper proteins in Table 5 account for vitally important but very small portions of the copper in tissues.

Much work has been done with recently available electron spin resonance and nuclear magnetic resonance techniques to: (a) confirm the presence of copper in a protein, (b) elucidate its valence state in the protein, and (c) show a possible change in the valence state of copper on addition of substrate. These aspects are beyond the scope of this review and are well summarized, together with other recent developments in the general field of metalloproteins, in a recent comprehensive review by Malmström and Neilands (M7).

It was pointed out earlier, but bears repetition here, that the mere existence of these copper proteins and the indispensable function of some of them are perhaps the best proof that copper is an essential nutrient for man.

Ceruloplasmin, the copper protein in plasma, deserves special attention. Human plasma contains approximately 32 mg/100 ml of this protein (see Table 6) (C2, C12, R4). According to the recent careful measurements of Kasper and Deutsch (K3), the molecular weight of ceruloplasmin is 160,000, somewhat higher than the 151,000 obtained by Pedersen (P3). Ceruloplasmin is an α_2-globulin and contains 8 atoms of copper per molecule (H14). In addition to being a metalloprotein, it is a glycoprotein containing 7% carbohydrate: hexose, hexosamine, and neuraminic acid (L4). Ceruloplasmin has been prepared in a pure form from human and pig sera (H14). Several newer methods are now available for its isolation and purification (B29, C20, D4, L4, S4, S7, S39).

Ceruloplasmin has an intense and characteristic blue color, with a strong absorption maximum at 610 mμ (H14). The extinction coefficient in a 1-cm cell is $E_{610}^{1\%} = 0.68$ (K3, S16), which is very similar to the extinction coefficient that can be calculated from the data in Holmberg and Laurell's paper describing the first isolation of ceruloplasmin (H14).

Ceruloplasmin is an enzyme exhibiting oxidase activity against several substrates with a pH optimum between 5.4 and 5.9. The best known substrate is *p*-phenylenediamine or its dimethyl derivative. The oxidase activity is much weaker against other substrates such as hydroquinone, catechol, pyrogallol, DOPA, adrenaline, noradrenaline, serotonin (L4). The physiological substrate of ceruloplasmin, if any, has not yet been found.

The blue color and the enzyme activity of ceruloplasmin disappear when copper is removed from the molecule or when the protein is treated with reducing substances. The change in color upon the addition of reducing

substances is attributed to a change in the valence of copper (L4). The 8 copper atoms in ceruloplasmin do not have identical properties. Scheinberg and Morell (S16) found that 4 of the 8 atoms of copper are exchangeable with ionic copper in the presence of ascorbic acid. Curzon showed that, by digestion of ceruloplasmin with chymotrypsin, 4 atoms of copper can be liberated per mole (C17). By electron spin resonance studies (B16, B30, K4) and by magnetic susceptibility measurements (M8), it was established that 4 atoms of copper in ceruloplasmin are in the cupric state, and the other 4 are in the cuprous state. The blue color and the enzyme activity are dependent on the presence of the cupric atoms (B16). The color is not dependent on the presence of molecular oxygen in the ceruloplasmin molecule (M33).

The nature of the copper-protein bond in ceruloplasmin is not known. Copper-binding agents, such as diethyldithiocarbamate at pH 5.7–8.8 (L4, S16) or EDTA at pH 5.5 (L7), do not remove copper from ceruloplasmin. At lower pH, however, in addition to the above two, a number of copper-chelating agents will remove copper (L4). Lowering the pH below 4.75 alone leads to loss of the characteristic blue color (G12, H14). This effect recently has been shown to be reversible; thus, under certain conditions upon raising the pH there is very definite, although not complete, return of color and enzyme activity, and the typical hyperfine structure in the electron spin resonance spectrum reappears (C19). A reversible change in the nature of the copper-protein bond seems to have been produced under these conditions. Morell and Scheinberg (M30), employing conditions under which exchange between ceruloplasmin and ionic copper was shown to occur (S16), successfully prepared an apoprotein of ceruloplasmin, which contained only 4 atoms of copper per molecule, had no color, and was devoid of enzyme activity. Recombination of the apoprotein with copper occurred in the presence of ascorbic acid (M30). Further studies of this apoprotein and similar apoproteins produced by exposure of ceruloplasmin to Tris-hydrochloric acid buffer at pH 7.4 were recently reported by Kasper and Deutsch (K3). They found these apoproteins to have the same molecular weight as the native ceruloplasmin, but to show an increased asymmetry of the molecule, which suggests that some or all of the 4 atoms of copper that were removed play an important role in the maintenance of the secondary or tertiary structure of ceruloplasmin. One may speculate that the mere treatment of ceruloplasmin with acid leads to partial unfolding of the molecule by affecting the copper-protein bond insofar as it is involved in the maintenance of tertiary or secondary structure and that copper-binding agents and reducing agents then have easier access to copper attached to the unfolded molecule. Scheinberg *et al.* (S18)

suggested that 16 free carboxyl groups, probably in aspartyl or glutamyl residues, are the sites of copper binding in ceruloplasmin. Kasper and Deutsch (K3) have recent evidence against this contention. Their results suggest that histidyl and either tyrosyl or lysyl residues or both are involved in the binding of copper in ceruloplasmin.

It has been suggested that the ceruloplasmin molecule is heterogeneous, as are haptoglobins, transferrins, and certain other proteins (B28, M1, M31, R15, R16, S7). A genetic basis for this has been proposed (M31), but could not be proven. It may well be that the observed heterogeneity of ceruloplasmin is the result of partial denaturation, polymerization, or dissociation of the protein into several nonidentical subunits rather than the existence of several molecular species (K3, P16, P17, P18, R15).

4. Intake, Elimination, and Transport of Copper

4.1. Nutritional Aspects

The average North American diet contains 3–5 mg copper per day. Because of the ubiquitous presence of copper in food constituents and even in drinking water, it is difficult to devise a balanced diet composed of natural foods that contains less than 1 mg copper per day. The daily minimal requirement of copper for the adult man is stated to be 2 mg per day (C5). Infants require 0.05 mg/kg body weight per day (N1). These figures are only approximate and most probably far too high, since copper deficiency has not been produced with much lower intakes of copper (W3).

Copper balance studies recently performed by Kleinbaum (K9) in full-term newborn infants showed minor over-all loss or only a minimal degree of copper retention. Premature infants, without exception, were found to have a slightly negative copper balance. These findings suggest that in this age group there may not be a dependence on dietary supply of copper. The increased copper depots present in newborn and premature infants are apparently being used preferentially for growth requirements in this age group. Full-term infants, after the first month of life up to 1 year, without exception showed positive balances: a retention of 0.1–0.4 mg copper per day (K9). This indicates a need for dietary copper. Not knowing the efficiency of absorption from the bowel, one cannot predict nutritional requirements from these studies.

Naturally occurring copper deficiency has not been proven to occur in man, most probably because of minimal requirements and plentiful supply in the diet. The copper deficiency, suspected on the basis of low serum copper levels in infants with various types of protein-losing enteropathy (S48, Z3), is of doubtful pathognomonic significance.

Copper deficiency occurs naturally and can be produced experimentally in a number of animal species. The chapter on copper in Underwood's "Trace Elements in Human and Animal Nutrition" is an excellent source of information pertaining to this subject (U2).

4.2. Absorption of Copper from the Intestine

Thompsett (T6) remarked in 1940 that the study of copper absorption seems to have been neglected. He reports a single experiment on copper absorption in mice and concludes that acid facilitates, high calcium intake inhibits, and low calcium intake facilitates absorption. Gitlin *et al.* (G6) found in mice that the higher the oral dose of copper the more was absorbed. Their results suggest the existence of two mechanisms for absorption: a mechanism following first-order kinetics, and a second mechanism suggestive of an enzymatic process. Crampton *et al.* (C15), using everted sacs of hamster intestine, found maximal transport of Cu^{64} from the mucosal to the serosal side in the lower middle part of the small intestine. There was little increase in the rate of transport above the initial concentration of 1 μg/ml of copper in the medium. Under anaerobic conditions transport was much reduced. Findings in intact dogs suggest that maximal copper absorption occurs in the upper parts of the small intestine (S1).

Radioactive copper given by mouth to human subjects appears very rapibly in the blood (B9, C5, J1), which indicates rapid absorption, probably from the upper parts of the small intestine and stomach.

There is evidence to suggest that the form in which copper is present in food greatly influences its availability for absorption. Mills has shown that copper in herbage is in the form of water-soluble, negatively charged organic complexes, and that copper administered in this form is more effective in increasing copper stores in the copper-deficient rat than inorganic copper sulfate (M21–M23). It was suggested that copper may in fact be transported through the intestinal mucosa in the form of a negatively charged organic complex as well as in ionic form (M24).

While absorption of the copper contained in foods of plant origin is seemingly efficient, it has been reported that rats fed on raw meat developed copper deficiency. This suggests that the copper present in raw meat is not avaliable for absorption. Experience in this direction is well summarized and the pertinent literature quoted in a recent article by Moore *et al.* (M29). Guggenheim (G16), on the other hand, presents recent evidence to the effect that the "meat anemia" in mice is due to the high zinc content of meat in the presence of low but, by itself, adequate amounts of copper, and that a concomitant lack of calcium further aggravates the situation. One must wait to see whether this applies to the raw meat copper deficiency of

rats, and it must be explained why copper deficiency does not develop on a diet containing cooked meat, as Moore *et al.* (M29) have shown. A great deal remains to be learned about the effect of the form of copper in the diet on its availability for absorption.

The absorption of copper from the bowel can be influenced by a number of inorganic substances. Anions, which have a tendency to form highly insoluble salts with copper under the conditions prevailing in bowel contents, tend to reduce the absorption of copper. Sulfide is the best known of these. Cupric sulfide is poorly absorbed by rats and pigs (B21, S23). Addition of sulfides to the diet markedly reduces copper absorption in animals (D5) and man (C5). Of the cations, molybdenum is known to influence copper retention in animals. It is suspected that molybdenum influences the membrane transport of copper in such a way that absorption is decreased and excretion increased (D6). For details of this problem the reader is referred to Underwood's monograph (U2) and a recent review by Mills (M25). Problems of this nature have not yet been encountered in man. Oral doses of potassium sulfide and certain ion-exchange resins are used to reduce the absorption of copper in certain pathological conditions in man (C5).

Although certain factors influencing the absorption of copper from the intestine are recognized, we have no concept of the mechanism of absorption nor do we know the regulatory mechanisms which influence the rate of absorption. The suggestion of Scheinberg and Morell (S16) that ceruloplasmin may be involved in the regulation of copper absorption could not be substantiated.

4.3. Excretion of Copper

The main route of excretion of copper from the body is via the feces. Urine contains extremely small amounts of copper (4–30 μg/day in man) (B33). Sweat contains only negligible quantities (M26). There is very little copper in saliva, and it is recirculated. Insignificant amounts are lost by menstruation (L6).

The amount of copper in feces depends to a great extent on the dietary intake (K9). Copper output via feces is not a reliable measure of excretion of copper from the body since some of the dietary copper may pass through the gastrointestinal tract without being absorbed and thus may constitute a significant portion of fecal copper. It has been shown, however, by several investigators that copper given by the intravenous route is excreted very efficiently and almost exclusively via feces. Gitlin *et al.* (G6) found that, short of giving lethal doses of copper intravenously to mice, the higher the dose the larger the amounts excreted in the feces. In man, up to 33% of an

intravenous dose of radioactive copper can be recovered from the stools, and only a few tenths of a per cent of the dose appear in the urine (B9).

Most of the copper excreted from the body via the faces reaches the intestine by the bile. In dogs, 7% of an intravenous dose of Cu^{64} was excreted in the feces; 81% of this came through the bile, 13% passed through the intestinal wall directly, and only 0.5% in the urine (M6). In rats, Neumann *et al.* (N4) recovered 7% of a 3-μg intravenous dose of copper in the bile during 10 hours following the injection. Owen (O5) reported more recently that, following the intravenous administration of 5–100-μg doses of labeled copper to rats, 20–50% of the injected dose was recovered in the bile during 24 hours following injection; the higher the dose, the greater the percentage recovered in the bile. Figure 1 is a scintillation scan of the upper abdominal region of a normal human subject who received 24 hours earlier a 1-mg intravenous dose of copper labeled

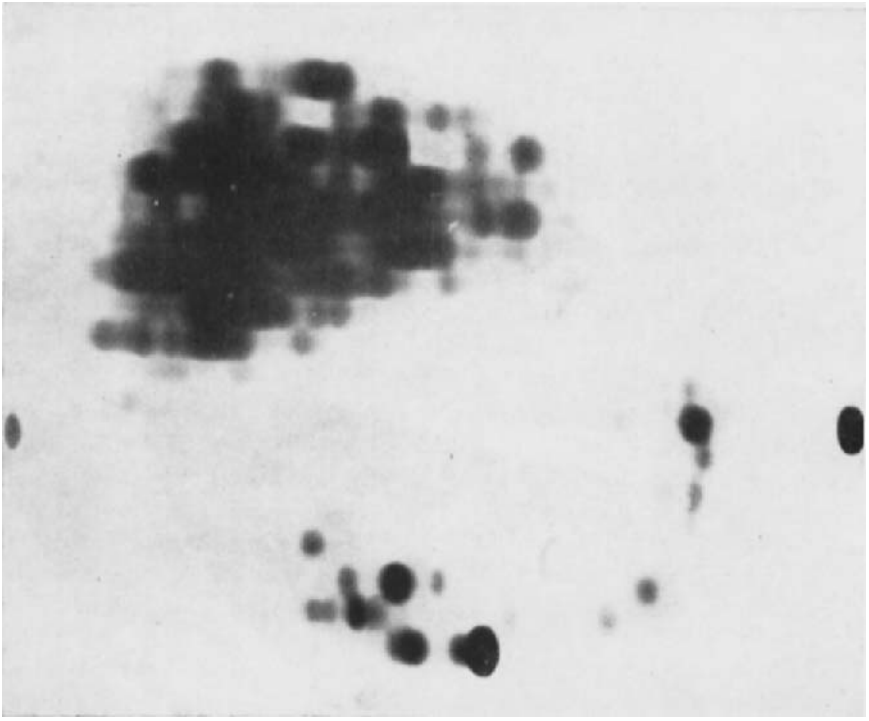

FIG. 1. Scintillation scan of the upper abdomen of a control human subject, taken 24 hours after the intravenous administration of 1 mg radioactive labeled copper. The triangular shadow represents the liver. The dots along the curved line below the liver represent radioactive material in the second part of the duodenum.

with Cu^{64}. The dark areas represent concentrations of radioactive material. The liver is outlined very clearly. In addition, a semicircular area of concentration of radioactive material can be seen, which most probably represents activity in the second and third parts of the duodenum. During the next 24 hours, this individual excreted 16% of the radioactive dose via the feces. It seems likely that the radioactive copper passed into the duodenum via the bile, probably following a sudden contraction of the gall bladder. It is hard to explain otherwise the presence of a high concentration of activity in such a localized segment of bowel.

Ceruloplasmin can be detected in the bile (J2), but the amounts found cannot account for more than a very small fraction of the copper in bile. Approximately 50% of the copper in bile is bound to various other proteins, and the rest is probably complexed to smaller organic molecules (H5). Hawke and Holmes (H6) found convincing evidence of an enterohepatic circulation of copper. To decide whether copper, once excreted via the bile, could be reabsorbed from the intestine, they cannulated the bile duct of a rat and connected it with a tube to the duodenum of a second rat. Following intravenous injection of radioactive copper into the first rat, activity could be detected in the blood, bile, and organs of the second rat. Obviously, the Cu^{64} excreted via the bile of the first rat was absorbed from the bowel of the recipient animal.

4.4. Transport of Copper and Distribution Following Oral or Intravenous Administration

Copper absorbed from the gastrointestinal tract is transported by the blood to the tissues. Following the ingestion of radioactive copper, there is a rapid increase in plasma radioactivity followed by a rapid fall (B9, B32, C5, E2, J1, M15). Copper administered intravenously as a single dose disappears extremely rapidly from the blood (Fig. 2). Half of a 0.1–1-mg dose of copper disappeared from the plasma compartment in less than 10 minutes. Bearn and Kunkel (B8) found that following the ingestion or intravenous administration of Cu^{64}, during the early stages, the Cu^{64} is bound exclusively to serum albumin. It seems that the portion of serum copper which serves as an immediate transport form of copper and is in equilibrium with tissue copper is not the large compartment (90–95%) bound to ceruloplasmin, but the 5–10% of copper loosely bound to albumin.

Ceruloplasmin apparently does not fulfill a function analogous to that of transferrin. It has been shown that exchange between ceruloplasmin-bound and ionic copper does not occur in the living organism, and that copper is incorporated into ceruloplasmin when the protein is formed and is released only in the course of decay of the protein molecule (S44). Con-

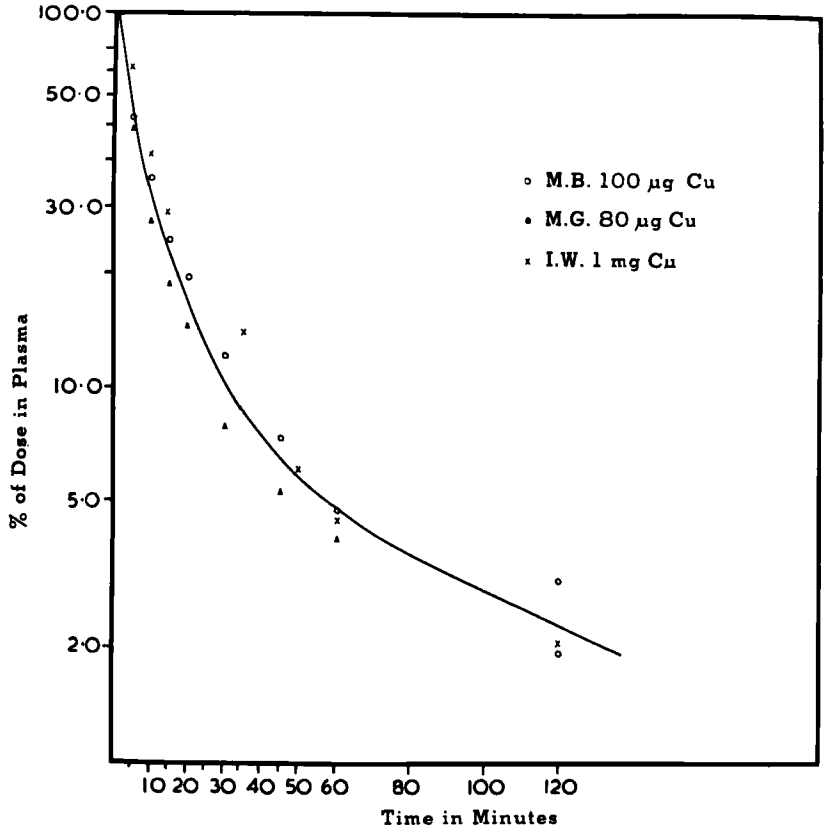

FIG. 2. Disappearance of Cu^{64} from the plasma compartment following intravenous injection of 100-μg to 1-mg doses of radioactive labeled copper in three human controls. The percentage of the dose remaining in the plasma compartment is plotted against time following injection.

sidering this together with the findings of Bearn and Kunkel (B8), outlined in the previous paragraph, it is apparent that the albumin-copper complex is the form in which copper is transported in the blood.

Moreover, Neumann and Sass-Kortsak (N3) have shown recently that, in equilibrium with albumin-bound copper, there is a third fraction of serum copper bound to amino acids. This fraction is rather small compared even to the albumin-bound fraction, but by virtue of its low molecular size it may have an important function in the transport of copper through membranes. Of the 21 amino acids present in human blood, histidine was the strongest binder of copper in competition with albumin. Some of the

evidence indicated that the true transport form of copper may be a complex formed by 2 different amino acids and 1 atom of copper. However, the isolation and chemical characterization of such complexes have not yet been achieved. Whether amino acid-copper complexes act as carriers facilitating the transport of copper to the cell membrane where acceptor sites with higher affinity for copper take over or whether copper is transported actually through the cell membranes as an amino acid-copper complex remains to be seen. In model experiments with dialyzing membranes, it was shown that, when an acceptor for copper (albumin) was present on both sides of the membrane and Cu^{64} was added to one side, the presence of amino acids greatly facilitated the transfer of copper through the membrane. Much remains to be learned about the actual transport mechanisms of copper from the blood to the tissue cells and through the tissue cells.

Returning now to the distribution of copper in the body, a large percentage of the copper which rapidly disappears from the blood is taken up by the liver. Bush *et al.* (B32) were the first to discover the concentration of activity in the liver following administration of radiocopper. Osborne *et al.* (O1, O4) in control human subjects found 66–95% of an intravenously injected 30–300-μg dose of copper in the liver 2–8 hours following injection. Results of a similar study (S8) in a control individual are shown in Fig. 3. The lower part of the graph shows that 86% of the intravenous 1-mg dose of copper was concentrated in the liver 4 hours after its administration. Thereafter, activity over the liver declined, a phenomenon also noted earlier by Osborne and Walshe (O1).

Two routes are available for the discharge of copper from the liver. One is excretion via the bile, for which circumstantial evidence is presented in Fig. 1; this is a scintillation scan obtained in the same normal individual whose results are shown in Fig. 3; it shows the concentration of radioactivity in the duodenum 24 hours after administration of the dose. This individual excreted 16% of the dose via the stool, most of which reached the bowel via the bile. The latter conclusion is probably correct on the basis of evidence presented in Section 4.3. The second route of discharge of copper from the liver is via ceruloplasmin. Ceruloplasmin is synthesized in the liver (O5); in the course of synthesis copper is incorporated into the newly formed ceruloplasmin which is then discharged into the blood stream.

It was shown by others earlier (B8, B9, B32, J1) and is demonstrated in Fig. 3 that in response to the administration of a single dose of Cu^{64} the initial rapid fall of activity is followed by a marked and sustained secondary rise in total plasma activity. During the first few hours following its administration, the Cu^{64} is exclusively in the albumin-bound form; during the secondary rise of activity, however, most of the Cu^{64} is bound to cerulo-

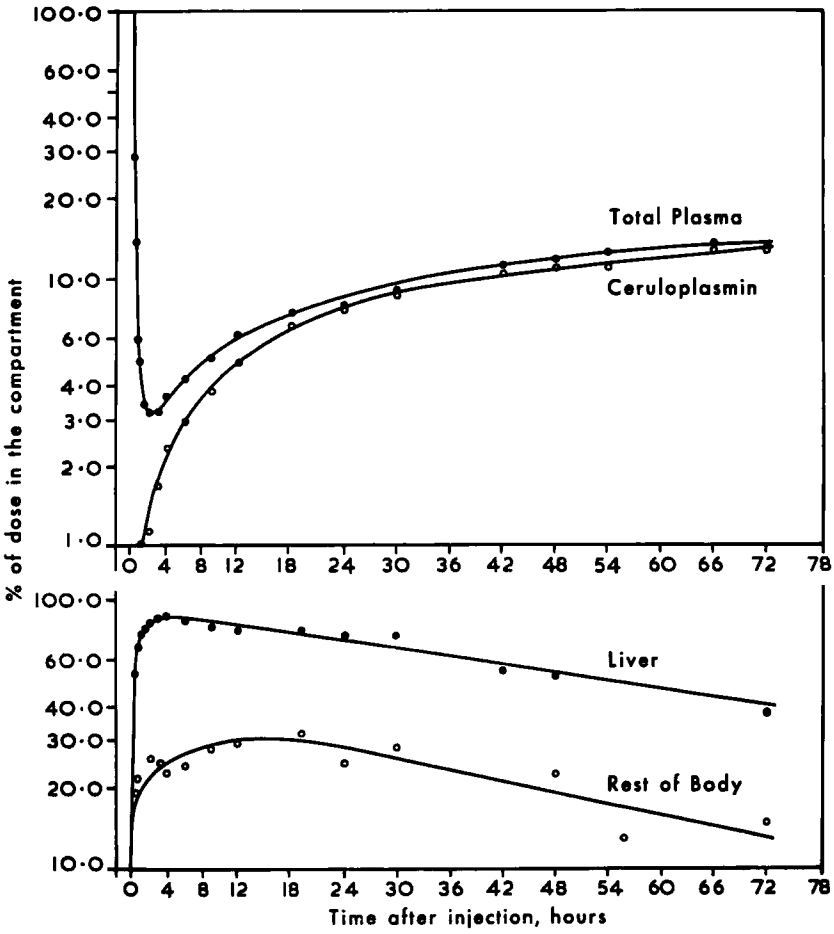

FIG. 3. The distribution of a single intravenous dose of 1 mg radioactive labeled copper (Cu^{64}) in the various body compartments of a human control. The percentage of the dose in the total plasma compartment, that bound to ceruloplasmin, that taken up by the liver, and that taken up by the rest of the body are plotted against time following injection.

plasmin (B8). The secondary rise of total plasma activity is due to the increasing appearance of ceruloplasmin containing labeled copper, most probably discharged from the liver.

During the course of the experiment shown in Fig. 3, the per cent of the dose contained in the liver decreased from 86% to 45%; thus there was a loss of approximately 40% of the dose from the liver. Fecal excretion,

probably via the biliary route, accounted for 16.4% of this, and by the end of the study a further 13.2% of the dose was in the circulating plasma, bound to ceruloplasmin. It seems that 30% of the 40% of the dose discharged from the liver is accounted for by the two routes. This is reasonable agreement, considering some margin of error in the results, as well as the fact that fecal reabsorption may have occurred and that some labeled ceruloplasmin most probably equilibrated into the extravascular pool of ceruloplasmin and thus escaped measurement.

Total body scintillation scanning following a single intravenous dose of radiocopper has not disclosed significant areas of concentration of activity except in the liver and, to a minor degree, in the kidney (S8). The latter is also evident from the earlier work of Maytum *et al.* (M17). In spite of a degree of concentration of activity in the kidney, only an insignificant part of this is excreted in the urine. In the course of the experiment shown in Fig. 3, only 0.6% of the dose was excreted in the urine. If an even distribution of the radiocopper in the body is assumed, except for the area of liver and kidney, and after correction of surface counts for activity in the circulating blood, it is evident that some of the injected radiocopper in the study shown in Fig. 3 was taken up by the tissues in the rest of the body. This uptake is transient, and the rapid rise of activity is followed by a more gradual decline (lower part of Fig. 2). It is probable that the copper from the tissues during this phase is gradually transferred to the liver.

Ceruloplasmin, with the copper locked in the protein, has a much slower turnover than the rest of the circulating copper. The half-life of ceruloplasmin in man was estimated as 4–7 days (S20, S42). Thus the turnover rate of this protein is faster than that of albumin or γ-globulin. Ceruloplasmin, as was mentioned previously, is most probably synthesized in the liver; however, the site of its degradation is not known. A most interesting recent suggestion by Broman (B27) ascribes to ceruloplasmin the function of a special copper carrier which transports copper from the liver to the various sites where cytochrome oxidase is synthesized. It is proposed that, "An elaborate prosthetic group comprising four cuprous-cupric pairs framed in a system of ligands may be transferred from the large carrier part of the ceruloplasmin molecule (which is catabolized) to the cytochrome oxidase unit under construction." This is a most intriguing suggestion that deserves further study.

5. Physiological and Pathological Variations of Copper Metabolism

From the foregoing considerations it is obvious that, in spite of considerable effort on the part of many investigators, the basic processes and mechanisms whereby copper is absorbed, transported, stored, built into body

constituents, and excreted by the living organism are by no means completely understood. How the various physiological and pathological factors lead to apparent alterations of copper metabolism is even less clearly defined. It is not possible for this reason to place these factors in logical order or to explain fully their effects or mechanism of action.

5.1. Effect of Age and Sex on Copper Metabolism

The copper content of fetal and neonatal tissues is much higher than in adults. This is well documented in man (F2, G1, G2, K11, N7) and in many animal species (U2). The most complete data on the copper content of human fetal tissues were reported recently by Fazekas *et al.* (F2). They measured the copper content of 29 different tissues in a total of 109 human fetuses of differing degree of maturity, including full-term newborn infants. The increased amounts of copper in fetal tissues are more impressive when compared on a dry weight basis, no doubt due to the higher water content of fetal tissues. If Fazekas' data in full-term newborn infants and the values of Tipton and Cook (T3) in adults on a dry weight basis are compared, it is evident that all newborn tissues contain higher amounts of copper. Liver, muscle, skin, and adrenals are particularly high, 18–20 times the mean adult concentration. The copper concentration of thyroid, testes, and uterus is over 10 times that of adults. The brain copper content, however, is only 2 times the adult's. Only in the liver is a gradually increasing concentration evident with increasing degree of maturity. In other tissues the copper concentration does not seem to change with age of the fetus. These increased copper depots in the newborn are obviously derived from the mother through the placenta. The copper content of the placenta is rather high (4–5 μg/g wet weight and 30–40 μg/g dry weight) (F2, K11).

The high tissue copper levels of the newborn decrease soon after birth. In three infants between 3 and 12 months of age Nusbaum *et al.* (N7) found the copper content of the liver and kidney to be within the normal adult range. On the other hand, Gerlach (G1) found high liver copper content in some infants up to 1 year of age. In children between 7 and 13 years of age the tissue copper concentrations were not different from those in adults (G1, S9).

The serum copper and ceruloplasmin levels vary quite markedly with age, as shown in Table 6. The mean levels and range of variation are well established in normal adults. The results obtained in three large series of subjects by three different groups of investigators show very close agreement. It has been known for some time that the serum copper content of newborn infants is much lower, about one-third of the normal adult range, and, more significantly, one-sixth of the values obtained in parturient

mothers (H9, H15, S2, S19). The low copper levels in the newborn are paralleled by similarly low serum levels of ceruloplasmin (Table 6). The neonatal levels of the small albumin-bound fraction of serum copper, which can be calculated as the difference between copper bound to ceruloplasmin (0.34%) and total serum copper content, are not significantly different from the maternal levels (S19) or from normal adult levels (C2). The placenta does not seem to permit the transfer of ceruloplasmin from mother to fetus, or only to a very small extent, and perhaps most or all of the fetal ceruloplasmin is synthesized by the fetus. The increased copper stores in

TABLE 6
CHANGES OF HUMAN SERUM COPPER AND CERULOPLASMIN LEVELS WITH AGE

	Copper		Ceruloplasmin		
	No.	(μg/100 ml)	No.	(mg/100 ml)	Ref.
Parturient mothers	12	216 (118–302)[a]	12	55.5 (39.4–89.0)[a]	(S20)
Newborns (cord blood)	12	36 (12–26)[a]	12	6.5 (1.8–13.1)[a]	(S20)
2 years	133	140 (95–186)[b]	134	42.6 (31.4–53.9)[b]	(C13)
6 years		129 (83–174)[b]		38.1 (26.9–49.2)[b]	(C13)
10 years		117 (72–162)[b]		33.5 (22.4–44.6)[b]	(C13)
Adults (21 years)	95	109 (69–150)[b]	136	30.8 (20.6–41.0)[b]	(C13)
Adults	—	— —	100	32.3 ± 4.9[c]	(R4)
Adults	235	114 (89–147)[b]	30	33 (25–43)[b]	(C2)

[a] Range.
[b] 95% limits.
[c] Standard deviation.

the newborn are apparently transferred from the mother via the albumin-bound fraction of serum copper, which seems to be in equilibrium on both sides of the placenta. The equilibration is probably further aided by the third, small-molecular size amino acid-bound fraction of the serum copper which has recently been shown to exist (N3). While this seems most plausible, the possibility cannot be excluded that ceruloplasmin is transferred from the mother through the placenta and that its rate of breakdown in the fetus exceeds the rate of transfer, therby leading to the marked difference in concentration on the two sides of the placental barrier.

Interesting age-dependent changes in the concentration of both serum copper and ceruloplasmin in children were recently found by Cox and Sass-Kortsak (C13). In a group of 134 healthy children from 2 to 15 years of age with close to equal numbers of males and females, there was no significant difference related to sex, but a highly significant ($P < 0.001$) negative regression coefficient with increasing age. The figures given in Table 6 for 2, 6, and 10 years of age were calculated on the basis of the

regression lines and their 95% confidence limits. From this it seems that by about 2 years of age the initially rather low levels of copper and ceruloplasmin rise above the normal adult range and then gradually decrease to the normal adult range, which is reached sometime between 13 and 16 years of age. That the method used for measurements is not the determining factor is shown by the fact that both serum copper and ceruloplasmin levels changed in a parallel fashion, and the values obtained by the same methods and the same investigators in adults approximate very closely the values found by two different groups of investigators in similar large series of normal adults. The reasons for and the physiological meaning of the marked changes in tissue copper content, serum copper, and ceruloplasmin levels with age are not known.

Cartwright and Wintrobe (C2) reported significantly higher levels of both serum copper and ceruloplasmin in normal adult females compared to males. Cox *et al.* (C13) in a somewhat smaller series could not confirm this. In the latter series there was no significant difference between the serum copper and ceruloplasmin content of males and females either in children or in adults.

5.2. Effect of Diet on Copper Metabolism

The copper content of the tissues and blood of animals is markedly dependent on the dietary intake (U2). This has not yet been reported in man. Premature infants could not be made copper deficient by being put on a diet containing very little copper (W13). This, however, may be explained by the presence of large copper depots in this age group (see Section 5.1). The presence of increased amounts of copper in drinking water derived from copper pipes has been shown not to influence the copper content of the liver in man (M2).

5.3. Effect of Pregnancy and Hormones

It is well documented that in normal pregnant women there is a gradual increase of both serum copper and ceruloplasmin levels during pregnancy, culminating in levels approximately twice normal at term (see Table 6) (H9, K15, M12, S2, S19). The copper content of the liver is also increased in pregnancy (G1). The high levels of serum copper and ceruloplasmin decrease rapidly after delivery to reach the normal adult range within 2 months (H9). The mechanism of the elevation of serum and tissue copper and serum ceruloplasmin in the pregnant woman is not known.

Administration of estrogens leads to elevation of serum copper and ceruloplasmin in both man and animals (E6, G4, J4, M20, R21, T9). Since there is an increased production of estrogens during pregnancy, it may be

assumed that this is the main reason for the increase in serum copper and ceruloplasmin. It has been suggested recently that the placenta is essential for the high levels of serum copper in pregnancy, and that hormones (estrogens or others) produced by the placenta are responsible for the alterations of copper metabolism in pregnancy (H7).

Administration of testosterone (C14, J4), thyroid hormones (M19), and ACTH (E5) has been shown to result in elevation of serum copper levels. The latter effect could have been due to contamination of the ACTH preparation with melanotropin (A2). Antithyroid drugs produce a fall in serum copper levels (F7).

5.4. Alterations of Copper Metabolism in Pathological States

More or less constant and in certain instances characteristic alterations of copper metabolism have been reported in a variety of human pathological states. Little is known about the mechanisms whereby the homeostasis of copper is disturbed in these conditions, although logical reasons can be given in a number of instances. These observations are restricted in most cases to a description of the alterations in the blood levels of copper and ceruloplasmin in the patients. Efforts to find an explanation for these changes and attempts to sort out the reasons for their development in the course of the disease are made infrequently. The reason is perhaps the tacit recognition that in most instances the disturbance in copper metabolism does not play a central role in either the causation or mechanism of the disease.

There is one notable exception. Hepatolenticular degeneration or Wilson's disease seems to be based on a genetically determined primary disturbance of copper metabolism. This condition is therefore in a class by itself.

Since there is no better classification, we shall discuss in the succeeding three sections the pathological disturbances of copper metabolism under three headings: conditions characterized by hypocupremia, by hypercupremia, and Wilson's disease.

5.4.1. *Diseases Characterized by Hypocupremia*

Hypocupremia and low blood levels of ceruloplasmin are one of the most characteristic biochemical alterations in *Wilson's disease.* This condition is discussed in a separate section below.

Hypocupremia and hypoceruloplasminemia of marked degree are found regularly in patients with *kwashiorkor,* a form of protein malnutrition with normal caloric intake and complicated by multiple vitamin deficiencies (E4, G9, L2, R8). Macdonald and Warren (M3) found that the copper content of the liver (on a fat-free dry weight basis) is significantly reduced

in these children, as compared to values in controls of the same age who died of other diseases. It is not known whether these changes are due to low dietary copper intake, decreased absorption from the gastrointestinal tract, or failure of the synthesis of ceruloplasmin. The finding of low copper stores in the liver seems to favor the former two factors. It is interesting to note that, in rats on a low-protein but normocaloric diet supplemented by essential minerals and vitamins, Allen and Schneiden (A8) found normal copper concentration in the liver. This suggests that copper deficiency in kwashiorkor is not due to the protein deficiency alone, but to the lack of some other essential dietary constituent, perhaps vitamins or minerals.

Low copper and ceruloplasmin levels have been reported in *marasmus* of infants (protein and caloric malnutrition) from Chile (M28) and India (G9).

Hypocupremia and low serum ceruloplasmin levels were reported in *celiac disease* (A13, L1), and in *tropical* (B36) and *nontropical sprue* (C1, S40). Oral administration of copper to the patients with tropical sprue resulted in a sustained rise of serum copper in only one of four patients (B36). Sternlieb and Janowitz (S40) have recently shown that in nontropical sprue the absorption of copper from the gut is impaired.

A syndrome consisting of *hypocupremia, hypoferremia, hypoproteinemia, edema,* and *hypochromic microcytic anemia* has been described in infants (K10, L1, S48, U1, Z3). The hypoferremia, anemia, and hypoproteinemia responded to treatment with iron; however, the hypocupremia required administration of copper as well. Nevertheless, dietary copper deficiency does not seem to be a factor in this condition (W13). These patients are most probably suffering from some form of protein-losing enteropathy, and are losing ceruloplasmin and transferrin along with other serum proteins through the gastrointestinal tract in excess of the ability of the synthetic mechanisms to replace these losses. Hypocupremia is found in protein-losing enteropathy in adults.

Low serum copper and ceruloplasmin levels are found regularly in the *nephrotic syndrome* with hypoproteinemia (C3, M12). These patients were found to excrete 46–75 mg ceruloplasmin per 24 hours in the urine, and this and perhaps the concomitant loss of albumin-bound copper most probably explain the development of hypoceruloplasminemia and hypocupremia in this condition.

Slight elevation of serum copper with significant reduction of serum ceruloplasmin levels was found in one large series of patients with *multiple sclerosis* (P7). What connection this has with the mechanism of the disease is not known.

In the majority of patients with *liver disease,* other than Wilson's disease,

the serum copper and ceruloplasmin levels are elevated. However, in one young patient with chronic hepatitis, paradoxical low blood levels of both copper and ceruloplasmin were found by Walshe and Briggs (W6); Wilson's disease was excluded in this patient on solid grounds. Very low ceruloplasmin and copper levels were also found by these authors in four cases of fatal hepatitis.

Low serum copper levels were reported in *vitiligo* (B13). However, the normal series of these authors exhibits a very wide range of variation of serum copper levels with 7 of 25 normal subjects having less than 50 μg/100 ml of copper. This is most unusual and points to either a methodical error or a peculiar type of population. The mean serum copper level of the group of 76 patients with vitiligo is lower than the mean of the normals subjects; however, statistical proof of the significance of this difference is not given.

5.4.2. *Diseases Characterized by Hypercupremia*

Hypercupremia is more commonly encountered in disease states than hypocupremia. It is well known that, in patients with *acute and chronic infections* due to a wide variety of viral, bacterial, or parasitic agents, the serum copper levels are elevated, probably due entirely to an increase in ceruloplasmin (B23, B24, H8, H13, L3, M12, R10).

Patients with *disseminated lupus erythematosus, rheumatoid arthritis, rheumatic fever,* and *glomerulonephritis* have increased blood levels of copper and ceruloplasmin (H8, H15). Other conditions with hypercupremia and hyperceruloplasminemia are *myocardial infarction* (A3, V2), various types of *anemia* (C6, H8, L3, P1, P2), *lymphogranulomatosis* (L3, P1, P2, R5), *leukemias* (L3, R5), *malignant neoplasias* of various forms (K5, K14, P1), and the *postoperative state* following major surgery (Z4). The mechanism by which serum ceruloplasmin and copper levels increase in these conditions is not known. According to one theory, ceruloplasmin is considered as one of the "acute-phase reactants" together with serum glycoproteins, seromucoid, mucopolysaccharides, and C-reactive protein. It is argued that increased blood levels of these, and of ceruloplasmin, are found whenever there is inflammation or other type of tissue destruction in the body. However, the argument in favor of this contention is weakened by the lack of positive correlation between serum ceruloplasmin and C-reactive protein levels in some of these conditions (R10).

Elevated serum copper and ceruloplasmin levels were recorded in various forms of acute and chronic *liver disease* (B10, G13, G17, P6, R2, R6, R14, S9). In a group of patients with portal cirrhosis the serum non-ceruloplasmin-bound copper was also elevated (G13). The elevation of serum copper and ceruloplasmin is most marked in biliary cirrhosis and, generally

speaking, in liver disease characterized by intra- or extrahepatic biliary obstruction. The blockage of the biliary excretory route of copper is most likely an important factor in the production of hypercupremia in these patients.

Finally, elevated serum copper and ceruloplasmin blood levels were reported in *schizophrenia* (A5, A11, B25), and it was postulated that this may be an important pathognomonic feature of this condition. Others, however, were unable to confirm this (F11, H17, S21). It seems that the higher copper and ceruloplasmin levels reported earlier in schizophrenic individuals can be explained, partly on the basis of a high incidence of acute and chronic infection in institutionalized mentally ill individuals and partly by the presence of low serum ascorbic acid levels which may influence the particular type of oxidase activity determination used in one of these studies (A5).

The copper content of tissues has been studied in some of the pathological conditions characterized by elevated serum copper and ceruloplasmin levels (B35, G1, G2, H21, I1, K11, N6, W16). Moderate but definite increase in the copper content of the liver and of the spleen was reported in chronic infections and malignant diseases, especially with metastases. Marked elevation of the copper content of the spleen was reported in patients with malignant disease complicated by infections (W16). Elevated copper content was also found in the spleen and to a lesser degree in the liver of patients who died of glomerulonephritis (W16). In patients with cirrhosis of the liver, the copper content of the liver and of other organs is quite variable. High liver copper content was reported in some cases, especially in some children with cirrhosis (B35). Unfortunately, Wilson's disease was not excluded in most of these instances. However, there can be no doubt that in some forms of cirrhosis, especially when biliary obstruction or intrahepatic cholestasis is an important part of the disease process, the copper content of the liver is elevated. For example, in a 6-year-old boy who died with a chronic biliary type of cirrhosis which developed following "neonatal giant cell hepatitis," we found 240 μg copper per gram (wet weight) of liver, a 20-fold increase over the upper limit of normal. Very high copper content of the liver was reported recently in adults with primary biliary cirrhosis (H21). Increased liver copper content in the presence of chronic biliary retention is not surprising, since the biliary tract is the main route of copper excretion.

5.4.3. *Wilson's Disease*

This condition was first described in 1912 by Kinnier Wilson (W14), an eminent English neurologist. It is characterized clinically by a peculiar

type of nervous system involvement, cirrhosis of the liver, and Kayser-Fleischer rings of the cornea. The disease seems to be based on an inborn error of metabolism, the exact nature of which is not clearly defined, but which leads primarily to a profound disturbance of the metabolism of copper.

5.4.3.1. *The Pattern of Inheritance.* The familial occurrence of the disease was recorded in Wilson's first description (W14), and has been confirmed by many others. Bearn's studies, first on 16 and later on 31 families with Wilson's disease, proved conclusively the autosomal recessive pattern of inheritance (B1–B3, B5). Accordingly both parents are as a rule heterozygous in respect to the gene of Wilson's disease; their children have a 1:4 chance to be homozygous normal, a 1:4 chance to be homozygous abnormal, and a 1:2 chance to be heterozygotes. There is a high incidence of consanguineous marriages among the parents (B3). The disease is somewhat more prevalent in males. The frequency of the abnormal gene in the American population was calculated to be approximately 1:2000 and the incidence of the disease 1:4,000,000 (B5). This may well be too conservative an estimate. It was suggested that the frequency of the abnormal gene may be higher in the population of southern Italy, among East European Jews (B2), and among Japanese (A12).

5.4.3.2. *Clinical Features.* The age of onset of the symptoms is highly variable. Late adolescence and the early twenties are the usual times of presentation, but the youngest patient was reported to have first developed symptoms at 4 years of age (C7) and the oldest during the fourth (S36) or even fifth decade of life (S14).

The initial neurological symptoms consist usually of clumsiness in the use of extremities followed by the development of tremor, rigidity, dysarthria, and dysphagia. Torsion spasms and abnormal postures may develop. Pyramidal signs may be observed rarely and epileptic seizures may occur. The electroencephalogram is normal until late stages of the disease (B26, S47). In the course of development of the organic neurological symptoms, psychic disturbances in the form of euphoria and other types of inappropriate behavior may appear. By careful testing, latent psychological impairment can be found before the appearance of neurological symptoms and signs (K12). Psychotic behavior occasionally may be the form of presentation of the disease, in which case the erroneous diagnoses of schizophrenia or hysteria often are made.

The early hepatic symptoms and signs are those of acute or chronic hepatitis, which then develops into a postnecrotic type of cirrhosis. There is nothing to distinguish the symptoms referable to involvement of the liver in the course of Wilson's disease from those of postnecrotic cirrhosis

due to a number of other etiological factors. Liver function tests are generally abnormal, with mild to severe jaundice of the regurgitation type, moderately elevated alkaline phosphatase, abnormal flocculation tests, low blood levels of albumin and elevated γ-globulin, low esterified cholesterol, and prothrombin deficiency and other clotting defects. Portal hypertension and bleeding from esophageal varices may develop relatively early in the course of the liver involvement in Wilson's disease.

In addition to the brain and the liver, the kidney is also regularly involved in the disease process, although this is clinically not obvious (B12, H12, W17).

A typical clinical feature of the disease is the presence of Kayser-Fleischer rings of the cornea. This is the only important clinical feature of the disease which Wilson missed in his original description. The rings were described, before Wilson's recognition of hepatolenticular degeneration, by Kayser (K6) and independently by Fleischer (F4) in patients with unrecognized forms of neurological disease and cirrhosis. Fleischer's case was properly diagnosed later as Wilson's disease (F5), and the rings were established as typical features of the disease. The rings consist of orange-yellow, sometimes greenish, pigment granules in Descemet's membrane close to the edge of the cornea; they are sometimes incomplete. During the early stages, the rings may be seen only with the aid of a slit lamp. Their presence is an absolute diagnostic criterion of Wilson's disease. They may nevertheless be absent during the early stages of the disease, and therefore their absence, especially in young patients, does not exclude the diagnosis. The pigment in Descemet's membrane contains copper; this has been proven histochemically (B22), spectrographically (G3), and recently by use of the electron probe (T7). A detailed histological, histochemical, and electron microscopic study of the rings was published by Uzman and Jakus (U6).

There are other less constant and characteristic clinical features of the disease. Azure lunulae were found in this condition (B11). Severe hemolytic crises were reported as early manifestations in some patients (G11). Some degree of increased destruction of red blood cells may be present throughout the disease, often leading to the formation of pigment stones in the gall bladder. Nonspecific changes in bones were reported by several investigators. These consist of generalized demineralization, spontaneous fractures, pseudofractures, osteomalacia, and even frank rickets (F3, M34, R18). Osteochondritis, periarticular fragmentation of bone, and osteoarthritis may also complicate the disease (F3, M34).

While many of the patients develop the full-blown clinical picture of the disease with involvement of the nervous system, liver, and kidney and with Kayser-Fleischer rings, a substantial number may have neurological

manifestations only and no demonstrable liver involvement. Others may present liver disease and die of cirrhosis or its consequences without developing the slightest sign or symptom of neurological involvement. In general, the younger the age of presentation, the more likely is the hepatic form of the disease. Patients with later onset, on the other hand, are more likely to present the neurological form of the disease. An acute and sometimes fulminating form of Wilson's disease may lead to death in a few weeks to a few months after onset of the first symptoms. This is more commonly seen in children (C8, L5, S28).

5.4.3.3. *Pathological Changes.* Pathological changes in the organs involved in the disease are numerous, but not specific for the disease. Detailed descriptions of the classical pathological features can be found in a number of earlier publications (D2, H1, W14). In addition to the classical picture of lenticular degeneration, the cerebral cortex and the cerebellum may also be involved, sometimes very severely (S22). The pathological changes in the liver are usually those of postnecrotic cirrhosis and sometimes Laennac's cirrhosis. Hepatic structural alterations (A10) and finer cytological changes in early stages of the disease were studied recently (G8, S12). Glycogen degeneration of nuclei, deposition of lipofuchsin pigment, and fine cytoplasmic fat droplets were found in early biopsy specimens (S12), but these changes can by no means be considered as specific for the disease. Electron microscopic changes consist mainly of an increase in the number and density of lysosomes, which are more numerous in the pericanalicular zone. Larger cytoplasmic pigment granules are also seen (S12). Schaffner *et al.* (S12) reported striking reduction of acid phosphatase activity by histochemical methods, but Goldfisher (G8) was unable to confirm this. Histological changes in the kidney—consisting of focal areas of degeneration, sloughing, and necrosis of tubular epithelial cells, and in other areas of tubular dilation and separation of the tubular cells from the basement membrane—were recently described (W17).

5.4.3.4. *Disturbance of Copper Metabolism.* A profound and characteristic disturbance of the metabolism of copper is the cardinal biochemical feature of Wilson's disease. The earliest evidence of this came from Germany, where in 1913 Rumpel (R20) found increased copper and silver content of the liver in patients with so-called pseudosclerosis. Siemerling and Oloff (S27) as far back as 1922 suggested that the "pseudosclerosis" of Westphal-Strümpell is actually due to accumulation of copper salts in the nervous system, liver, and eye. These early observations were not followed up for over 20 years, until Glazebrook (G7) in 1945 reported high copper content in the liver and brain of a patient with Wilson's disease, and increased

excretion of copper in the urine was found 3 years later by Mandelbrote *et al.* (M9).

It is now well established that patients with Wilson's disease have grossly abnormal amounts of copper in the liver, brain, kidney, and cornea (B19, C16, U6). The copper content of other tissues may increase, but much less than in these four organs that are the site of the pathological changes in this disease. Increased urinary copper excretion is also characteristic of the disease; as evidence for this a large number of references to pertinent literature can be found in Cumings' monograph (C16).

Scheinberg and Gitlin (S15) and independently Bearn and Kunkel (B7) reported in 1952 that in patients with Wilson's disease the blood levels of ceruloplasmin are very significantly lowered. This was established by the use of both immunochemical (S15) and enzymatic (B7) techniques and has been confirmed by many investigators. With increasing numbers of patients, however, it became obvious that, although the majority of patients had extremely low, sometimes undetectable, amounts of ceruloplasmin in the serum, a significant number of firmly diagnosed patients had half or three-quarters of the normal level (M12). Moreover, a small but significant number of patients with Wilson's disease were reported more recently to have perfectly normal blood levels of ceruloplasmin (E8, R19, S9). Rosenoer (R17) reported the interesting observation that the initially normal levels of ceruloplasmin in his patient (R19) decreased markedly to very low levels during treatment and efficient decoppering with penicillamine.

It has been suggested that certain environmental factors, particularly the increased estrogenic activity which may develop in the course of liver disease, could be responsible for the finding of normal ceruloplasmin levels in some of the patients with Wilson's disease. However, in two of these patients, normal serum ceruloplasmin levels were present at a relatively early stage of the disease when no other physical sign of estrogenic overactivity could be detected (S9, S10). In another patient, clinical evidence of liver impairment was completely absent (R17, R19). The suggestion (S13) concerning a qualitative abnormality of the ceruloplasmin in a patient with normal blood levels (S9) could not be substantiated by more careful retesting, including members of the patient's family (S10). Walshe and Carpenter (W7) reported recently that ceruloplasmin in sera of patients with Wilson's disease responds as an enzyme to certain inhibitors in a manner different from ceruloplasmin in sera of normal individuals. While this finding suggests that the ceruloplasmin in patients with Wilson's disease is different from normal ceruloplasmin, it is also possible that another factor present in sera of patients with Wilson's disease and absent

from normal sera is the reason for the altered response to the inhibitors. Thus no conclusive evidence has been presented so far to prove the contention that the ceruloplasmin present in the patient with Wilson's disease is qualitatively abnormal, although this possibility cannot be excluded.

The total serum copper levels are also usually reduced in patients with Wilson's disease (B1, C4). Considering the low ceruloplasmin levels, this is not surprising. While the amounts of copper bound to ceruloplasmin are reduced, the non-ceruloplasmin-bound, direct-reacting fraction of serum copper is elevated in these patients (C5). Those with normal levels of ceruloplasmin have a high serum copper, since they have an elevated blood level of non-ceruloplasmin-bound copper together with normal amounts of ceruloplasmin-bound copper (S9).

In order to explain the presence of vastly increased copper stores in the tissues of patients with Wilson's disease, it was suggested that the absorption of copper from the gastrointestinal tract is increased. The finding of markedly positive copper balances in seven patients by two groups of investigators (C4, Z2) was used as an argument in favor of this contention. However, the validity of this argument is questionable, since decreased excretion via the bile can equally well explain the experimental data. Whether the patient with Wilson's disease absorbs copper in excess of the normal amounts is an unanswered question.

Studies with radioactive copper have helped to delineate more lucidly the disturbance of copper metabolism in the patient with Wilson's disease. Numerous investigators have performed such studies (B8, B9, B32, E2, J1, M15, M16, M17, O1, O4, S8, S9, S10, S44). This type of investigation consisted mainly of the oral or intravenous administration of a single dose of radioactive copper, followed by a study of the fate of such a dose in the patients compared to controls.

After oral administration, the blood levels of Cu^{64} rise more rapidly and subsequently decrease more slowly in the patients than in the controls. The peak blood level is usually higher in the patient. This was also used as an argument in favor of increased copper absorption, but again the results can be equally well explained by decreased rate of removal of Cu^{64} from the blood. Following intravenous administration, the disappearance from the blood is slower in patients than in controls, which bears out the point above. The secondary rise of activity in the blood, due to the release of ceruloplasmin-bound Cu^{64} probably from the liver, is regularly seen in the controls, but never in the patients. The patient with Wilson's disease seems to be unable to incorporate copper into ceruloplasmin, and, interestingly, this is the case even in those rare patients with normal blood levels of ceruloplasmin (S9).

The distribution of an intravenous dose of Cu^{64} in the various tissue compartments was studied by several investigators (B32, M17, O1–O4, S8). The uptake of Cu^{64} in the liver, the main organ where it is concentrated, is slower than in controls (S8), which parallels the decreased rate of initial disappearance of Cu^{64} from the blood. In addition, the patient's liver takes up a smaller fraction of the dose than the normal liver (O1, O2, S8). Finally, activity in the liver of controls reaches a peak 4–10 hours after administration of the dose and is followed by a gradual and significant decrease of activity over the liver (Fig. 2), while in the patient with Wilson's disease a gradual slow increase of activity over the liver continues, and throughout the 3-day observation period a peak is never attained and apparently Cu^{64} is not being discharged from the liver (O1, O2, S8). By a scintillation scanning technique (S8) and use of a scintillation probe directed toward the lower pole of the left kidney (M17), a minimal degree of concentration of Cu^{64} can be detected in the kidney in the controls. In patients with Wilson's disease a much higher degree of concentration occurs in the kidney (M17, S8). It was also found in the patients that the rest of the body tissues take up a much higher fraction of the dose than in the controls (S8).

The pattern and extent of the excretion of Cu^{64} are markedly altered in patients with Wilson's disease. Following both oral and intravenous administration, the urinary excretion of Cu^{64} is higher in patients than in controls, whereas fecal excretion is much reduced in the patients (B9, B10, B32, E2).

5.4.3.5. *Other Biochemical Abnormalities.* Other biochemical abnormalities in Wilson's disease consist of renal aminoaciduria, glycosuria, and sometimes intermittent proteinuria (B6, B12, C12, S38, U4). Increased urinary excretion of urate and phosphate coupled with low uric acid blood levels and hypophosphatemia has also been reported (B12). These abnormalities are considered to be manifestations of renal tubular damage. The clearance of glucose, urate, phosphate, and α-amino nitrogen are increased, and the secretory capacity for *p*-aminohippurate is decreased (B12). In addition to these manifestations of tubular dysfunction, it was shown that the renal plasma flow and glomerular filtration rate are decreased (B12, H12). The degree of functional renal involvement roughly parallels the severity and duration of the overt disease (B12).

5.4.3.6. *Biochemical Findings in Patients during the Early Asymptomatic Phase and in Heterozygotes.* A question with both practical and theoretical implications is whether all these biochemical changes are present during the early stages of the disease when the patient is completely asymptomatic and all clinical signs and symptoms of the disease are absent. The practical implication of this question is the obvious importance of early diagnosis in

order to permit the institution of treatment before permanent damage has developed. Theoretically it is important to answer this question, since the earlier a manifestation of the disease is present, the more closely it must be related to the basic abnormality. The findings pertinent to this problem are summarized in Table 7. Findings in heterozygotes are included in a separate column for comparison.

TABLE 7

METABOLIC ABNORMALITIES IN SYMPTOMATIC AND EARLY ASYMPTOMATIC HOMOZYGOUS PATIENTS AND HETEROZYGOTES OF WILSON'S DISEASE

Abnormality	Homozygotes		Heterozygotes
	Symptomatic	Asymptomatic	
Serum copper	Usually low	Usually low	Rarely low
Serum ceruloplasmin	Low[a]	Low[a]	Rarely low
Non-ceruloplasmin-bound copper	High	Normal	Normal
Urinary copper	High	Normal	Normal
Copper in tissues	Elevated	Elevated	?
Handling of radiocopper			
Rate of clearance from blood	Slow	Normal	Normal
Incorporation into ceruloplasmin	None	None	Slow
Uptake in liver			
Initial rate	Slow	Normal	Normal
Extent	Low	Normal	Normal
Discharge from liver	Impaired	Impaired	Impaired
Excretion			
Urinary	High	Normal	Normal
Fecal	Low	Low	?
Other abnormalities			
Aminoaciduria	Present	Absent	Absent[b]
Phosphaturia + low serum P	Present	Absent	Absent
Uricosuria + low serum urate	Present	Absent	Absent

[a] Occasionally homozygous patients with Wilson's disease may have normal blood levels of ceruloplasmin (E8, R19, S9).

[b] Aminoaciduria was found in members of 2 families with Wilson's disease only (S35, U5).

Low serum ceruloplasmin levels, if present, are a constant feature of the disease. Very low levels have been found in completely asymptomatic patients as early as infancy (H3). The author has investigated two completely asymptomatic patients with Wilson's disease (3 and 21 years of age) and found very low serum ceruloplasmin and reduced serum copper levels. Asymptomatic patients similarly to the symptomatic ones show no evidence of incorporation of Cu^{64} into the ceruloplasmin. The copper content of the

liver is elevated early, during the asymptomatic phase of the disease. This was found by Harris and Scheinberg (H3) as well as by the author.

On the other hand, urinary copper excretion is surprisingly normal in the asymptomatic patient with a normal non-ceruloplasmin-bound copper level in the serum, and the urinary excretion of labeled copper following intravenous administration is not increased. Since in these individuals the initial rate of disappearance of an intravenously injected single dose of Cu^{64} is normal, together with a normal rate and extent of uptake of Cu^{64} in the liver, it seems logical to assume that cupruria and elevated non-ceruloplasmin-bound copper levels in the serum are secondary biochemical alterations in Wilson's disease which only appear at a relatively late stage, probably consequent to a high degree of saturation of possible binding sites for copper in the tissues, particularly the liver.

An early feature of the disease is the apparent inability to discharge copper from the liver; this is also present to a moderate extent in heterozygotes, but there are not sufficient numbers of observations to consider this proven. On the other hand, manifestations of renal tubular damage are not present in the asymptomatic patient, pointing to the secondary nature of these changes.

The finding of abnormalities in heterozygotes is also of both practical and theoretical importance. Low serum copper and ceruloplasmin levels are a rather inconstant finding in heterozygotes and the decrease is not marked (S10, S35, S43). A far more constant feature is the reduced rate of incorporation of Cu^{64} into ceruloplasmin noted by both Scheinberg's and the author's group (S10, S43, S45). Osborne and Walshe (O1) suggest that the maximum percentage of an intravenous dose of radiocopper taken up by the liver may be lower in heterozygotes than in normal individuals; this has not yet been confirmed. The author found in a few heterozygotes that following intravenous injection of Cu^{64} the secondary discharge of Cu^{64} from the liver was slower than in the normals.

5.4.3.7. *Treatment.* Effective methods of treatment are available for the patient with Wilson's disease. The most important are decoppering agents: dimercaprol (BAL) (B4, D3), ethylenediaminetetraacetic acid (EDTA) (B6, C5, M15), penicillamine (S41, W2–W4), and lately dithiocarbamate (S49). Adjuncts to the above are the administration of a diet low in copper and potassium sulfide (C5) or certain resins (B6) to decrease copper absorption from the gastrointestinal tract. Of the decoppering agents, penicillamine is both the most effective and the easiest to administer and is therefore the drug of choice. As a rule, patients should be treated continuously with an adult dose of 1 g/day. An important aspect of the treatment is that sometimes the response in the form of clinical improvement

may not be apparent for several months, and therefore reports of failure of this therapeutic approach based on trials shorter than 4–6 months (as a minimum) should be regarded with skepticism.

5.4.3.8. *The Nature of the Basic Metabolic Defect.* Finally, a few words should be said about the mechanism of Wilson's disease. There are several theories; however, none has been proven conclusively and the nature of the genetically determined basic defect is still not known.

Uzman (I1, U5, U7) suggested that the primary disturbance in Wilson's disease is one of protein metabolism, and that an abnormal protein or polypeptide with a very high avidity for copper is formed in these patients, the disturbances of copper metabolism being secondary in nature. Aminoaciduria and peptiduria, consequences of the altered protein metabolism, are suggested as the primary biochemical manifestations of the disease. This theory could not gain general recognition for several reasons. The main objection is the failure of the proposer of this theory or others to isolate and characterize the hypothetical abnormal protein and/or polypeptides. Secondly, aminoaciduria and peptiduria were not found to be particularly early manifestations of the disease, nor could aminoaciduria or peptiduria be found with regularity in relatives of patients, except in a family investigated by Uzman (U5) and a family investigated by Soothhill *et al.* (S35). Finally, the theory does not adequately explain many of the characteristic abnormalities of copper metabolism in this disease, in particular the presence of low serum ceruloplasmin levels and deposition of abnormal amounts of copper in the tissues at a very early age, long before aminoaciduria or peptiduria or other signs of a disturbed protein metabolism are evident.

A second general theory which contends that Wilson's disease is due primarily to a disturbance of copper metabolism is gaining wider recognition. The functional and morphological changes in the tissues and organs involved are regarded as consequences of the toxic action of the excess copper deposited in these organs. It fits in very well that the largest amounts of copper are found in those organs clinically and morphologically involved in the disease process (liver, brain, kidney). The fact that the removal of copper from the patient by the use of decoppering agents is followed by clinical and laboratory improvement is a good argument in favor of this theory. It has also been shown that copper in the amounts present in the tissues will interfere with essential intracellular metabolic processes (H2, V5).

By what mechanism the abnormal amounts of copper are deposited in the body is not known. Scheinberg and Gitlin (S15), finding very low serum ceruloplasmin levels in these patients, suggested that an inability of the patient to synthesize this protein is basically at fault. Absorption of copper

from the gastrointestinal tract was suggested to be controlled in some way by ceruloplasmin (S16, S17) and in the presence of low blood levels of ceruloplasmin, control over absorption to be lost, and copper to be absorbed in excess of the needs, leading to accumulation of abnormal amounts of copper in the tissues. The ceruloplasmin theory of Wilson's disease is most attractive but—in this reviewer's opinion—untenable for several reasons. Patients with normal blood levels of this protein nevertheless died of the disease. It has so far, not been possible to prove that the ceruloplasmin in these patients was in any way abnormal qualitatively. Second, in a number of pathological states other than Wilson's disease, ceruloplasmin levels may be as low as in some patients with Wilson's disease without development of clinical or biochemical changes similar to those in patients with Wilson's disease. It has also been found that patients with Wilson's disease, during treatment, will show further marked decrease in serum ceruloplasmin levels and, in spite of this, show marked clinical improvement. Finally, the control of copper absorption by ceruloplasmin was predicated on the assumption that an equilibrium exists *in vivo* between ceruloplasmin-bound and free copper. This, however, could not be proved (S44). A disturbance in the synthesis of ceruloplasmin is no doubt present in most patients with Wilson's disease; however, it seems to be an effect rather than the cause of the disease.

Evidence is accumulating to suggest that, in addition to the defect in the synthesis of ceruloplasmin, another mechanism whereby copper is discharged from the liver is also disturbed, namely, excretion of copper via the bile. There is only indirect evidence for this, consisting mainly of two observations:

1. Several investigators found normal amounts of copper in the bile of patients with Wilson's disease (C5, D3). Gitlin *et al.* (G6) suggested that in the presence of the large excess of copper regularly found in the liver of these patients much higher copper content of the bile should be expected, since this type of response can be obtained in animals overloaded with copper intraperitoneally. Gitlin *et al.* rightly conclude that the finding of merely normal amounts of copper in the bile of patients who are evidently overloaded with copper must mean that they are not able to excrete copper via the bile under conditions of overloading.

2. Following intravenous administration of Cu^{64}, patients with Wilson's disease excrete much less of the Cu^{64} in the stools than normal individuals (see Section 4.3). Since a high percentage of fecal Cu^{64} is excreted into the gastrointestinal tract via the bile, this also suggests that the biliary excretion of copper is impaired in Wilson's disease.

There is no reason why the defect in the synthesis of ceruloplasmin should

influence the excretion of copper via the bile, and vice versa. For this reason it seems logical to assume that the basic defect in Wilson's disease probably concerns a mechanism by which copper is made available, or presented in a suitably activated form, both for excretion via the bile and for incorporation into ceruloplasmin. Conceivably there are mechanisms of intracellular transport of copper in the liver cell, and a disturbance of these at one point may constitute a block which results in accumulation of copper in the cell and prevents excretion of copper from the cell both via the bile and via synthesis into ceruloplasmin and discharge back into the circulation. The problem, of course, is that next to nothing is known about intracellular transport mechanisms of copper. Further experimental work along these lines is needed.

5.4.3.9. *The Question of Genetic Homogeneity.* The section on Wilson's disease in this review was introduced by considering the genetic aspects of this condition. Following detailed discussion of clinical, pathological, and biochemical features of the disease, it seems pertinent to return to the problems of inheritance. The question has been rightly asked, "Is the great variability of the clinical, pathological, and biochemical features of Wilson's disease explainable as an effect of changing environment or are we dealing with a genetically heterogeneous condition?" (B3, B5). Conceivably what we now recognize as Wilson's disease is due to the abnormality of different alleles which may or may not be at the same locus. It is also possible that the variability of the manifestations of the disease is due to one or several modifying genes. While these possibilities cannot at present be excluded, it seems equally possible that the biochemical features studied to date are not the primary products of a single abnormal gene, and the inconstancy of these secondary manifestations need not be interpreted as evidence of genetic heterogeneity. This latter possibility was suggested above. The answer will perhaps be given by more detailed metabolic and genetic studies on large families with Wilson's disease, and by further inquiry into the nature of the basic abnormality in this condition. The latter will be possible only by increasing our existing meager knowledge of the intricate mechanisms whereby copper is handled by the human and animal organism.

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HYPERBARIC OXYGENATION

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1. Introduction

1.1. History

All nature is art, unknown to thee
All chance, which thou canst not see;
All discord, harmony not understood;
All partial evil, universal good.

Pope

During the past 10 years a progressive interest has arisen concerning the use of oxygen under pressure in the treatment of a variety of disease states. The idea of using pressures greater than one atmosphere is not new. Paul Bert's (B26) classic book on pressure physiology summarized virtually all experience obtained to 1878. In various centers at the end of the eighteenth

and beginning of the nineteenth centuries, pressure chambers were constructed for treating a variety of conditions. However the rationale was not easily discernible and the degree of pressure was not of an order of magnitude to result in therapeutic benefit. In 1928 an investigating team (B46) reported the construction of the Timken-Cunningham tank, in Cleveland, Ohio, to be 64 ft in diameter and five stories high. According to this report, Dr. Cunningham claimed that high pressure therapy had been useful in treating a variety of diseases including cancer, syphilis, tuberculosis, pleurisy, diabetes mellitus, and pernicious anemia. Dr. Cunningham never produced records to substantiate his claims, and 100% oxygen was never used. Apparently the tank was dismantled during World War II (J3).

Physiologists since the time of Paul Bert worked intensively on two important aspects of pressure physiology, oxygen toxicity and dysbarism. The dangers of inspired oxygen partial pressures ($P_{I_{O_2}}$)[1] greater than that breathed at one atmosphere were extensively investigated. This work formed the nidus of present day hyperbaric oxygen therapy. The contributions of Haldane (H1) and de Almeida (D5) deserve special mention.

Yet the idea that partial pressures of oxygen greater than one atmosphere absolute might have clinical usefulness does not appear to have germinated until the 1950's. At that time workers in Amsterdam, Glasgow, and London independently and simultaneously conducted experiments that have led to the enormous current interest in this aspect of high pressure physiology. The early work in Amsterdam has been reviewed by Boerema (B32), that of the Glasgow group by Illingworth *et al.* (I1), and that of the English group by Churchill-Davidson *et al.* (C14).

The resurgent interest in high pressure oxygen therapy in the United States can be traced in part to the early publications of these three European centers and in part to a visit made to this country by Drs. Boerema and Brummelkamp of Amsterdam. Interest in hyperbaric oxygenation increased at an astounding rate. Even Congress was informed and appealed to for funds (H11).

In July 1962 a Workshop on Oxygen Administration under Increased Atmospheric Pressure was held in Washington, D.C., under the auspices of the National Research Council (A1). There have been numerous symposia pertaining to high pressure oxygen therapy on local, regional, national, and international levels. Industries that never before manufactured pressure chambers suddenly became involved in this venture; some have dropped

[1] The symbols used throughout the text are consistent with the recommendations of the Pappenheimer Committee [*Federation Proc.* **9**, 602–605 (1950)]. $P_{I_{O_2}}$ should be read as the inspired partial pressure of oxygen. P_B should be read as the barometric pressure. $P_{a_{O_2}}$ should be read as the partial pressure of oxygen in arterial blood. $P_{A_{O_2}}$ should be read as the partial pressure of oxygen in the alveoli.

it with almost the same suddenness. Hospitals are vying with one another for funds to construct large pressure therapeutic and research centers; having a pressure chamber appears to have become a new status symbol.

Is high pressure oxygen therapy a fad that will soon fade? Is there a sound basis for current enthusiasm? What are the prospects for its future? What are the present problems and limitations associated with this new form of therapy? What areas require more intensive investigation if the full therapeutic potentialities of hyperbaric oxygenation are to be realized? It is the purpose of this review to discuss various aspects of this subject in an attempt to answer many of these questions.

Before launching into the subject, a word on nomenclature is required. Oxygen under high pressure (OHP) for use in therapy has many synonyms, and numerous abbreviations have been suggested: hyperbaric oxygenation, high pressure oxygen (HPO), high pressure oxygen therapy (HPOT), high oxygen drenching or hyperbaric oxygen drenching (HOD), and hybaroxic therapy.[1a]

1.2. Physical and Physiological Basis for Hyperbaric Oxygenation

A knowledge of the gas laws is an essential prerequisite for comprehending the physical basis of hyperbaric oxygenation.

Gases are composed of discrete particles in constant motion moving in straight lines at a high velocity. The pressure or tension of the gas is due to the bombardment of the containing vessel by the constantly moving particles. Pressure may be expressed in a number of ways; some of the more common terms are centimeters of water (cm H_2O), millimeters of mercury (mm Hg), pounds per square inch (psi), torr, or atmospheres (atm). Pounds per square inch can be expressed either in absolute pressure or as gauge pressure. Gauge pressures (psig) generally do not take into account the existing ambient atmospheric pressure at sea level. Similar confusion exists for the terminology of atmospheres (atm); another designation is atmospheres absolute (ata). It is advisable to refer to pressures in terms of either mm Hg, or pounds per square inch absolute (psia), or atmospheres absolute (ata). The relation between these terms is as follows:

[1a] This unnecessary proliferation of terminology also has a counterpart in confusion as to what hyperbaric oxygenation implies. Strictly speaking, any respiratory milieu containing oxygen at a partial pressure greater than 153 mm Hg may be co nsidered hyperbaric oxygenation. Generally, however, hyperbaric oxygenation implies use of increased atmospheric pressure, i.e., pressure greater than 760 mm Hg. Thus investigators employing 2 atm O_2 use the term hyperbaric oxygenation with the same accuracy as those employing 3, 4, or more. However, the physiological, biochemical, pathological, and clinical sequelae will vary markedly, depending on the pressures employed. It would appear that, whenever the term hyperbaric oxygenation or any of its synonyms is used, it should be accompanied by a parenthetical value for the specific pressure under consideration.

14.7 psia = 0 psig = 760 mm Hg = 760 torr = 1 ata = 30 inches Hg

One atmosphere is also equal to 33 feet of water. The gas law, based on the work of Boyle, Charles, and Gay-Lussac, states that when temperature is constant, the volume of a perfect gas is inversely proportional to the pressure; when the pressure is constant, the gas volume is directly proportional to the temperature. Avogadro's Law states that equal volumes of gases at the same temperature and pressure contain the same number of molecules. The actual number of molecules in a mole of gas is 6.023×10^{23}, and at 0°C and 760 mm Hg will occupy a volume of 22.4 liters. Dalton's Law states that each gas in a mixture of gases behaves as if it alone occupied the total volume and exerts a pressure, its partial pressure, independently of the other gases present. Thus the barometric pressure (P_B) or total pressure = $P_1 + P_2 + P_3 + \ldots + P_x$. The total pressure is equal to the sum of the individual partial pressures. Water vapor is a gas and obeys Dalton's Law. The vapor pressure tendency of a liquid to enter the gaseous phase depends upon the rate of escape of molecules from the surface of the liquid. Gases in contact with water receive water molecules by evaporation until the number of molecules leaving the liquid phase is equal to the number of molecules leaving the gas phase to return to the liquid phase. This evaporation, or escape of molecules from the surface of the liquid, is a function of the temperature of the liquid. The temperature of air in the lungs is 37°C, and alveolar air is thought to be in equilibrium with respect to water; the P_{H_2O} at 37°C is equal to 47 mm Hg.

Henry's Law states that the amount of gas dissolved by a given liquid, with which it does not combine chemically, is directly proportional to the partial pressure of the gas: if the pressure of a gas is doubled then the amount of gas physically dissolved in the solution is doubled. The constant which converts the proportionality to an equality in the Henry's Law equation is called the Henry's Law constant: this constant is the solubility coefficient of the gas in the particular solution. The solubility coefficient varies with the nature of the gas and liquid, the presence of solutes in the liquid, and inversely with the temperature. Thus at a constant pressure, but under hypothermic conditions, more gas can be dissolved in a given amount of fluid (tissue).

One of the most important physical laws upon which hyperbaric oxygenation and the decompression tables rests is the law pertaining to diffusion. Diffusion is defined as the passive transfer of a gas through a membrane from a region of high partial pressure to a region of relatively low partial pressure. It is a spontaneous process accompanied by a decrease in free energy; the sole cause of the transfer of the gas from one region to

another is the inequality of the partial pressure of the gas. The physical factors involved in diffusion were quantified by Graham: the rate of diffusion is directly proportional to the pressure and temperature of the gas and inversely proportional to the square root of the density or molecular weight of the gas. There are many factors that affect the rate of diffusion. The first is the thickness of the membrane through which the gas must diffuse—the greater the distance the slower the rate of diffusion. The second factor is the surface area available for diffusion—the larger the surface area available for diffusion the greater will be the rate of diffusion. The third factor is the solubility of the gas in the medium through which it has to diffuse—the more soluble the gas in the material through which it has to diffuse the faster will be the rate of diffusion. In living tissues this problem is compounded since one must contend with differential solubilities in the various tissues. Edema, by increasing both the distance of diffusion as well as affecting the solubility of the tissues for the gas, imposes a barrier to diffusion. The fourth factor involves the rate at which the gas is brought to the area for diffusion; this is dependent upon the blood supply to the area and would constitute a perfusion limitation. The fifth factor exists primarily for oxygen and is dependent upon the oxygen requirements of the tissues through which the oxygen is diffusing. If the tissues are actively metabolizing and utilizing oxygen, then it can be readily seen that the rate of oxygen diffusion across a given distance will be markedly decreased.

At sea level, the normal inspired partial pressure of oxygen is approximately 150 mm Hg. The alveolar partial pressure of oxygen ($P_{A_{O_2}}$) is approximately 100 mm Hg. The arterial blood in equilibrium with the alveoli contains very little oxygen in physical solution, approximately 0.3 ml/100 ml plasma. However, the blood contains approximately 20 ml O_2/100 ml blood. The explanation for the disparity between the volumes of oxygen dissolved in the plasma and blood at the same temperature and pressure is the presence of hemoglobin in the erythrocytes. This pigment has a great affinity for oxygen; 1 g hemoglobin will combine with 1.34 ml oxygen. Thus a normal hemoglobin concentration of approximately 15 g/100 ml blood is potentially capable of binding approximately 20 ml oxygen. At a P_{O_2} of 100 mm Hg, the hemoglobin is 97–98% saturated with oxygen. When 100% oxygen is administered at 1 ata to a normal individual, the $P_{A_{O_2}}$ will be increased and as a consequence so will the P_{O_2} of the arterial blood ($P_{a_{O_2}}$). The $P_{a_{O_2}}$ will rise to approximately 670 mm Hg. The hemoglobin saturation will be increased by 2–3% to become fully saturated; the total oxygen content of the blood will increase to approximately 22 ml O_2/100 ml blood. At the same time the volume of oxygen physically dis-

solved in the plasma will be increased 6–7-fold to approximately 2.0 ml O_2/100 plasma (Henry's Law). If this normal individual continues to breathe 100% oxygen and is now placed in a chamber in which the total ambient pressure is increasing, the $P_{A_{O_2}}$ and $P_{a_{O_2}}$ are increased correspondingly. During this compression there is no further increase in the percent saturation of the hemoglobin (since it is already saturated), but the amount of oxygen physically dissolved in the plasma is increased correspondingly (Henry's Law). At a pressure of 3 ata, for example, the $P_{I_{O_2}}$ is 2280 mm Hg, the $P_{A_{O_2}}$ is greater than 2100 mm Hg. This corresponds to an approximately 20-fold increase in the $P_{a_{O_2}}$ as compared to breathing air at 1 ata. In addition, the amount of physically dissolved oxygen is also increased 20-fold to approximately 6.0 ml O_2/100 ml plasma. The total oxygen content of the blood will be approximately 26 ml O_2/100 ml blood.

There is one very important shortcoming of the high oxygen partial pressure associated with physically dissolved oxygen at increased barometric pressures. As the oxygen is removed from solution the P_{O_2} decreases at a very rapid rate. At 3 ata and a normal oxygen consumption, the decrease in P_{O_2} from the arterial to the venous end of a capillary would be reflected by a change from 2100 mm Hg O_2 to 100 mm Hg. In contrast, an equivalent oxygen consumption in the presence of hemoglobin would reflect a change from 100 mm Hg O_2 at the arterial end to approximately 60 mm Hg at the venous end of the capillary. The marked difference in changes in the partial pressure of oxygen in the two situations is due to the peculiar properties of the hemoglobin molecule and its ability to bind molecular oxygen. In the presence of a low blood flow and/or abnormally active tissue metabolism, it would be possible to obtain a low venous P_{O_2} even though the arterial P_{O_2} is very high. If tissues should extract more than 6 volumes oxygen per 100 ml blood, then the 6 volumes of physically dissolved oxygen would not meet the oxygen requirements of the tissue, in terms of either the volume of oxygen available or the partial pressure required for its diffusion. In the case of shunts (failure to oxygenate returning venous blood before its return to the arterial circulation), OHP provides a means of adding sufficient oxygen to that portion of the blood which was normally aerated so as to compensate fully for the venous admixture. However, OHP, i.e., 3 ata oxygen, may provide in some cases just sufficient oxygen to result in a normal arterial value. In the case of severe shunts, to achieve the arterial oxygen tensions theoretically expected in hyperbaric oxygenation would require using pressures almost twice that now used clinically, i.e., at least 6 ata would be required.

The physical and physiological considerations discussed above as well as

other physiological factors (to be discussed under the appropriate topic) form the foundation upon which clinical hyperbaric oxygenation is built.

2. Some Specific Uses of Hyperbaric Oxygenation

2.1. Hypoxia

Oxygen lack not only stops the machine but wrecks the machinery.
Haldane

The implications of the preceding discussion are that, at the P_{a_2} greater than 2100 mm Hg that is attained by breathing oxygen at 3 ata, there should be an adequate volume of physically dissolved oxygen (equal to the average arterial-venous difference) as well as a sufficiently high oxygen pressure gradient for oxygen diffusion through the tissues. Theoretically almost any form of hypoxia could be overcome by breathing oxygen at 3 ata. Hypoxia due to carbon monoxide poisoning, hemorrhage, methemoglobinemia, anemia, alveolar diffusion barriers (edema, hyaline membrane disease), right to left cardiopulmonary shunts, ventilation-perfusion inequalities, inadequate tissue perfusion, etc., theoretically should be amenable to hyperbaric oxygen therapy.

The use of oxygen under pressure for satisfying the body's oxygen requirements was demonstrated by the dramatic experiments of Haldane (H1) 70 years ago and by the more recent experiments of Boerema (B32). Haldane successfully kept mice alive at 2 ata oxygen while their hemoglobin was completely saturated with carbon monoxide. Boerema has shown that, at 3 ata oxygen, decreasing the hemoglobin concentration of pigs to less than 1.0% resulted in no electrocardiographic evidence of myocardial anoxia. Certain physiological advantages accrue from eliminating the need for red blood cells; the resulting decreased viscosity could result in an increase in flow and decrease the work of the heart.

2.2. Respiratory Poisoning

His cup of joy is full when the result of his observations is put to immediate practical use.
Pasteur

2.2.1. *Carbon Monoxide*

Carbon monoxide is among the leading causes of death from poisonings. Subsequent to the experiments of Haldane, 1 ata oxygen alone or in com-

bination with carbon dioxide was used for the treatment of carbon monoxide poisoning (K8). The use of oxygen under pressure for treating carbon monoxide poisoning seems to have remained dormant until 1942 when End and Long (E3) successfully used 3 ata oxygen for treating carbon monoxide-exposed dogs and guinea pigs. Pitts and Pace (P16) showed that the half-life for the elimination of 20 vol% carbon monoxide in the presence of 2.5 ata oxygen was 19 minutes, in contrast to the half-life of 68 minutes in the presence of 2.5 ata air. Pace *et al.* (P1) found that the elimination of carbon monoxide under standard conditions followed the equation

$$COHb_t = COHb_0 e^{-kt}$$

where $COHb_t$ is the concentration of CO in the blood at time t, $COHb_0$ is the blood carbon monoxide concentration at time 0, and K is the rate constant. The K values for males and females (30-years old) are, at 0.2 atm oxygen, 0.0028 (min^{-1}) and 0.0039 (min^{-1}); at 1.0 ata oxygen, 0.0150 (min^{-1}) and 0.0199 (min^{-1}); at 2.5 ata oxygen, 0.0315 (min^{-1}) and 0.0460 (min^{-1}), respectively.

The Glasgow group in recent years has successfully used 2 ata oxygen routinely for treating carbon monoxide poisoning in man (D18, D19, L8, L9, S18, S22). The clinical success of this therapy has been confirmed (S14).

Douglas *et al.* (D18) have shown that 2 ata oxygen is more efficient than 1 ata oxygen plus 5% or 7% carbon dioxide in oxygen for eliminating carbon monoxide from the blood. The least efficient of the gases was 1 ata oxygen.

The theoretical reason for using hyperbaric oxygenation in carbon monoxide poisoning is based on Le Chatelier's theorem (if a system at equilibrium is disturbed, the system will shift in such a way as to minimize the effect of the disturbance) and the fact that it provides a rapid means for overcoming the hypoxemia. High oxygen tensions will favor the association of hemoglobin with oxygen and thereby increase the rate of dissociation of carboxyhemoglobin into its components, reduced hemoglobin and carbon monoxide. By this means the elimination of carbon monoxide is markedly facilitated. The most important aspect of hyperbaric oxygenation in this therapy is the alleviation of hypoxemia. For HPOT to be successful, the patient must be brought to a hyperbaric facility quickly (portable hyperbaric oxygen chambers are available and in use). The experience of the Glasgow group confirms the prediction that once anoxic damage occurs, it will not be reversed by oxygen. The ultimate prognosis of a given carbon monoxide poisoning case will depend upon many factors, not the least of which are the extent and duration of the poisoning prior to institution of therapy. All available evidence indicates that hyperbaric oxygenation is the treatment of choice in carbon monoxide poisoning.

2.2.2. *Cyanide*

There has been some indication in recent years that oxygen may be useful in the treatment of cyanide poisoning (P6). Ivanov (I2) showed that 2.8 ata oxygen can restore the cortical electrical activity of mouse cerebrum depressed by KCN. In the United States, Levine (L13) and Cope (C27) suggest the use of 100% oxygen in the treatment of cyanide poisoning. Bond (B33) found that cyanide-induced respiratory inhibition in insects (*Sitophilus granarius* adults and *Tenebroides mauritanicus* larvae) "could be partially overcome by increasing the oxygen concentration."

In contrast to mammalian experience, rapid resumption of aerobic metabolism by the insects after cyanide treatment was detrimental to the insect. However, the surprising deleterious effects appeared to be due to one of the products of the aerobic metabolism of cyanide (B34) rather than to oxygen or peroxide formation. The inhibition of catalase by cyanide appeared to have no obvious relationship to the enhancement of toxicity by oxygen.

A possible explanation for the beneficial effects of hyperbaric oxygen in the presence of cyanide, permitting survival of tissues and animals, may be the delivery of a sufficient volume of oxygen at a sufficiently high tension to the tissues to provide for a minimal level of extracytochromal metabolism.

2.3. Myocardial Hypoxia

There are numerous papers citing experimentally induced as well as clinical cases of hypoxia in which hyperbaric oxygen was successfully used to overcome the hypoxia and prevent the occurrence of untoward sequelae —and thereby permit a more rapid recovery. Nelson and Woodward (N2), using Cunningham's first chamber in Kansas, reported successful use of compressed air (21 psig) in relieving anoxemia caused by either pleurocentesis or injection of gum arabic into the lungs. Hyperbaric oxygen has been used in recent years to overcome the ischemia due to damage to, or occlusion of, peripheral, retinal, cardiac, and mesenteric arteries (I1, J1, M9, S9, S17, S19–S21, T1).

One aspect of hypoxia which has and still is receiving much attention, because of its potential therapeutic value, is coronary ischemia. Smith and Lawson (S21) reported that the administration of oxygen at 2 ata protected dogs against ventricular fibrillation during acute interruption of the main left circumflex coronary artery. Although the ligation resulted in ischemia to 40% of the ventricular muscle mass, the administration of OHP was able to maintain more of these hypoxic cells in a viable state. In essence these results were confirmed by Meijne *et al.* (M10), Petropoulos (P12),

Roshe and Allen (R3), and Van Elk and Trippel (V8). Harris and Hitchcock (H4) did not find OHP to effect the release of serum glutamic-oxalacetic transaminase (SGOT; a widely accepted diagnostic aid for clinically assessing suspected cases of myocardial infarction) in dogs following ligation of the anterior descending branch of the left coronary artery. On the surface, these data would tend to be at variance with the data indicating a protective effect on the heart against ventricular fibrillation. It appears, on the basis of Harris and Hitchcock's work, that destruction of heart muscle occurs following acute myocardial infarction even in the presence of OHP. One does not know whether OHP protected some of the cells in the ischemic area against hypoxic destruction. The question arises of how much myocardial mass must be ischemic (hypoxic) to trigger fibrillation. What degree of ischemia (hypoxia) induces fibrillation? What is the importance of the site of ischemia (hypoxia) in inducing fibrillation? What degree of ischemia will result in destruction of cardiac muscle? What degree of ischemia will permit the release of SGOT into the circulation and still prevent fibrillation? Harris and Hitchcock's results possibly indicate that less ischemia is required for SGOT release than for triggering fibrillation.

Hunter *et al.* (H16) were unable to confirm the findings of the beneficial effects of oxygen as reported by other investigators, but point out that their techniques for producing myocardial ischemia differed somewhat from those of other investigators. However, they feel, "There is sound theory for the idea of placing a human patient with ischemic myocardial damage under compression."

The importance of collateral circulation for the possible successful use of OHP in cases of myocardial infarction is stressed by Harris and Hitchcock as well as by Meijne *et al.* (M13). The role of the Thebesian vessels in maintaining myocardial metabolism should be considered. Since the arterial pressure distal to an occlusion approaches 0 mm Hg and the venous pressure, though low in absolute numbers, is higher than the arterial pressure, one can envision a retrograde circulatory oxygen supply to the hypoxic tissues. The efficiency of this venous blood in providing oxygen for ischemic tissues is markedly increased by hyperbaric oxygenation.

The promising results obtained in animal experimentation unfortunately have not had their counterpart in controlled clinical studies (C1): the mortality figures for the control and experimental groups were virtually identical. The marked variance between the theoretical and the practical requires an explanation. The most obvious factor with which to begin the search is the administration of oxygen, and here one finds the technique of administering oxygen to have been inefficient. If one assumes no dif-

ference between the techniques for administering oxygen to the cardiac cases and to the infectious disease cases at the same institution, one can readily see that the arterial oxygen tensions attained in cardiacs were much lower than the theoretically calculated maximum for 2 ata (K1). Improvement in the techniques of oxygen administration coupled with higher ambient pressures should result in improved clinical results. Further, the use of hyperbaric oxygenation should be coupled with techniques for improving the rate of opening of collateral circulatory pathways.

2.4. Asphyxia Neonatorum

As has been emphasized, regardless of the underlying cause, oxygen must be supplied to the tissues at a pressure of either one or more atmospheres in order to prevent both the early and later sequelae of acute hypoxia. Other areas in which hyperbaric oxygen may have potential use are the treatment of the respiratory distress syndrome of the newborn (RDS) and asphyxia neonatorum. Hutchison *et al.* (H17) did not find hyperbaric oxygen per se to be of significant value in the treatment of RDS; correction of the acidosis was superior to hyperbaric oxygenation in increasing survival: 1 of 6 patients treated with OHP survived, whereas of 27 patients in which the biochemical disturbance was corrected 16 survived; of the 16 survivors, 5 had also received oxygen under pressure. The finding of Hutchison *et al.* (H17) that hyperbaric oxygenation is not of value in treating suspected cases of respiratory distress syndrome of the newborn was recently confirmed by Cochran *et al.* (C18a). Hyperbaric oxygenation may relieve the hypoxia but has no effect on the respiratory acidosis. In addition it does not effect a more rapid dissolution of the hyaline membrane. A complex disease entity such as the respiratory distress syndrome would seem to require a therapeutic approach that combines some of the individual techniques currently used in various centers for the treatment of this disease. This combined therapy is envisaged as including the use of intermittent positive pressure breathing in conjunction with hyperbaric oxygenation, hypothermia, and proteolytic enzyme therapy. As more is learned about the chemical nature of the pulmonary surfactant, it too can be included in the therapeutic regimen. Although hyperbaric oxygenation may have to be limited because of the ever present dangers of oxygen toxicity (including retrolental fibroplasia), intermittent positive pressure breathing combined with hypothermia and proteolytic enzyme therapy can be continued at one atmosphere pressure in the absence of the pressure chamber. Although this discussion centered on the mechanical aspects of ventilation, consideration must be given to the role of the

circulation in the pathology of the disease. Any therapeutic advances made in the perfusion aspect of the disease should be considered for incorporation into the combined therapeutic regimen.

The use of hyperbaric oxygen for resuscitation of the newborn has created much controversy. Moss (M20) treated 20 infants with OHP. Apparent success was reported in 2 cases of neonatal asphyxia and in 3 other cases treated within 3 days of birth; what happened to the other 15 cases is not mentioned. Hutchison *et al.* (H18) concluded from their experiences in treating 65 infants, "Apnea neonatorum is not an emergency in which a controlled trial would be permissible, but it is our firm impression that hyperbaric oxygen is the most effective method of resuscitation yet devised for the severely asphyxiated newborn infant." The work has been criticized by Barrie (B8) and Davis and Tizard (D2) and defended by Hutchison *et al.* (H19) and Donald (D15). Cross *et al.* (C31) recently compared the efficacy of hyperbaric oxygenation and intermittent positive pressure breathing (IPPB), using 100% oxygen for resuscitation of asphyxiated mature fetal rabbits delivered by caesarean section. Their data clearly indicate that IPPB was superior to OHP for resuscitative purposes: of 17 rabbits treated with 4 ata oxygen there were no survivors, whereas 10 of 12 animals survived when given IPPB. These experiments also indicated that, contrary to the viewpoint of Goodlin (G14, G15) and Hutchison *et al.* and in agreement with the comments of Davis and Tizard (D2), cutaneous diffusion of oxygen was not of sufficient magnitude to influence the oxygenation of the animal. Hutchison *et al.* (H20) questioned the relevance of Cross's results to asphyxia neonatorum by claiming, "The efficacy of hyperbaric oxygen as a method of resuscitation in the human infant can only be decided by observations made on human infants under everyday conditions of obstetric practice. These conditions have not been sufficiently closely simulated in animal experiments to justify direct extrapolation of the results to humans." In contradiction to a previous statement (*vide supra*), Hutchison *et al.* stated plans to conduct a controlled experiment using human infants. A number of investigators (C30, D3, G25) subsequently pointed out that asphyxia implies not only hypoxia, but also respiratory acidosis, and that IPPB will not only improve oxygenation, but, in contrast to hyperbaric oxygenation, will assist respiratory exchange. In addition to pointing out that consideration should be given in the treatment of asphyxia neonatorum to the use of hypothermia alone or in conjunction with IPPB, with or without compression to 2, 3, or more ata, Gottlieb (G25) emphasized the importance and relevancy of comparative physiological studies.

2.5. Surgery

Skepticism is a useful tool of the inquisitive mind, but it is scarcely a method of investigation.

Thurber

The hypoxia resulting from deliberate occlusion of blood vessels during certain surgical procedures severely limits the time surgeons have available for performing connective or palliative operations. Boerema *et al.* (B30) in 1956 introduced the use of high pressure oxygenation to help ameliorate many of the technical difficulties by prolonging the safe period of circulatory arrest. In order to prolong the margin of safety for circulatory arrest, they suggested increasing the oxygen reservoir of the body by inhalation of oxygen at 3 ata. It would be theoretically possible, by combining hyperbaric oxygenation with hypothermia, to maintain relatively normal tissue oxygen tensions despite a diminished blood supply. Hypothermia not only increases the solubility of the gas, thereby permitting a given mass of tissue to hold a greater volume of gas, but decreases the tissue demand for oxygen; there is an approximately 6% decrease in oxygen consumption per degree fall in body temperature. The decreased metabolism also minimizes the chances of metabolic acidosis (providing shivering is prevented). OHP has an additional value in conjunction with the extracorporeal circulation; it increases the oxygenation capacity of the oxygenator and permits smaller priming volumes to be used.

Further benefits accrue from combining these techniques: the safety of procedures requiring low blood flow techniques is markedly increased, deep levels of hypothermia may be employed without irritation to the myocardium, and adequate oxygenation should allow for an undisturbed rewarming of the body.

Experimentally and clinically, hyperbaric oxygenation has been used in conjunction with hypothermia and extracorporeal circulation for numerous successful corrective and palliative cardiovascular procedures (B4, B25, B29, M14, R2, W4). Meijne (M12) emphasizes the need to separate the value of OHP during the actual period of cardiac arrest and the value of OHP "after the arrest period during lowered cardiac output." Because of the moderate oxygen stores of the body at 3 ata and the requirement for the large oxygen tension gradients between the capillaries and the mitochondria, he recommends that OHP "be used mainly in conditions where at least some circulation is present."

2.6. Shock

To understand the nature of disease
is the fundamental object of medicine,
for knowledge about a thing is the best
way to acquire power over it.
Selye

Hypoxia is one of the important factors that intensify the development of shock. The decreased blood pressure results in the production of a general ischemic hypokinetic anoxia (stagnant anoxia). Secondary anoxic effects such as hypotonic anoxemia only tend to compound the pathological effects of the shock syndrome. Boothby *et al.* (B36) emphasized the importance of oxygen administration to prevent the deleterious effects of the hypokinetic anoxia. On the basis of this suggestion, Wood *et al.* (W14) found 100% oxygen at 1 ata to be effective in treating dogs with mild peripheral circulatory failure produced by hemorrhage, trauma, or histamine injection. Probably because it can be more easily controlled, the most consistent results were obtained with hemorrhagic shock. Schnedorf and Orr (S5, S6) also reported on the beneficial action of 1 ata oxygen on the course of hemorrhagic shock produced in dogs. These studies stimulated Frank and Fine (F7) to study the effectiveness of oxygen under pressure in preventing the course of events in hemorrhagic shock. They found that preventing venous anoxemia by the administration of 3 ata oxygen in no way altered the events of the shock and concluded, "Oxygen therapy in shock is therefore of doubtful value." It was not until 1959 that OHP was resurrected for the treatment of shock. Burnett *et al.* (B47) found that 2 ata oxygen did not improve the survival of rats subjected to traumatic or histamine shock; it did protect rats during the acute hemolytic crises produced by glycerol. Esmond *et al.* (E4) and Moulton *et al.* (M21) found that 3 ata oxygen is beneficial in increasing survival of dogs subjected to experimental hemorrhagic shock and rats following Noble-Collip drum shock. The beneficial effects of OHP on the subsequent events of shock were confirmed in subsequent experiments (A12, C28, C29) and were attributed to the maintenance of the cellular oxidative processes despite the reduction of the normal oxygen transport mechanism.

There are conflicting reports concerning the value of OHP in endotoxin or bacteremic shock. Evans *et al.* (E7) found OHP to be beneficial in improving survival of dogs subjected to *Escherichia coli* endotoxin shock, whereas Blair *et al.* (B27) found no beneficial effects of oxygen on bacteremic shock (instillation of saline suspension of feces) in dogs; in other experiments they found that OHP increased survival of Noble-Collip drum-shocked rats and hemorrhage-shocked dogs.

The reasons for the discrepancies in the results between investigators is difficult to ascertain. Perhaps the manner in which the shock was produced, the extent and duration of the shock, the technique of administering the oxygen, and possible differences in premedication of the animals account for the differences. Some of these possibilities derive strong support from the work of Weale (W3), who found that in late stages of shock oxygen therapy becomes ineffective. Also Burnett's group used 2 ata oxygen whereas Esmond's group used 3 ata oxygen. In the case of endotoxin shock, Evans *et al.* employed an endotoxin obtained from a single organism whereas Blair *et al.* used a complex mixture of materials. Other marked differences were noted in the technique of anesthetization and maintenance of animals: Evans employed pentobarbital anesthesia and artificially ventilated the animals with a respirator via an endotracheal tube, thus assuring adequate oxygenation, whereas Blair *et al.* employed chloralosed animals and did not assist ventilation. There is need for more detailed studies using standardized techniques (anesthesia, premedication, and assisted ventilation should be used whenever possible) before final judgment is made on whether OHP has beneficial effects on bacteremic shock. The utilization of standardized techniques should simplify comparison of results from different laboratories and of the effects of oxygen in different types of shock, as well as aiding in the identification of underlying differences in the pathophysiology of different types of shock.

2.7. Infectious Diseases

The area in which hyperbaric oxygenation has produced the most dramatic results is probably in the treatment of anaerobic infections. The theory behind the use of oxygen as an antimicrobial agent is based on the fact that certain species of microorganisms are unable to grow in the presence of nascent oxygen. Therefore oxygen is applied to the site of inflammation to destroy the invading parasite. This was the basis for earlier oxygen and hydrogen peroxide treatments (*vide infra*) and for the present use of hyperbaric oxygen in the treatment of anaerobic infections. Boerema and Brummelkamp pioneered the development of present-day hyperbaric oxygenation in the treatment of clostridial infections (B31). They reasoned that an effective mode of therapy would be to envelop the advancing inflammation with oxygen through the capillaries and tissues by virtue of the high pressure oxygen gradient produced by the inhalation of pure oxygen under pressure. The result would be an oxidizing environment deleterious to the invading anaerobic microbe.

Cunningham (C33) in 1927 used compressed air for treating syphilis, diabetes, etc. As noted earlier, his work was not well documented and fell into disrepute. However his basic idea for using oxygen in the treatment

of syphilis is sound since the causative agent of syphilis, *Treponema pallidum*, is anaerobic. White (W9) described a case of gas gangrene which he successfully treated with oxygen and hydrogen peroxide. Hoge (H12) described six cases of gas gangrene which he treated with oxygen and hydrogen peroxide; not one case showed extension beyond the oxygen barrier. Hinton (H10) in 1947 described a case of gas gangrene which he successfully treated by the use of oxygen injections. He injected 1000 ml of oxygen every 4–5 hours in order to maintain a barrier around the phlegmon.

The use of oxygen under pressure for the treatment of experimental gas gangrene was not encouraging (D6), even though *in vitro* cultures of *Clostridium welchii* exposed to 3 atm oxygen were almost completely killed within 3 days. The spores survived this oxygen exposure (P2). Brummelkamp *et al.* (B42) reported on the successful treatment of *Clostridium perfringens* infections in guinea pigs by the use of OHP. In a later series of experiments they were not as fortunate in their results; their data were similar to those obtained by De Almeida (D6). Fortunately, the use of OHP for the treatment of clostridial infections in man was dramatically successful. The clinical work of the Amsterdam group has been summarized (B43, B44). This group administers seven sessions of hyperbaric oxygen therapy in a 3-day period, three exposures to HPOT on the first day and two sessions on each of the next two days. Each treatment consists of exposure to 3 ata oxygen for approximately 2 hours. Neither antibiotics nor anti-gas gangrene serum is used routinely. Antibiotics are used to prevent secondary infections. They prefer to perform any surgery for the repair of necrotic tissue after oxygen therapy, when the patient is nontoxic and improved. The use of OHP facilitated demarcation of the necrotic tissue. The successful use of HPOT in the treatment of clostridial infections has been confirmed by others (M22, S23, W2).

The Glasgow group concluded on the basis of their experience that HPOT "is an aid to, but does not replace, adequate surgery." They do not mention the treatment of gas gangrene without surgery. Also they used a therapeutic regimen different from that of Boerema's group; the Glasgow group used 2 ata for 2 hours as compared to the 3 ata used by the Dutch. It would seem that before a group comes to a conclusion about a certain technique or mode of therapy, different from that of those who devised and used it successfully, it should be incumbent upon them to employ the same conditions used by the innovators; otherwise unnecessary confusion results. There is a marked difference between hyperbaric oxygenation at 2 ata as compared with hyperbaric oxygenation at 3 ata. The diffusion of oxygen is greatly enhanced by the extra 760 mm Hg oxygen obtained by increasing

the pressure another atmosphere. This pressure difference may account for the somewhat divergent attitudes of the two groups. In addition, recent data have indicated that the Glasgow patients were not receiving the theoretical amount of oxygen; arterial oxygen tension varied in the range 600–1000 mm Hg (K1). The need for early and adequate oxygenation can be surmised from the studies of Glover and Mendelson (G12) and Kelley and Page (K6).

The use of HPOT in the treatment of tetanus has not met with the same success as for gas gangrene. The rationale for the use of HPOT in the treatment of tetanus is similar to that for gas gangrene. In addition to being caused by an anaerobic organism, the tetanolysin is supposedly oxylabile (W17). Kelley and Page (K6) were unable to show beneficial effects of HPOT in tetanus infection in mice, but were very successful in treating gas gangrene. On the basis of clinical and experimental results to date (B45, L16, P3, P4, S13, W2, W12), the consensus appears to be that HPOT may exert some beneficial effect. Whether any derived benefit is due to the oxygen working alone, or in conjunction with tetanus antitoxin, antibiotics, and sedatives, is at present unknown.

In an attempt to determine the mechanism whereby oxygen may be beneficial in the treatment of tetanus, Brummelkamp (B45) oxygenated tetanus toxin by bubbling oxygen through it for 2 hours at 1 and 3 ata. He found no diminution of its toxicity in mice. There is virtually no information at present concerning the oxygen tensions attained in infected tissues when treated with OHP. Since the *in vitro* lethal P_{O_2} for *Clostridium welchii* is approximately 90 mm Hg, it is surmised that in the clinical cases the minimal tissue P_{O_2} is of the same order of magnitude (B45).

The deleterious effects of oxygen is not confined solely to obligate anaerobes. Oxygen inhibits the growth of aerobic protista as well (A2, C18, G21, G24, K3, K4, M17, M18, W11). Gottlieb (G20) recently proposed the use of oxygen for the treatment of tuberculosis and Hansen's disease. This was followed by a detailed report (G24) in which it was shown that *in vitro* exposure of the tubercle bacillus to 3 ata oxygen for 2 hours twice daily resulted in marked retardation of its growth. The growth of drug-resistant strains of *Mycobacterium tuberculosis* as well as scotochromogenic and Battey-type organisms was also adversely affected. An interesting finding was the enhanced effectiveness of antituberculosis agents in the presence of high oxygen tensions. These effects of oxygen on the tubercle bacillus in *in vitro* experiments were confirmed in part by the *in vivo* studies of Allison *et al.* (A7). Although the *in vitro* data suggest a direct effect of oxygen on the microbe, the *in vivo* experimentation must include a consideration of possible physiological and biochemical alterations in the host

which may make the host more or less resistant to invading pathogens and thereby alter the course of an infection. In the case of experimental tuberculosis in rabbits, there was evidence of fewer tubercles in the lungs of oxygen-treated animals. In addition, the tubercles were of smaller size and with less caseation than those of the controls.

The possible use of oxygen under pressure for the treatment of sepsis has stimulated further *in vitro* investigations (H13, M6, N4). The pitfalls encountered in *in vitro* bacterial experimentation have been discussed in detail by Gottlieb *et al.* (G23). Some of the earlier findings on oxygen inhibition of bacteria, fungi, and protozoa (A2, C18, K3, K4, M17, M18) should be explored further for possible clinical development. There are a few isolated reports, but no systematic study designed to explore the possible existence of synergistic action between antimicrobial agents and oxygen tension in either *in vitro* or *in vivo* systems. In addition to the projected use of OHP for the treatment of Hansen's disease and tuberculosis, Gottlieb *et al.* postulated that OHP might be useful in combating other mycobacterial infections as well as infections caused by related organisms, e.g., actinomycetes. Pascale *et al.* (P4) used OHP for combating a fusiform infection. Nuckolls and Osterhout (N4) showed that OHP will inhibit the growth of Bacteroides species *in vitro* and *in vivo;* however, death occurred because of secondary infections. Bacterial infections caused by members of the genus *Neisseria* might prove amenable to control by hyperbaric oxygenation. Although *N. meningitidis*, unlike *N. gonorrheae*, is an aerobic catalase-positive organism, oxygen under increased pressure inhibits other aerobic catalase-positive organisms. In addition, the susceptibility of tissues to oxygen toxicity does not appear to be related to their catalase content (*vide infra*). If conventional antibiotic therapy is effective in controlling a disease, there is of course no need to employ hyperbaric oxygenation. Hyperbaric oxygenation may be the most propitious therapy for treating the fulminating type of meningitis, often found in military installations. In addition to a direct effect on the organism, hyperbaric oxygen may alter the blood-brain barrier (W16) and make it more permeable to antibiotics. Also, the use of agents that alter cell permeability, e.g. chlorpromazine, in conjunction with OHP and drugs (*sensu lato*) should be explored for facilitating the transport of drugs across the blood-brain barrier.

The use of small mammals for studying the effects of oxygen on sepsis presents many difficulties, as demonstrated by the experiments of Brummelkamp (B45). Small animals are quite prone to the pulmonary damaging effects of oxygen under pressure. Part of this susceptibility may be due to a change in the chemical state of the pulmonary parenchyma due to the

presence of an endemic pneumonia-type condition found in virtually all laboratory strains of small mammals. Another possibility is an exacerbation of the disease process by the oxygen. Such considerations point to the necessity for controlled studies using gnotobiotic animals.

The ability of an organism to grow in the presence or absence of nascent oxygen is one of the physiological determinants of bacterial taxonomy. On the basis of the relative susceptibilities of aerobic organisms to grow in the presence of 100% oxygen, at various total pressures, it is suggested that the concept of "aerobiosis" be extended to include the ability of protista to grow under various oxygen tensions. Such information with its ensuing ramifications may provide another physiological parameter for distinguishing between microbial species (G24).

2.8. Neoplasia

Another use of hyperbaric oxygenation, which has met with a certain amount of success, is in conjunction with radiotherapy for the treatment of neoplasias. This technique is based on the well-documented observation that normal and malignant tissues are less susceptible to the deleterious effects of X-irradiation when they are relatively hypoxic (G28, G29). This is true not only for mammalian cells, but for lower forms of life as well. The increased sensitivity of cells to X-irradiation in the presence of an increased oxygen tension approaches a factor of 3. This means that a smaller dose of radiation is required in the presence of oxygen to produce an effect equal to that obtained during anoxia.

A graphic representation of the oxygen tension–response relationship reveals a very steep rise in the curve with an almost sudden flattening. At the point of flattening there is no further significant increase in tissue sensitivity to X-irradiation in the presence of oxygen. It is at this point also that the increased sensitivity of the tissue to X-irradiation in the presence of oxygen is approximately equal to a factor of 3 (G28). The sensitivity factor is not identical for all cells; Trowell (T5) reported a sensitivity of 11 for rat lymph-node lymphocytes. Trowell's experiments also tend to confirm the well-known observation that sensitivity appears to be better correlated with oxygen availability than with oxygen utilization. According to Gray, the radiosensitivity of cells changes exceedingly rapidly in response to changes in oxygen tension. In some tissues, changes in sensitivity may occur within 2 seconds. The chief function of hyperbaric oxygenation is to provide a sufficiently high oxygen gradient so that by diffusion the entire tumor, especially the relatively hypoxic central portion, will be oxygenated and thereby rendered more susceptible to the damaging effects of X-irradiation.

On the basis of the above reasoning, Gray *et al.* (G29) irradiated Ehrlich

ascites tumor cells in mice breathing oxygen at 3 ata at the time of irradiation. In virtually all cases, tumor regression was greater in animals inspiring oxygen than in animals breathing air.

The preliminary studies of Gray *et al.* stimulated the English group at St. Thomas' Hospital in London to determine whether the auspicious laboratory experiments can be extrapolated for human use in clinical radiotherapy. Churchill-Davidson, Sanger, and Thomlinson first reported their results on 8 patients in 1955 (C13, S1). The successful destruction of parts of tumors which received both OHP and X-irradiation as compared to X-irradiation alone inspired them to undertake a more extensive trial using curative radiation doses on whole tumors. Periodic reports of their work have appeared (C14, C15, S2). As of April 1963 they had treated 160 patients with advanced disease; "38 (24%) of them had developed metastases outside the treated area and no less than 10 (6%) had developed a second primary tumor. . . . Although not uniform the overall response to treatment has been better than would have been expected from conventional radiotherapy, and in 15–20% of the cases has been quite dramatic."

Due to technical difficulties, Churchill-Davidson's group was unable to use the normal method of irradiation. Emery *et al.* (E2) overcame these technical problems by developing a portable "Perspex" chamber. Van den Brenk's (V7) group in Australia has successfully used OHP in conjunction with fractionated megavoltage irradiation in treating advanced malignant disease.

Wildemuth (W10) recently reported his experiences in treating 100 cancer patients and is very favorably disposed toward the combined use of hyperbaric oxygenation and radiotherapy in treating various neoplasias.

A note of caution should be introduced. Oxygen administration does not enhance significantly the radiosensitivity of well-oxygenated tissues. Hyperbaric administration of oxygen increases the radiosensitivity of tumors by providing oxygen to an otherwise hypoxic tissue. This implies that hyperbaric oxygenation would increase the radiosensitivity of poorly vascularized normal tissues. Radionecrosis of bone and cartilage is a reality (G29, H14). The dangers of radionecrosis may be overcome by the use of higher energy radiation since comparatively less radiation would be absorbed by bone. Another possible means of circumventing this difficulty would be through the use of a regional perfusion system which can oxygenate the infusion fluid either by an oxygenator or by means of hydrogen peroxide (M1). Irrespective of the mechanics of oxygenating the infusion fluid, it should be stressed that this type of procedure could be executed at increased ambient pressure. By use of a perfusion technique it may be

possible also to avert many of the toxic side effects of oxygen, alone or in combination with X-irradiation.

Many interesting animal experiments essentially confirm the initial animal experiments of Gray *et al.* and the subsequent clinical experience (G13, G29, T4, V1, V2, V5). Du Sault (D21, D22) recently has shown that the cure rate of spontaneous mammary adenocarcinomas in C3H mice, irradiated while breathing a mixture of 95% oxygen and 5% carbon dioxide at 1 ata, is the same as in mice breathing 100% oxygen at 3 ata. He found no increase in normal tissue reactions. In view of these findings he suggests that the technique of choice is irradiation while breathing O_2-CO_2 mixtures at 1 ata. The enhanced effectiveness of the O_2-CO_2 mixture was postulated as being due to the CO_2 stimulation of respiration and vasodilatation resulting in more oxygen being delivered to the tumor. Unfortunately tissue oxygen tensions were not obtained.

In general, the results of radiotherapy in the presence of oxygen have been promising. However, complete "kill" apparently is not obtained, albeit there have been a number of "cures." Histological and pathological studies suggest that within tumors there are areas which are poorly vascularized and thus poorly oxygenated. The hypoxic environment renders some of the cells relatively radiation-resistant and permits them to survive radiotherapy. The surviving cells are thought to be the cause of later recurrence of tumor growth. Even with the use of hyperbaric oxygenation there may be areas of the tumor that remain relatively hypoxic; in addition, one is dealing with a population of cells with varying degrees of radiosensitivity. The fact that portions of tumors are poorly oxygenated and that, even in the presence of 3–4 ata oxygen, the theoretical oxygen tension in the tumor is not attained has been amply confirmed by oxygen tension measurements made with the oxygen electrode (C9, C10, C15, E5, E6, V1, V7).

At present, there is usually a 15-minute equilibration period of the patient with oxygen prior to irradiation. In the future, pressures greater than 3 or 4 ata may have to be employed to assure adequate tumor oxygenation, and confirmation of the degree of oxygenation by micropolarographic electrodes may be required. Ultimately, such measurements would control the degree of pressurization required for each specific tumor to be irradiated.

2.8.1. *Radiomimetics and Other Antitumor Compounds*

Because of the increased sensitivity of tumors to X-irradiation therapy in the presence of increased tumor oxygen tension, and because nitrogen mustard has an effect on tumors similar to that of ionizing radiation, some investigators have postulated that the tumoricidal effect of cytotoxic drugs

may be enhanced by the presence of an increased oxygen tension in the tumor.

Before discussing the subject of radiomimetic drug effects in the presence of high oxygen tensions, one should know first the effect of oxygen alone on the *in vivo* growth of tumors. De Almeida (D5) found that increasing oxygen tensions results in increasing destruction of the "fuso-cellular sarcoma of Roffo." In order to increase this differential effect of oxygen between tumors and the host, de Almeida resorted to fasting the animals; fasted animals were found to be less susceptible to oxygen toxicity. Campbell (C3), also using fasted animals, was unable to show oxygen destruction of the Walker rat tumor, mouse sarcoma 63, or the Twort mouse tumor. Kluft and Boerema (K11), using the mouse malignant mammary tumor TM 8013, found that intermittent hyperbaric oxygenation (2 hours, 3 ata, twice daily) retards the rate of growth of the tumor and depresses lung metastases. However, there was no significant increase in life expectancy of the mice. Antopol *et al.* (A9) studied the effects of pressure on the growth of carcinoma 755 and sarcoma 180 in mice. They exposed tumor-bearing animals to air at 70 psig for 2–6 hours on 5 consecutive days for 2–5 weeks and found a slower rate of tumor growth. They attributed the slower growth rate to the increased pressure. However, slower tumor growth rate also can be interpreted as being due to the effect of oxygen, rather than to pressure. The puissance of this interpretation is based on the fact that at 70 psig air the P_{O_2} is equivalent to that of 1.1 ata 100% oxygen. In light of the possible effects of oxygen on tumors found by others, this interpretation appears to be valid (A10). In order to differentiate between the effects of pressure and the effects of oxygen, it would be necessary to expose one group of tumor-bearing animals to 1.1 ata oxygen (866 mm Hg) and another group to 70 psig of pressure but maintaining the partial pressure of oxygen at 152 mm Hg. It cannot be stated at present what the effects of pressure are on the growth of tumors *in vivo* or *in vitro*. Neither can it be said that the increased tensions of the other gases, especially nitrogen, are without effect. Possible synergistic effects of these physical parameters remain to be studied.

The conflicting effect of increased oxygen tensions on the destruction of tumors is due in part to the different therapeutic regimens and oxygen tensions employed. The remaining explanations ascribe the different findings to differences in the physiology and biochemistry of these tumors as well as to differences in the unique host-parasite relationship that exists between each tumor and the affected organism. All these factors will have to be studied in sufficient detail before a comprehensive explanation for the successes and failures emerges.

The effect of oxygen in combination with tumoricidal agents has been studied by a number of workers. Gray *et al.* (G29), in contrast to their results with X-irradiation, did not find the effectiveness of mechlorethamine to be enhanced by the increased oxygen tension. Krementz and Knudson (K12) reported a potentiation by oxygen of the tumoricidal effects of nitrogen mustard (mechlorethamine) on Ehrlich ascites tumor and sarcoma 37 in mice. Back *et al.* (B1–B3), studying the effect of varying oxygen tensions on the *in vitro* and *in vivo* action of nitrogen mustard on normal animal tissues and tumor cells, found that the sensitivity to nitrogen mustard in the ascitic and solid forms of sarcoma 180, leukemia 1210, and 6C3 HED Gardner lymphosarcoma remained unaltered even in the presence of 100% oxygen at 4 ata. In addition they were unable to detect oxygen potentiation of cytotoxic effects of nitrogen mustard on the growth rate of *Vicia faba* bean root tips. The conflicting results reported by Gray (G29) and Krementz and Knudson (K12) and by Back *et al.* (B1–B3) have each received support from other workers. Leather and Eckert (L11) found that the maintenance of oxygen saturation of the tumor by OHP at the same time that nitrogen mustard is infused through the tumor results in an oxygen potentiation of the tumoricidal effects of nitrogen mustard on the spontaneous mammary carcinoma of Swiss-Webster mice. Kinsey (K10) reported a potentiation of the tumoricidal effects of 5-fluorouracil on the Cloudman S-91 melanoma in DBA/2 mice.

In contrast to these positive findings, Marshall *et al.* (M3) reported no increase in the therapeutic ratio for nitrogen mustard with increases in oxygen tension. However, these experiments were performed at 1 ata with a partial pressure of oxygen of 0.2–0.6 ata. De Cosse and Rogers (D7), in separate experiments using nitrogen mustard and amethopterin, were unable to detect synergistic effects between these cytotoxic agents and oxygen, 6 hours daily at 2 ata, on the AMel 4 hamster melanoma. In 6 patients with advanced malignant disease, Adams *et al.* (A4) found no clinical benefit from combined OHP (2 ata) and chemotherapy.

At the present time no concrete generalizations emerge concerning the effectiveness of combined OHP and cytotoxic agents in tumor therapy. Obviously there is room for further exploration. There is no a priori reason for believing that a synergistic action will exist between oxygen and any drug or combination of drugs. Too little is known of the mechanism of action of all these substances and the physiology and biochemistry of the tumors to make valid predictions. Further inquiries into dose-effect relationships between different drugs at varying oxygen tensions are required. In order to assure concentration of the cytotoxic agent in the tumor, it is suggested that perfusion techniques be used. The use of drugs and radio-

therapy in conjunction with oxygenation may be another fruitful area of investigation.

It would be desirable to have oxygen tension (availability) measurements of tumors at the time of therapy. Such information would be invaluable in deciding whether the success or failure of certain forms of therapy could be correlated with tumor oxygen tensions. In addition, such measurements would permit varying the total pressure from animal to animal to assure a constant tumor oxygen tension. When interpreting data, attention should be given to whether the tumor is a spontaneous tumor or an implanted tumor; oxygen may exert an effect on the immunological response of the homograft rejection mechanism. Such possible difficulties may be circumvented by using spontaneous tumors. New insights into immunological mechanisms may be obtained with the implanted tumor technique.

Up to this point the discussion has centered on the possible usefulness of oxygen—alone or in combination with radiotherapy or cytotoxic agents—for the treatment of neoplasias. However, the question arises of the effect, if any, of oxygen alone or in combination with drugs (*sensu lato*) on inducing tumors in animals. There is a marked paucity of information in this area. Heston and Pratt (H8, H9) found that oxygen alone does not increase the rate of spontaneous pulmonary tumors in genetically susceptible strain A mice. However, in the presence of a carcinogen (dibenzanthracene) there is a positive correlation between increased oxygen tension and increased induction of pulmonary tumors. The oxygen had to be administered at the same time as the drug. Di Paolo (D10, D11) found the incidence of lung tumors to increase in the offspring of pregnant strain A mice given urethan 24 hours prepartum and immediately exposed to a hyperoxic or hypoxic environment.

2.9. Hyperoxygenated Solutions

> . . . *that effective research requires an environment that is not distracted by the immediate needs of industry.*
> Bienfait

In recent years there has been an interest in a new and unique area of respiratory physiology—"breathing fluids." The concepts and experiments to be discussed seem to involve an apparent reversal of evolution—animals breathe a superoxygenated liquid, exchanging oxygen and carbon dioxide as if their lungs were gills (G14, K14–K19, P8). These investigators have shown that mice, rats, or dogs submerged in hyperoxygenated solutions (8–20 atm) will survive for hours, as compared to control animals which die quickly. A balanced salt solution increases the efficiency of this pro-

cedure, as does adding a carbon dioxide buffer such as THAM (tris(hydroxymethyl)aminomethane, 2-amino-2-(hydroxymethyl)-1,3-propanediol) to the breathing fluid. The implications in drowning are obvious. Indeed, Kylstra recommends that 0.9% saline solution be used in all swimming pools in order to increase the number of successful resuscitations after accidental submersions.

Kylstra found that adult white mice, drowned in a balanced salt solution, ceased respiratory movements after 1 minute of submersion at 25°C. If 0.1% hydrogen peroxide is added to the drowning solution the animals will survive for an additional minute or two. On the other hand, animals placed in drowning fluid equilibrated with 8 ata oxygen will survive for 2 hours. On the basis of the observation that animals so immersed make respiratory movements, Kylstra interprets his data to mean that gas exchange in the lungs occurs. Goodlin ascribes survival to diffusion of gases across the skin. A much stronger case is made for pulmonary gas exchange as compared to cutaneous respiration.

Pegg *et al.* (P8) found that rats survived up to 4 hours while spontaneously breathing fluid equilibrated with 5–10 atm oxygen. The expirations were labored, the respiratory frequency was 40% of normal, and the heart rate was 60% of normal. Upon removal from the chamber the animals died within 5 minutes. Letting the animals breathe 100% oxygen in conjunction with intermittent positive pressure breathing (15 cm water) prolonged postsubmersion survival as long as 21 hours. Lungs of animals dying immediately after removal from the chamber were atelectatic and contained serosanguinous fluid. The lung extracts had a surface tension greater than 18 dynes per centimeter, indicating a loss of pulmonary surfactant. Animals that survived for more than 7 hours had hyaline membranes. The loss of pulmonary surfactant can also be inferred from the work of Kylstra and Lanphier (K19), who found that the pressure-volume diagrams of dogs, obtained during inflation of the lung with air following fluid breathing, were suggestive of marked increases in alveolar surface tension. These investigators also reported that fluid ventilation rates of 4200 ml/min and an inspiratory partial pressure of oxygen of approximately 2200 mm Hg resulted in an arterial oxygen content of 14.7–21.6 vol% at rates of oxygen consumption equal to 120 ml/min.

Postexposure survival remains a perplexing problem. In the early work, death was probably due to many factors such as exhaustion from the work of breathing, contamination of lungs by urine, feces, and skin oils, atelectasis due to loss of pulmonary surfactant, hyaline membrane, and possibly electrolyte imbalances, especially in the lung parenchyma. However, Kylstra and Lanphier devised techniques to obviate the difficulties en-

countered in complete submersion of the animal in the breathing fluid and thereby eliminated the difficulties from lung contamination by excretory substances.

These highly imaginative studies may duplicate some of the changes that occur during the liquid-to-gas transition at birth, and thus may explain some of the complications encountered and possibly provide new insights for the therapy of surfactant deficiency in idiopathic respiratory disease of the newborn.

3. Limitations Associated with Pressure and Hyperbaric Oxygenation

3.1. Physiological Limitations

Science is built up with facts, as a house is with stone. But a collection of facts is no more science than a heap of stones a house.
Poincare

The pressures that may be required to treat certain forms of hypoxia, i.e., those due to severe shunting, ischemia, or neoplasias, may not be practical. In severe shunts it is conceivable that, in order to alleviate the condition and to hyperoxygenate the patient, pressures greater than the 3 or 4 ata used clinically today would be required. Depending on the high pressure requirement (which may be impractical for a clinical facility) and the duration of exposure (until the abnormal condition is corrected), inconveniently long decompression times might result for the attending personnel in the chamber with the patient.

3.2. Oxygen

Oxygen, although essential for life and given under pressure to help sustain life, under certain circumstances exerts harmful effects. Oxygen toxicity is probably one of the most important limiting factors in high pressure oxygen therapy. Before continuing the discussion, some definitions are in order. *Hyperoxia* is an increase in the oxygen concentration of the respirable gaseous environment above the usual 150 mm Hg found at sea level. *Oxygen toxicity* is any variation from the normal structure or function attributable to the action of oxygen which produces deleterious effects. These terms are not interchangeable: the former refers to the physical composition of the gaseous environment; the latter refers to a pathological state of an organism resulting from a change in a specific physical characteristic of the gaseous environment to which the organism was exposed.

Oxygen toxicity is a time–pressure-dependent phenomenon, the two being inversely related (B11). The tolerance to oxygen decreases sharply with increasing pressure. Although the deleterious effects of oxygen at 1 ata (OAP) and OHP share many features, they are not identical. For the sake of brevity they may be considered together. Inspired oxygen concentrations up to 380 mm Hg may be employed for relatively long periods of time without harmful effects (M16). At inspired partial pressures greater than this the physiology of an animal may be disrupted in a number of ways: local irritation of the respiratory tract, derangement of the cerebral nervous system, visual disturbances, circulatory and hemorrhagic disorders, reproductive organ degeneration, complete or partial obliteration of air-containing spaces of the body (lungs, sinuses, middle ear).

Local irritation of the respiratory tract is encountered primarily when the $P_{I_{O_2}}$ is greater than 500 mm Hg (S26). The studies of Ohlssen (O1) have shown that in man breathing 78–88% oxygen at 1 ata, pulmonary symptoms occur between 12–24 hours and include nasal congestion, coughing, substernal stress, sore throat, decreased vital capacity, ear trouble, general fatigue, and mental disturbances. As the $P_{I_{O_2}}$ is increased, the time of onset of pulmonary irritability decreases. However, in man oxygen under pressure causes marked cerebral irritability which supervenes and forces the exposure to be terminated before the respiratory aspects become apparent. Small animals (mice, rats, and guinea pigs) die within 4–6 days of continuous exposure to OAP (W5). The initial toxic effects, dyspnea and lethargy, are manifested within 15–20 hours. In larger animals (dogs) the initial toxic effects occur in 36–42 hours, in the monkey in 72–96 hours. It is interesting to note that animals surviving 240 hours of continuous oxygen exposure show thickening of the pulmonary arterioles. Smith *et al.* (S16) had found thirty years earlier that rats surviving continuous exposure to 4 ata air ($P_{I_{O_2}}$ of 610 mm Hg) showed severe pulmonary arteriosclerotic changes.

Interruption of oxygen administration decreases the toxic effects on the pulmonary and cerebral nervous system, especially if the periods of exposure to oxygen are of short duration (K5, P9). However, there is evidence that even routine oxygen therapy may give rise to pulmonary alterations (P18).

The predominant involvement of the respiratory tract in oxygen toxicity is probably due to the fact that the lungs are the first and only organ to come in contact with the full $P_{I_{O_2}}$. Dyspnea, capillary congestion, alveolar exudation, hemorrhage, atelectasis, swelling of alveolar walls with encroachment on alveolar spaces, fragmentation of basement membranes between alveolar and endothelial cell layers, accumulation of exudate between the basement

membrane and the alveolar or endothelial cells, immobilization of cilia, appearance of periodic acid–Schiff-positive membranes and changes frequently resembling those seen in human neonatal hyaline membrane disease, and vacuolization of mitochondria constitute the damage done to the lung by exposure to high oxygen tensions (B23, B24, C11, K2, P18, S8, S24, V3). Present evidence seems to indicate that man may be more resistant to pulmonary damage than are small mammals. Because of the greater sensitivity of small animals to pulmonary damage, studies on the effects of OHP on infectious diseases, neoplasias, and other phenomena are greatly hindered (B45); part of the increased pulmonary susceptibility may be due to the previously mentioned existence of an endemic pneumonia-type condition in these animals

In man, as opposed to small animals, pressures greater than 3 ata terminate in convulsions long before pulmonary damage becomes manifest. The precipitation of convulsions constitutes the most striking and inexplicable event of OHP. In small animals, pulmonary damage is noted along with the central nervous system (CNS) involvement. No positive correlations have been shown between the degree of pulmonary involvement and the CNS effects. The CNS symptoms of oxygen toxicity in man and small animals are extremely variable and unpredictable (D16, D17, G22); there is as much variation in an individual from day to day as there is between individuals. There is no warning, and under certain conditions there is no detectable lasting damage (D16, D17). The clinical use of oxygen convulsions in the treatment of schizophrenia produced no detectable harmful or beneficial effects (L3).

The CNS manifestations of oxygen toxicity may include muscle twitchings of fingers, toes, and mouth, nausea, dizziness, vertigo, nervous irritability (positive Chvostek sign), Jacksonian-type seizures, and grand mal-type seizures (D17). Seizure onset may be sudden and not preceded by an aura. Up to the time of seizure, there may be apparent normality, unimpaired judgment, and capacity for work (D16, D17, F9). The cortical electroencephalogram (EEG) during and following a convulsion is indistinguishable from that of grand mal epilepsy. In oxygen toxicity, peripheral motor discharges may predominate and cause generalized jactitation without electrical or clinical evidence of cortical disturbance (D16, D17).

In Donald's experiments (D16, D17), those not reaching a convulsive end point showed a tendency toward a slight increase in beta activity and increased synchronization of delta activity in the EEG. Coupled with this was a decrease in the amount and voltage of the dominant alpha frequency (6–12 cps). Single high-voltage spikes appeared infrequently; these were symmetrical and bilateral.

In small animals, the CNS manifestations of oxygen toxicity have been shown to include foreleg tremors, hindleg tremors, head tremors, or a combination of these as the first visually detectable signs of oxygen toxicity; other animals revealing no apparent overt symptoms of CNS involvement may suddenly manifest mild or violent whole-body seizures (G22, K13).

There appears to be no sharp line of demarcation between the effects of oxygen at different inspired concentrations. Although the respiratory and CNS manifestations predominate during relatively short-time exposure to oxygen, longer exposures reveal physiological derangements of other organs and tissues, i.e., visual disturbances, circulatory and hemorrhagic disorders, liver and reproductive organ degeneration, and teratogenic effects (A6, A11, B18, C4, D4, D13, F4, G6, G31, M5, M15, R4, S4).

The incidence of convulsions in breathing oxygen at 2.8 atm for 30 minutes (U. S. Naval Oxygen Tolerance Test) is approximately 2.0%. Onset of convulsions has occurred within 15 minutes (F9). Forster and Churchill-Davidson (F6) have shown that subjects at rest will not safely tolerate 4 ata O_2 for a half hour but are unlikely to show signs of toxicity at 3 ata for the same length of time. Clinical experience with O_2 convulsions is difficult to assess. In Amsterdam, large numbers of patients have been exposed to 3 ata O_2 for 2 hours; in Glasgow, patients have been exposed to 2 ata O_2 for 2 hours. In both centers there have been few ill effects. However, the manner in which the oxygen is administered to the patient is critical. In most cases the type of mask used does not deliver pure oxygen to the patient. An additional difficulty in interpreting clinical results is that patients are often either anesthetized or sedated. These procedures tend to "protect" against the overt CNS symptoms of oxygen toxicity. However, the use of depressants as protective agents (P13) creates a false sense of security. One may only be masking the outward manifestations of oxygen toxicity without preventing cellular damage. That this is likely was shown in the work of Van den Brenk and Jamieson with pentobarbital sodium (V4). These investigators found that pentobarbital sodium accentuated delayed damage to the CNS by OHP while supposedly protecting against convulsions. One might argue that different mechanisms are involved in producing convulsions and permanent damage. Gilbert (G9) has discussed the possibility that a substance may play a dual role, in one instance protect against and in another enhance oxygen toxicity.

That oxygen produces permanent motor damage has long been known (B9, B10). Van den Brenk and Jamieson (V6) reported that in over 200 patients treated with 45 psig oxygen in conjunction with radiation for neoplasia and given pentobarbital sodium only 5 cases with convulsions and no deaths were encountered. This incidence of convulsions is similar

to that reported by the U. S. Navy (*vide supra*). There were no subsequent clinical signs of brain damage, paresis, or psychological disturbances. This is not to imply that permanent CNS damage may not occur. Whether permanent, detectable CNS damage ensues is dependent upon such factors as P_{O_2}, duration of exposure, P_{CO_2}, circulation, respiration, body temperature, endocrine balance, diet, individual susceptibility, etc. In addition, it is not wise to assume that the body's reaction to a drug (*sensu lato*) would be the same under conditions of hyperbaric oxygenation as under normotensive oxygen conditions (B11). One must approach the use of oxygen under pressure with caution.

There are many predisposing factors to oxygen toxicity. Increasing activity or exercise while breathing oxygen under pressure decreases the time required for the symptoms of oxygen poisoning to become manifest (U2, Y2). The reason for the decrease in latency is not known, but may be due to a combination of factors such as increased body temperature, increased sympathetic nervous system activity, and/or increased $P_{a_{CO_2}}$. Campbell (C2) has shown that increased ambient temperature decreased the tolerance of rats to oxygen poisoning. These findings were confirmed for rats by Hulpieu and Cole (H15), and for bacteria by Thayson (T2). Popovic *et al.* (P17) have shown recently that in ground squirrels lowering of body temperature protects against OHP by decreasing metabolism. These data imply that patients with high fever may be more prone to oxygen toxicity than patients with normal temperature; similarly, hypothermic patients should be less susceptible to oxygen toxicity. There is evidence that diet may affect tolerance to oxygen poisoning. Campbell (C2) reported that fasted animals were more likely than nonfasted animals to survive exposure to OHP. This has been confirmed for mice by Gilbert *et al.* (G8).

Endocrine effects also play an important role in oxygen toxicity. As one would expect, oxygen under increased pressure is a stressor. Gerschman and Fenn (G3) found a decreased ascorbic acid concentration in adrenal glands of rats following exposure to 1, 2, 3, and 6 ata oxygen. This was considered evidence that oxygen as a stressor may increase adrenal cortical hormone secretion. These findings were confirmed by Hale *et al.* (H3).

Bean (B12) found that hypophysectomy provided appreciable protection against the acute and chronic adverse effects of OHP; he noted increased latency of onset of neuromuscular reactions, decreased incidence of reactions, decreased severity of reactions, decrease in permanent effects, decreased mortality, and less severe pulmonary damage. Part of the protective action of hypophysectomy could be ascribed to loss of adrenocortical function. Cortisone augments oxygen toxicity (B13). The rest of the protective effect of hypophysectomy is probably attributable to the loss of thyroid

hormone. Grossman and Penrod (G30) found in rats that reduction of thyroid secretion by propylthiouracil afforded protection against 5.5 ata oxygen. Animals fed desiccated thyroid had more severe symptoms. These results were confirmed in rats breathing oxygen at 1 ata by Smith *et al.* (S15). Epinephrine markedly enhances the incidence and severity of the CNS components of oxygen toxicity, as well as intensifying pulmonary damage and increasing mortality (B14).

One of the functions of the hypophyseal-adrenocortical axis is generally considered to be a hormonal defense mechanism against a stressor. However in the presence of OHP as a stressor the hypophyseal-adrenocortical axis becomes a liability in that its increased activity augments oxygen toxicity (B12).

One of the most important factors affecting an animal's susceptibility to oxygen toxicity is the partial pressure of carbon dioxide. At low inspired concentrations, carbon dioxide decreases the latency of onset of oxygen-induced convulsions, whereas at higher concentrations it inhibits convulsions (C12). The inhibiting effect of a high $P_{I_{CO_2}}$ may be related to the anesthetic properties of CO_2. The manner in which CO_2 enhances oxygen toxicity has been extensively studied, but there appears to be no agreement on the mechanism of the effect. Lambertsen *et al.* (L3) believe the most probable explanation of CO_2 enhancement of oxygen toxicity to be related to the cerebral vasodilatory properties of CO_2; dilatation of cerebral blood vessels would result in contact of the tissues with an increased oxygen tension. Gesell (G7) has proposed that the toxic effects of oxygen upon the CNS might be due to autointoxication by CO_2. The postulated increased tissue CO_2 was supposed to result from the fact that a large supply of physically dissolved oxygen would meet the tissue requirements for oxygen without the usual reduction of hemoglobin. The maintenance of hemoglobin in the oxygenated state would limit the base available for CO_2 transport, thereby causing CO_2 to be carried at a higher than normal P_{CO_2}. The resulting tissue hypercapnia and/or acidosis would be sufficiently severe to cause the symptoms of oxygen toxicity. Gesell did not perform gas analyses on the blood of his experimental animals. However, Lambertsen *et al.* (L1, L3) have shown that in unanesthetized man there is no gross accumulation of CO_2 in the brain during oxygen administration even up to the time at which convulsions begin. Behnke *et al.* (B19) and Lambertsen *et al.* (L1) showed that it was highly improbable that the rise in cerebral CO_2 tension due to the failure of reduction of oxyhemoglobin is sufficient to account for the symptoms of oxygen poisoning. The inhalation of CO_2 in concentrations such that the blood CO_2 tension is elevated to a degree greater than that observed during OHP does not cause convulsions in man. Lambertsen

et al. (L1) proposed that the slight endogenous increase in tissue CO_2 brought about by the absence of reduced hemoglobin formation would serve to protect the animal against oxygen poisoning in contrast to what occurs with exogenously supplied CO_2. The mechanism of this protection is envisioned as occurring as follows: the increased cellular P_{CO_2} would stimulate the respiratory center and cause hyperventilation, which in turn would bring about a decreased arterial P_{CO_2} with resultant cerebral vasoconstriction and decrease in cerebral blood flow. The decreased cerebral blood flow would result in a decreased mean brain P_{O_2}, thereby protecting the brain against oxygen poisoning.

There is some controversy concerning the cerebral vasoconstrictive effects of oxygen. Lambertsen *et al.* (L2) believe that the vasoconstrictive effect is due to the decreased $P_{a_{CO_2}}$ brought about by hyperventilation (*vide supra*), rather than by a direct vasoconstrictive effect of oxygen. Jacobson *et al.* (J2) found that, under constant CO_2 tensions, oxygen exerts a direct cerebral vasoconstrictive effect.

The recent work showing that CO_2-adapted mice were more resistant to oxygen toxicity than unadapted mice (W1) and the studies on the protective properties of THAM[2] (B15, G22, S3) have reopened the question of the role of CO_2 in oxygen toxicity. In addition it should be pointed out that CO_2 does not appear to enhance the toxic effects of OHP on brain tissue slices (S28) or on the conduction of the nerve impulse in frog sciatic nerve (P10).

In most cases, breathing oxygen under pressure, as now used for therapeutic purposes, is not likely to have marked effects on gas transport or acid-base balance. The CO_2 transport system is unlikely to become a limiting factor in gas exchange for a number of reasons: (1) carbon dioxide is much more soluble than oxygen in body fluids and therefore able to diffuse through tissues more readily; (2) the increased solubility permits CO_2 to be carried in more significant amounts in physical solution in the plasma; (3) the shape of the CO_2 dissociation curve of oxygenated blood indicates that it is adapted for buffering some changes in P_{CO_2}. Theoretically, there is at least one set of circumstances under which maintenance of hemoglobin in the oxygenated state may enhance an acidotic state: in the presence of a severe right-to-left shunt as found in many congenital cardiac anomalies. In the presence of a 50% shunt and OHP, half the venous blood which is acidic and oxygenated will bypass the lungs and go directly into the arterial circulation without having released the metabolically produced CO_2. Due to the absence of the pH buffering effect of reduced hemoglobin, a profound acidosis may be anticipated.

[2] THAM = tris buffer [tris(hydroxymethyl)aminomethane].

3.2.1. *Mechanism of Oxygen Toxicity*

There is no result in nature without
a cause; understand the cause and you
will have no need for the experience.
Da Vinci

From the time of its discovery, oxygen has been known to exert toxic biological effects (B11), yet the underlying mechanism remains to be elucidated. Oxygen undoubtedly exerts its toxic effects at either the cellular or subcellular level. One of the more popular concepts is that oxygen reversibly or irreversibly inhibits some key enzyme system(s) in a manner such that serious impairment of essential cellular metabolic functions results. This concept had its origin in the classic studies of Bert (B26) on oxygen inhibition of putrefaction of meat and oxygen consumption of muscle. The early work in this field was reviewed by Stadie *et al.* (S26). Since the time of Bert, it has been observed that respiration of tissues decreases irreversibly when exposed to OHP (D8, D9, S29). Not all tissues show the same degree of susceptibility to oxygen toxicity. Stadie (S29) found in the rat the descending order of susceptibility to be brain > kidney > liver > lung > muscle. Dickens (D8) found the order to be brain cortex (finely divided) > brain cortex (slices) > spinal cord > liver > testis > kidney > lung > muscle. Both groups of investigators agree that brain is the most susceptible tissue while muscle is the most resistant, and that lung tissue is more resistant than other tissues except muscle. This is a surprising finding since *in vivo* the lungs show marked oxygen effects (*vide supra*). The finding lends credence to the concept that the respiratory tract is predominantly involved probably because it is the only tissue exposed to the full inspired oxygen tension. The reason for the discrepancy in the relative susceptibility of liver and kidney is not known.

In the 1940's Stadies' group in the United States and Dickens in England undertook a detailed analysis of numerous enzyme systems, and attempted to correlate the speed of onset of the acute CNS symptoms of oxygen toxicity with the rate of inactivation of these enzymes exposed to the same gaseous conditions as the animals. They were unable to correlate the rate of enzyme inactivation with speed of onset of oxygen toxicity; in each system studied, the time required to inactivate the enzymes was greater than the time required for the onset of oxygen toxicity in the intact animal (D8, D9, S27, S30–S32). Jamieson and Van den Brenk (J4) recently reported a decrease in total dehydrogenase activity in the lung tissue of rats exposed to OHP before overt manifestations of oxygen toxicity appeared. This is the first positive correlation reported between enzyme inactivation

and time of onset of oxygen toxicity. Because total dehydrogenase activity was measured they were unable to specify a particular enzyme system. However, this is an isolated observation that must be confirmed.

The available evidence suggests the possible involvement of a few particularly sensitive enzyme reactions. That general depression of enzyme activity is not involved can be inferred from the fact that many enzyme systems are unaffected by high oxygen tensions, and that not all tissues show equal susceptibility to oxygen toxicity. It is interesting that those enzymes which are affected by oxygen require in general a sulfhydryl group for their functioning. The sulfhydryl group may be either an integral part of the enzyme molecule or a component of a coenzyme (D8, D9, H6, H7). Among the enzymes adversely affected by oxygen and requiring sulfhydryl groups are those involved in oxidative metabolism, i.e., the enzymes of the tricarboxylic acid cycle. In addition, glyceraldehyde-3-phosphodehydrogenase, pyruvic oxidase, and choline acetylase, which require as coenzymes the sulfhydryl-containing compounds glutathione, lipoic acid, and coenzyme A, respectively, have been implicated as possible sites of oxygen inhibition. Pyruvic oxidase is of particular interest and importance because of its central role in carbohydrate metabolism (T3). Gordon *et al.* (G16) found increased fructose-1,6-diphosphate in liver and muscle of rats but not in the brain following a 20-minute exposure to 6 ata oxygen. Aldolase and glyceraldehyde phosphodehydrogenase measurements in liver and muscle indicated that these enzymes did not become rate-limiting during exposure. The existence of a Pasteur effect was proposed to explain these data.

Wood and Watson (W15) found that the concentration of γ-aminobutyric acid (GABA) in the brain of rats exposed to 6 ata O_2 for 33 minutes was markedly decreased as compared to unexposed controls. The degree of reduction of GABA was related to the severity of symptoms of oxygen poisoning. The levels of glutamic acid, aspartic acid, and total α-amino acids were not altered by exposure to OHP. These investigators proposed that the GABA metabolic shunt would become a major pathway in the tricarboxylic acid cycle of the brain if oxygen inhibits the α-ketoglutaric dehydrogenase step (T3). If this occurs, a greater requirement for GABA would result. If the rate of formation of GABA cannot keep pace with the increased demand, the concentration of GABA in the brain will decrease and impair the functioning of the oxidative metabolic pathway via the GABA shunt. These investigators view the cause of oxygen toxicity as an oxygen inhibition of α-ketoglutaric dehydrogenase and a decreased GABA concentration. This hypothesis is strengthened by the observation

that intraperitoneally administered GABA protects rats against the toxic effects of OHP (W16).

Oxygen-induced inhibition of enzymes of carbohydrate metabolism as the primary cause of mammalian oxygen toxicity is strongly supported by comparative biochemical considerations. There is a marked parallelism between the mammalian oxygen-sensitive enzyme systems implicated in the mechanism of oxygen toxicity and the botanical enzyme systems similarly sensitive to oxygen (B5–B7, P19, T6).

An alternative proposal to the oxygen-induced enzyme inhibition theory for the mechanism of oxygen toxicity (*vide supra*) seems to present itself. In view of the fact that oxygen enhances the activity of some enzymes and can function as an enzyme inducer (D12), the enzyme theory should not be centered only around oxygen-caused enzyme inhibition; it also should be concerned with possible derangement of the kinetics of interrelated enzyme systems, caused either by oxygen enhancement of enzyme reactions or by oxygen induction of new enzyme systems or stimulation of further enzyme production of existing and functioning enzymes.

The theory concerning the oxidation of essential metabolites, resulting in a relative depletion of key substance(s) to certain critical concentrations, might possibly explain the existence of a preconvulsive latent period in animals subjected to OHP. This hypothesis also could explain individual as well as species differences in susceptibility to oxygen toxicity. Recovery from the convulsion might be explained on the basis of regeneration or resynthesis of the key metabolite(s). This follows from the existence of mechanisms for regeneration of sulfhydryl groups. The hypothesis is strengthened by the fact that the administration of reducing substances (G4, J4) or end products of metabolic reactions (W16) serves to prolong the preconvulsive latency in animals exposed to OHP. However, there is a major drawback to these theories: the supporting data were obtained either from *in vitro* experiments or from animals following exposure to OHP. The question arises of whether the decrease either in enzyme activity or in concentration of one or more metabolites is a primary effect of oxygen and thus a possible cause of oxygen toxicity, or a secondary effect due to some other primary disturbance. For example, the possibility that these changes are a result of the convulsion rather than a cause of the convulsion has never been obviated. In addition, it seems to be mandatory that alternative means be found to inactivate the suspected enzyme system(s) in order to show that the symptoms and pathology of oxygen toxicity will still become manifest. Any proposed theory of oxygen toxicity must also account for the recovery from the acute convulsive phase of oxygen toxicity

and the variable time of recurrence. Such a theory will have to include information on local blood flows, respiration, gas concentrations, pH, etc.

The extrapolation of *in vitro* data to *in vivo* occurrences is extremely difficult in regard to the enzymatic mechanisms of oxygen toxicity. One difficulty in relating *in vitro* data to *in vivo* occurrences pertains to the P_{O_2} of the affected tissues. *In vitro* preparations exposed to OHP receive the full impact of the high oxygen tension, which may not be mirrored *in vivo*. Conceivably the affected system may not be receiving the full oxygen tension as reflected in the $P_{a_{O_2}}$. Due to local blood flow, diffusion, tissue consumption, etc., it is possible that the particular system is affected by oxygen tensions hundreds to thousands of mm Hg less than in the arterial circulation. That the oxygen tension in the brain varies during OHP exposure was shown by Bean (B16). Because an *in vitro* enzyme system is inhibited by 4, 5, or 6 ata oxygen does not necessarily imply that this is the same enzyme system affected *in vivo*. Another possibility exists, namely, that just a few cells in a minute localized area of the cortex are adversely affected and that this results in a disruption of the electrical activity of the cortex. This disruption manifests itself as a local or general convulsion. Homogenates would not reveal this because of the complete destruction of the structure-function relationship ordinarily found in the intact CNS. The few cells in the susceptible focal area may be subjected to oxygen tensions varying from a few millimeters of Hg to a few thousand millimeters of Hg. Little is known about the actual oxygen tension required to upset normal cortical function.

Because of the presence of catalase in virtually all tissues, it is unlikely that hydrogen peroxide is involved in the mechanism of oxygen toxicity. In addition, Dickens (D8) showed that the susceptibility of tissues to oxygen poisoning is not related to catalase content. Neither was he able to detect peroxide formation in brain tissue exposed to OHP in the presence of cyanide or azide. Cyanide or azide inhibits the breakdown of hydrogen peroxide by catalase. These observations were essentially confirmed by Bond (B34) for insects. Gilbert and Lowenberg (G10) found that frog skeletal muscle exposed to 140 atm O_2 for 2 hours produced the equivalent of 1.03 m*M* H_2O_2/kg muscle. Unlike the 140 ata O_2, the presence of 1 m*M* H_2O_2 did not seem to influence the resting muscle membrane potential. Wolman and Zaidel (W13) suggested that unsaturated phospholipids and other unsaturated lipids are the primary target of oxygen. Becker and Galvin (B17) found a significant increase in cerebral lipid peroxides (CLP) in rats exposed to oxygen pressure greater than 2 ata. However, the CLP levels did not differ between convulsing and nonconvulsing animals, and at 2 ata, when occasional convulsions occurred, there was no corresponding

increase in CLP. They concluded that CLP elevation probably does not play a major role in oxygen-induced convulsions, but did not dismiss the possibility of local increases in CLP, which would not be detected in whole brain determinations but would be capable of triggering a seizure. In vitamin E-deficient rats, abnormal lipid peroxidation appears to be responsible for the hyperoxic hemolysis (M15).

The effect of oxygen on transport mechanisms has received limited attention. Mullins (M23) reported that exposure of hen erythrocytes to 70 atm oxygen decreased potassium ion exchange, but that this oxygen tension was without effect on rabbit erythrocytes. In view of the findings that nitrogen behaved similarly to oxygen, it was concluded that the pressure per se, as opposed to a specific gas effect, was responsible for altering the cell potassium ion exchange. The difference in responsiveness to pressure between the two types of erythrocyte may have been due to differences in metabolism; the hen erythrocyte is metabolically more active than the rabbit erythrocyte. Bruce and Stannard (B40) found that X-irradiation of yeast cells resulted in marked disturbance of the ability to retain potassium ions; this effect was dependent on the presence of oxygen during irradiation. Perhaps such changes in membrane electrolyte permeability may in part account for the lethal effects of radiation in the presence of oxygen in tumor therapy. Direct effects of oxygen on sodium ion transport were observed by Falsetti (F1), who found that oxygen tensions greater than 8 ata markedly inhibited sodium ion transport across the frog skin. That this inhibition was due to oxygen and not to pressure was shown by the absence of an effect when the experiment was performed under increased nitrogen pressures with the P_{O_2} remaining constant at the normal value. These results were interpreted as being consistent with the concept that high oxygen tensions interfere with the oxidative enzyme systems, thereby decreasing the supply of energy available for ion transport. The possibility of a direct interference with the transport mechanism has not been disproven. Stuart *et al.* (S34) found that exposure of baker's yeast to high oxygen tensions results in inability to retain potassium ions. Recently, Glinka and Reinhold (G11) have shown that oxygen and carbon dioxide have opposite effects on the movement of water into and out of plant cells. Increased oxygen tensions enhanced cell permeability to water, whereas carbon dioxide acted to decrease water flux. Carbon dioxide partially reversed the oxygen effect of increasing membrane permeability to water. Although none of these investigators studied the effects of gas tensions on membrane permeability to other substances (amino acids, glucose, etc.), there is no evidence to suggest a selective effect on sodium, potassium, and water permeability. In view of the transudations found in the alveoli (*vide supra*), increased

oxygen tensions seem to exert marked permeability effects on a wide variety of substances. Indeed, Wood and Watson (W16) found that OHP increases the amino acid permeability of the blood-brain barrier.

Gerschman *et al.* (G5) proposed a provocative theory concerning a common mechanism for radiation injury and oxygen toxicity. The theory is based on the formation of oxidizing free radicals. The role of trace metals in the genesis of oxygen toxicity has been reviewed by Haugaard (H7a). The teratogenic effects of oxygen, alluded to previously, may be due to oxygen breakage of chromosomes (C21a). Conger and Fairchild have shown that chromosomal aberrations in *Tradescantia paludosa* increase with increasing partial pressures of oxygen. Although the mechanism by which oxygen causes breakage of chromosomes is not understood, the work of Gilbert *et al.* (G7a) may provide a possible explanation. These investigators found that high oxygen tensions increased the viscosity of deoxyribonucleic acid in the presence of reducing substances, such as reduced glutathione.

The underlying mechanism of the local respiratory irritation caused by oxygen, although unknown, is undoubtedly related to an abnormally high P_{O_2}. Nonspecific effects from the tanks, pipes, regulators, and masks have been excluded by a study of controls given room air through the same system used to demonstrate pulmonary irritation due to oxygen. No untoward symptoms resulted from this procedure (C21). Questions recently have been raised concerning the role of pulmonary surfactant in the genesis of pulmonary manifestations of oxygen toxicity (C17). Bondurant and Smith (B35) were not able to demonstrate an effect of oxygen on the pulmonary surfactant extracted from rats following exposure of the animals to 6 ata oxygen. However, Collier's (C20) findings, using rabbit lung extracts, support the hypothesis that lung damage in oxygen toxicity is due to a loss of surfactant, which results from damage to the normal mechanism for its production. The fact that a suitable quantitative technique for measuring pulmonary surfactant is as yet unavailable makes results from experimentation on the effects of oxygen on pulmonary surfactant difficult to interpret.

In most experiments on OHP and in the clinical use of OHP, the animals and patients breathe 100% oxygen for varying periods of time. Since man has evolved in an atmosphere of high gaseous nitrogen content, during the course of evolution he may have become dependent upon nitrogen and thus in some way this gas may exert subtle physiological effects. On the basis of these considerations, it may be conjectured that the removal of nitrogen leads to deleterious physiological reactions: the inference is that oxygen toxicity may be due to the denitrogenation associated with the inhalation of pure oxygen. However this hypothesis is untenable. Bert (B26) showed

that oxygen toxicity is a function of the partial pressure of oxygen even in the presence of other gases. Dickens (D8, D9) found no protective effect of nitrogen other than as an oxygen diluent in tissue studies. However, it should be pointed out that, although human experiments using 100% oxygen at total ambient pressures less than 760 mm Hg for relatively short periods revealed no derangement attributable to nitrogen lack (C21), and although Rahn and MacHattie (R1) demonstrated that mice can live and reproduce in a pure oxygen atmosphere at reduced pressures, there have been reports that chick embryos will not develop normally if nitrogen is absent or replaced by another chemically inert gas (A5, A6, V11). The observation on the chick embryos has been challenged by Boriskin *et al.* (B37).

3.3. Inert Gases

Experiment is the sole source of truth.
It alone can teach us anything new, it
alone can give us certainty.
Poincaré

Although the absence of an inert gas does not appear to be involved in the mechanism of oxygen toxicity, it should be pointed out that, in the absence of an inert gas in the respirable milieu, atelectatic changes may be favored. However, in clinical hyperbaric oxygenation it is not the absence of nitrogen that is of immediate importance, but rather the presence of nitrogen in concentrations 3–4 times that to which man is ordinarily exposed. This is not so much a hazard to the patient as to the attending personnel. At 3 ata, nitrogen is not exerting much effect (M11). However, pressures greater than 3 ata conceivably will have to be employed, i.e., in treatment of severe shunts or neoplasias (*vide supra*). Nitrogen at increased pressure has peculiar physiological effects. Cousteau refers to it as "the rapture of the deep." Lanphier refers to it as Martini's law—"The mental effects of each 50 feet of descent breathing air are approximately equivalent to those of one dry martini, assuming the stomach to be empty."

The physiological effects of nitrogen as well as its physical properties have been studied extensively (B20–B22). That CO_2 is the cause of nitrogen narcosis was shown to be highly unlikely by the work of Lanphier and Morin (L5) and Lanphier and Busby (L6).

The insidious nature of nitrogen narcosis with its resultant impairment of judgment and fine movement may portend grave dangers for surgeons and attending personnel working under certain hyperbaric conditions. Thus the question of appropriate breathing mixtures for medical attending

personnel becomes an important consideration for those contemplating the use of hyperbaric oxygenation.

A brief excursion into the physiological effects of the inert atmospheric gases that may substitute for nitrogen as an oxygen diluent appears to be in order. Among these are helium, neon, argon, krypton, and xenon. Hydrogen is generally not included, since in the presence of high oxygen tensions the breathing mixture would be potentially explosive. Conceivably hydrogen may be combined with one or more inert gases and thereby obviate the explosion hazard. Radon, because of its radioactive properties, is an extremely toxic gas and therefore excluded from further consideration.

Helium, neon, argon, krypton, and xenon, apparently nonessential to life, produce certain readily recognizable biological effects when the normal inspired partial pressure of the gas is greatly exceeded. Some of the stable isotopes of the helium group of gases act upon organisms, producing narcosis, decreased response to stimuli, metabolic alterations, decreased oxygen-dependent sensitivity to radiation, and altered rate of development. In general the activity of the gases varies in direct relation to molecular weight. Lazerev *et al.* (L10), Lawrence *et al.* (L7), and Cullen and Gross (C32) have postulated and demonstrated the anesthetic properties of xenon in man. Further studies on xenon narcosis were carried out in monkeys (P14), dogs (D14), and rats (G19). The anesthetic potency of xenon is greater than that of nitrous oxide and nitrogen and thus could not substitute for nitrogen in hyperbaric oxygenation. Argon and krypton also exert greater anesthetic properties than nitrogen and thus cannot function as nitrogen substitutes in breathing mixtures for man in high pressure environments.

Investigators have observed a number of biological effects of the helium group of elements on the organizational units of the organism, organ, and cell. These effects included altering the rate of metamorphosis in insects (C23, F8), inhibition of muscular contraction (G26), altering the rate of metabolism of the whole animal and tissue slices (C25, C26, L12, L14, S35, S36), protecting cells against the oxygen-dependent effects of X-irradiation (E1), protecting animals against electroshock convulsions (C5, C6), and their use as nitrogen replacements in compression of divers and caisson workers (B21). The usefulness of the inert gases as models for studying the mechanism of anesthesia has recently been reviewed by Featherstone and Muehlbaecher (F2).

Helium, according to present knowledge, would be the most likely gas to replace nitrogen in a breathing mixture for use in high pressure environments. However, this leads to difficult problems in vocal communication (S10). There is insufficient information concerning the physiology of neon to suggest it as a definite alternative to helium and thus dispense with the

communication problem. On the other hand, it is difficult to invoke a strong argument against the use of neon in a breathing mixture. There is no reason to suspect that neon will have peculiar physiological properties out of line with the effects theoretically predictable on the basis of comparative physiology and biochemistry.

The advantages of the inert gases, helium and neon, as nitrogen substitutes are their comparatively lower narcotic properties and lower solubilities in body fluids. Their major disadvantage is the tendency toward faster diffusion into bubbles, thereby tending to prolong decompression time. Nitrogen has the primary advantage of requiring shorter decompression time at the pressures currently used in hyperbaric medicine. By using a mixture of one or more of these gases, with the oxygen kept constant at P_{O_2} of 150 mm Hg, a product conceivably could be obtained that would provide a respirable atmosphere with the advantages of rapid decompression time and low narcotic ability. Of course the ultimate proportions of the gases used will depend upon the projected time-pressure requirement of the therapeutic procedure.

The physiological and biochemical effects of the atmospheric inert gases should not be excluded from consideration in high pressure physiology studies.

3.4. Physical Effects of Pressure

What effects can be expected from the increase in pressure per se on human physiology and biochemistry? Increasing pressure should accelerate chemical processes leading to a decrease in volume, whereas any biochemical reaction involving an increase in volume will tend to be inhibited (J6). Reactions involving small molecules most likely will have small volume changes, whereas reactions involving large molecules, such as proteins and nucleic acids, may reveal large volume changes (J5). Pressure may affect cell, tissue, or organ function by increasing solation of the cytoplasm (M4). The effects of the increase in cytoplasmic solution may result in acceleration of interaction of particles, denaturation of critical enzymes, or changes in pH (M19). Factors such as these must not be overlooked in the mechanism of oxygen toxicity and inert gas effects (e.g., nitrogen narcosis).

3.5. Dysbarism

Compression and decompression of mammals bring about physical changes that could have serious medical consequences and result in complex legal problems. Compression difficulties may involve both patient and attending personnel, decompression difficulties are primarily a problem

for the attending personnel. Patients will be breathing pure oxygen; dysbarism caused by oxygen is rare due to the rapid metabolic utilization of the oxygen by the tissues. There is no metabolic utilization of inert gases.

Dysbarism, a syndrome common to those working in either a pressure or altitude chamber, is a general term connoting adverse effects resulting from changes in barometric pressure. The term includes barotitis, barosinusitis, gastrointestinal distention, air embolism, and decompression sickness ("bends," caisson disease). The first three abnormalities may be considered to be caused by "trapped" gases, and the remaining symptoms to result from the evolution and expansion of dissolved gases. Any body cavity (paranasal sinuses, middle ear, periodontal abscesses, and intestines) unable to equilibrate with the ambient pressure during either compression or decompression could be a source of serious difficulty. During compression, the greatest volume change occurs within the first 7.5 psig. If the personnel can be compressed to this point without experiencing difficulty, the likelihood is that they can be completely compressed without experiencing untoward reactions. About 5% of the general population cannot successfully equalize pressure in the middle ear or paranasal sinuses. Barotitis can be, and in fact has been, circumvented by myringotomy (F6). Barotitis also can be overcome by swallowing, yawning, or Valsalva maneuvers. Barosinusitis may be severe and if compression is continued result in rupture of the blood vessels with subsequent filling of the sinuses. The significance of these potential complications when choosing staff to man the chamber can be surmised from Boerema's experiences. He found that one half his staff could not work in the chamber (B32). Problems may be encountered in unconscious patients as well as in infants unable to communicate the reasons for, or sites of, discomfort and pain. During decompression, the expansion of the gases in the closed body cavities may also give rise to severe problems involving the paranasal sinuses, middle ear, improperly inserted tooth fillings, and intestines. Conditions causing inflammation or edema of the mucous membranes may result in obstruction of sinus orifices and eustachian tubes, with consequent failure to equilibrate pressure and development of barotitis and/or barosinusitis. Special attention may have to be given to the expansion of gases in the gastrointestinal tract. According to Boyle's Law, one would expect the gases to expand during decompression and to enlarge the abdominal circumference. This enlargement will occur until flatus or gaseous eructation causes deflation. Severe abdominal pain will result if normal adjustments do not occur. Either the amount of gas in the gastrointestinal tract is reduced or the individual must be recompressed to an ambient pressure providing relief. This problem may be of particular significance in individuals who have

swallowed significant amounts of air during performance of Valsalva maneuvers on compression.

Air embolism is not synonymous with nor a manifestation of decompression sickness. It refers specifically to air embolization of cerebral vessels subsequent to the rupture of lung parenchyma by expanding gases. Air embolism should not be confused with the bubble formation that occurs in the circulatory system and tissues in decompression sickness (*vide infra*).

Air embolism could be expected to result from transient blockage of expiratory pathways by general (closed glottis) or localized gas trapping in the lungs of patients and attending personnel during decompression. The therapy for this type of accident consists of immediate compression to 6 ata. Emphysema patients, asthmatics, persons with lung cysts or tuberculous lesions, etc., may be particularly prone to this phenomenon. That does not imply that such persons may not benefit from hyperbaric oxygenation, if they manifest symptoms of disease states which may be alleviated by OHP; it is implied that extreme caution must be taken during decompression of individuals so afflicted. The statement also implies that individuals exhibiting one or more of these symptoms should be considered a poor risk for the job of attending personnel. Yanda *et al.* (Y1) recently showed that there is no evidence of increased hazard in exposing emphysema patients to pressure if one uses special decompression schedules combined with drug therapy. However, decompression times are relatively long as compared to the standard U. S. Navy decompression tables. They reported a transient (2–28 days) but significant improvement in residual volume and clinical condition of three emphysema patients, and improvement in residual volume of four emphysema patients.

Decompression sickness is thought to be the result of intravascular, intracellular, and/or extracellular bubble formation. Bubbles tend to form in any tissue saturated with an inert gas whenever the ambient atmospheric pressure is decreased to a point where the tissue pressure of the gas is more than twice that in the surrounding atmosphere. This results in a marked pressure gradient which drives the gas out of solution. Unlike oxygen and carbon dioxide, which are actively metabolized or transported, the rate of diffusion of the inert gas from the tissues into the expired air is too slow to cope with the volume of inert gas evolved. Thus, the gas comes out of solution locally in the tissues to form bubbles. The number and size of the bubbles in general will be proportional to the diffusion gradient existing between the partial pressure of the inert gas in the tissues and its partial pressure at the lower ambient pressure. The greater the deviation from the 2:1 pressure gradient postulated by Boycott *et al.* (B38), the greater will be the number and size of the bubbles. For a more extensive discussion

of the factors involved in bubble formation the reader is referred to the excellent paper of Harvey (H5).

The inert gas generally concerned in hyberbaric medicine is nitrogen. It has an oil-water solubility ratio of approximately 5:1, which indicates a greater solubility in adipose tissue than in blood. The amount of nitrogen that will dissolve in the tissues depends in part upon the depth and length of the compression as well as the composition of the particular subject. All things being equal, the greater the compression and length of exposure, the greater will be the amount of nitrogen dissolved in the tissues. Obese individuals will dissolve more nitrogen than thin individuals. During decompression the nitrogen will evolve from all the tissues. However, in order to be eliminated, the nitrogen must be transported via the blood. If a large amount of nitrogen has been dissolved, a watery tissue such as blood, capable of containing only about one-fifth as much nitrogen as fat, will not be able to transport in solution the large amount of evolving nitrogen. Superimposed on this is the fact that adipose and bony tissues are poorly vascularized, thus compounding the perfusion limitation in gas transport. Consequently, intravascular (capillary) and intracellular bubble formation occurs. Recent data indicate that, *inter alia*, humoral agents such as bradykinin may have a role in the pathogenesis of dysbarism (C12a).

There are various factors that predispose to decompression sickness. Some of these are obesity, age, exercise, and CO_2 accumulation. In general, older individuals are more susceptible to decompression sickness than younger persons; this is apparently related to the status of the circulatory system. Increased physical activity results in more rapid saturation of the tissues per unit time than in a resting individual. This increased rate of tissue saturation is due to the increased rate of ventilation and circulation resulting in more rapid transport of nitrogen to the tissues. Due to their very rapid circulation and ventilation, small animals are more resistant than man to decompression sickness. Blinks *et al.* (B28) found that increased CO_2 tension in the tissues lowers the threshold for bubble formation. Although the mechanism of this phenomenon is not known, there is reason to suspect that it may be due to the high solubility and diffusibility of carbon dioxide (H5).

Other factors, such as temperature, water balance, and drugs, may affect the susceptibility to decompression sickness. Results were inconclusive (C24, G27).

The symptoms of decompression sickness include bends (local pain), skin rashes, itching, neurological disturbances (scotomata, hemianopsia, diplopia, paresis, paralysis, abnormal reflexes, dysesthesia, aphasia, vertigo), respiratory distress ("chokes"), nausea, and shock (neurocirculatory

collapse). Detailed accounts of the clinical manifestations, predisposing causes, and prevention of decompression sickness have been published recently (D20, L4, N1).

The principal manifestation of the bends is a deep boring ache or pain in the bones of muscles around the joints. The intensity of the pain can vary. This symptom may be accompanied by neurocirculatory collapse. The cause of the pain is thought to be due to excitation of mechanoreceptors as a result of tissue distortion by the expanding bubbles, or to ischemic excitation due to interference with vascular flow. The role of humoral agents in the production of pain should not be overlooked (K9). Even following the standard U. S. Navy Decompression Tables the incidence of bends may be as high as 4%, depending on age, physical condition, etc.

Skin rashes and itching occur fairly often upon decompression. The most common lesion is a mottling of the skin which spreads peripherally. The centers become erythematous and show a slight elevation in temperature. The skin manifestations are found primarily in the pectoral region, back of the shoulders, upper abdomen, forearm, and thigh. The lesions appear to be related to spasm and secondary hyperemia of the small vessels of the skin, apparently caused by bubble formation in the deeper fatty layers of the skin (F5).

Gersh and Catchpole (G2) have summarized the CNS symptoms of decompression sickness. The most prominent feature is the occurrence of areas of softening in the spinal cord, usually small and more circumscribed in the white than in the gray matter. Necrosis is more common in the white than in the gray columns. In the white columns, the anterior columns appear to be more susceptible. There is also ascending and descending degeneration of nerve tracts. Hemorrhage is generally not a significant factor in the CNS manifestations of decompression sickness. Neurocirculatory collapse is associated with the more serious cases of CNS involvement in decompression sickness.

Another distressing syndrome of dysbarism is "chokes" or respiratory distress. This syndrome is characterized by substernal pain, a nonproductive cough, and dyspnea. There is exacerbation of both the substernal distress and cough when the affected individuals attempt to breathe deeply. To avoid the burning pain the patient may resort to rapid and shallow breathing with subsequent decreases in the arterial P_{O_2} and P_{CO_2}. The incidence of this syndrome is approximately one-tenth that of bends. It may accompany bends; also it may result in neurocirculatory collapse.

For virtually all cases of decompression sickness, save perhaps neurocirculatory collapse which may require oxygenation and blood transfusions, (B41, C19) the most expeditious therapy is recompression. Afflicted indi-

viduals are generally recompressed to 6 ata. The reasoning for compressing to 6 ata is based on the fact that it is the diameter and not the volume of the gas bubble which is the critical dimension determining whether symptoms will occur. It is known from the gas laws that the bubble volume is inversely proportional to the pressure; however, the bubble diameter is proportional to cube root of 2π times the pressure. Compression to 6 ata results in a reduction of the bubble volume to one-sixth of its volume at 1 atmosphere. At the same time its diameter is reduced by a factor of 0.299; in order to reduce the bubble to one-tenth of its diameter at 1 ata it would be necessary to compress the patient to approximately 150 ata. Compression to 8 ata reduces the bubble diameter only by a factor of 0.27. From these calculations it is obvious that compression beyond 6 ata is of little therapeutic value.

One aspect of improper decompression that has been reported is aseptic bone necrosis. Bone is poorly vascularized and poorly innervated; thus, gas bubbles trapped between the periosteum and the bone may lead to necrosis. Being poorly innervated there is no warning until too late. It is insidious. Bone necrosis has been associated with high pressures, hard work, prolonged exposure, and most important inadequate decompression! Thus far bone necrosis has been seen only in caisson work. Aseptic bone necrosis at a later date could provide a basis for compensation claims.

3.6. Precautions

The protection of medical and ancillary personnel associated with the pressure chamber against the ever present danger of dysbarism makes it mandatory that they be thoroughly grounded in pressure physiology. Mastery of the use of the U. S. Navy or comparable decompression tables is a must, as is knowledge of the various techniques used to facilitate decompression while avoiding the deleterious effects of dysbarism. In addition, all hyperbaric facilities should have an associated chamber with a capability of attaining 6–7 ata. It may even be desirable for the entire hyperbaric facility to have the flexibility of attaining 6–7 ata. Increase in cost of construction for such a flexible facility is less than 1% of the total cost of construction of a hyperbaric chamber capable of only 3–4 ata. The dysbaric experiences of the hyperbaric personnel at Duke University have been discussed (A8).

Because of the increased partial pressure of oxygen in the chamber atmosphere there is the remote possibility of an enhancement of combustion. There are reports indicating that the flammability of given materials under variations of pressure remains essentially constant, providing the ratios of the various gases in the system remain constant. Only nonflam-

mable or noncombustible anesthetics should be used. The special problems of anesthesia in a pressure chamber have been reviewed by McDowall (M7), Pittinger (P15), Smith *et al.* (S25), and Vermeulen-Cranch (V10). All instruments must be explosion-proof and tested for operation under high pressure conditions. Ink-loaded pens will leak upon decompression; vacuum tubes may burst; wrist watches and other instrumentation that will equilibrate with the high pressure may not permit gas to escape as readily upon decompression, with the resultant "gas embolism of instruments."

4. Analytical Techniques

In recent years immense interest has developed in the use of various analytical techniques for measuring respiratory and anesthetic gases in both the gaseous and dissolved states. The ultimate goal is the determination of gas tensions of specific cells and parts of cells *in vivo*. A thorough knowledge of the instantaneous gaseous composition of various tissues during health and disease, functional and quiescent states, drug and X-irradiation therapy, anesthesia, etc., would undoubtedly lead to new insight into tissue function, mechanism and prevention of oxygen toxicity, efficacy of chemotherapy, control of respiration, theory of narcosis, etc.

The gases of primary concern for measurement are oxygen and carbon dioxide. Although it would be invaluable to have techniques available for measuring instantaneous inert gas tensions *in vivo*, it is difficult to conceive of a property of the individual gases confering sufficient specificity to be exploited at the present time for the routine differentiation of one of the atmospheric inert gases in the presence of the other atmospheric gases.

Specificity is the most important requirement in gas analysis. Techniques dependent on the physical properties of the gas molecules, such as thermal conductivity, density, viscosity, and sound velocity, generally have insufficient specificity to differentiate a single gas in a mixture of gases, and therefore must incorporate in the procedure some type of preliminary separation. Vapor phase fractionation (gas chromatography) is an example of a popular analytical technique based upon a physical property (thermal conductivity) of the gas that requires preliminary separation of the gases by means of special columns (molecular sieve, silica gel, etc.).

The detecting and measuring component of a gas chromatography unit contains four heat-sensitive thermistors whose resistances change as a function of temperature; two are used as references and two as detector. The four thermistors form the arms of a Wheatstone bridge. A constant current passing through the bridge heats the thermistors to their effective working range. Thermal equilibrium is attained by passing pure carrier gas

(helium) over the four arms of the bridge. A contaminant (gaseous) in the carrier gas will alter the thermal conductivity properties of the carrier gas. When the carrier gas containing this contaminant comes in contact with a detecting thermistor it alters the rate at which the thermistor loses heat and thereby causes an imbalance in the bridge. This electrical imbalance is recorded. The degree of change (height of peak or area under the curve) is proportional to the concentration of the contaminant.

There are a few techniques depending upon certain physical properties of the gases that can be exploited for analytical measurement. The first is the measurement of oxygen by virtue of its paramagnetic properties (P7). The second technique is based on the absorption and emission spectra of the gases. A substance is paramagnetic if its atoms or molecules possess a permanent magnetic moment. The substance will become magnetized in the same direction as the applied magnetic field due to the alignment of the individual magnetic moments in this field. Diamagnetic substances do not possess a permanent magnetic moment. For practical purposes oxygen is the only respiratory gas which is paramagnetic and, because of this property, can be measured specifically and accurately in the presence of the other atmospheric gases. Instruments are available commercially for this type of analysis.

The absorption and emission spectra of oxygen and nitrogen are well separated but lie in the region of the vacuum ultraviolet. The present cost of this instrumentation for routine gas analysis makes it prohibitive. Carbon dioxide as well as other polyatomic gases (CO, N_2O, H_2O vapor, organic anesthetic gases) can be measured by nondispersion infrared spectrophotometry. The speed of response and the degree of accuracy obtained by this technique are adequate for routine use during surgery, in clinical cardiac-pulmonary laboratories, and even in many research projects. Nitrogen tension can be measured by ultraviolet emission from ionized nitrogen gas. Nilsson (N3) has recently reviewed the subject of oximetry.

The mass spectrometer is theoretically capable of analyzing any gas in a mixture of gases with a speed, specificity, sensitivity, and accuracy that cannot be matched by other known means of analysis. However, the initial cost of the instrument and accessory equipment has thus far precluded its widespread use. Fowler and Hugh Jones described a mass spectrometer specifically for respiratory physiological studies. Extensive use has been made of this instrument (W6–W8).

If neither speed of analysis nor continuous analysis is required, the volumetric or manometric techniques may be employed. The volumetric method of gas analysis is based on the selective chemical absorption of components of the gas mixture, first CO_2 and then O_2. The change in volume, at constant

temperature and pressure, is a measure of the gas concentration (H2). The partial pressures of the gases are calculated from the volume changes, temperature, and ambient pressure, using the gas laws. Manometry involves the measuring of pressure changes while maintaining a constant volume (C7, U1, V9). Ultramicrorespirometry can be employed for studying gas exchange in very small numbers of isolated cells or tissues by use of the cartesian diver (K7, L15, P5). Manometry can also be used for measuring dissolved gas tensions by first evolving the gases from solution (P11).

The techniques discussed briefly above do not permit instantaneous gas analysis of *in vivo* tissue gas tensions in unanesthetized animals, although some of them may be useful in predicting tissue gas tension. The requirement for a technique capable of recording instantaneous gas tensions in tissues was partially obviated by the introduction of the oxygen cathode by Davies and Brink (D1). In essence, the principle upon which the oxygen electrode functions is as follows: as the applied voltage across an anode and cathode in a conductive aqueous solution is increased, the current produced from the electrolytic reduction of the oxygen plateaus within the range 0.6–0.8 volt. This implies that at a constant voltage the rate of reduction of the oxygen dissolved in the solution will be directly proportional to the amount of current flowing between the electrodes. The rate of reduction is dependent upon the concentration of the oxygen; thus, a measure of current is a measure of the concentration of dissolved oxygen. All oxygen electrode systems (platinum and silver–silver chloride) operate in this voltage range and show a current flow proportional to the concentration of the oxygen near the electrodes. The oxygen is irreversibly consumed in the measuring process.

The early electrode systems were subject to "poisoning" by proteins; in addition, the diffusion geometry was ill-defined. A major breakthrough in the use of oxygen electrodes occurred with the development of a membrane-covered electrode by Clark *et al.* (C16). The membrane protects the electrode surface against poisoning (aging). It also prevents reduction of substances other than oxygen and provides a measure of stability and reproducibility. Excellent discussions of the theory and limitations of the oxygen cathode can be found in the papers of Connelly (C22), Cater *et al.* (C8), Evans and Naylor (E5), and Severinghaus (A3, S12). The special problems associated with the preparation of the nonpolarizable electrode are discussed by Feder (F3). An extensive source of suppliers of oxygen electrode systems was published recently (M8). The use of the oxygen electrode for measuring oxygen tension under hyperbaric conditions is discussed by Schoemaker (S7).

In the same year that Clark modified the oxygen electrode, Stow *et al.*

(S33) introduced the CO_2 electrode. Severinghaus and Bradley modified the CO_2 electrode to increase its sensitivity and response time (S11). The technique consists essentially of recording the pH of a thin layer of isolated standard solution (KCl and $NaHCO_3$) in contact with the electrode but separated from the tissues by a semipermeable membrane. The membrane is permeable to CO_2 but impermeable to other ions which might affect the pH of the standard solution. The pH of the solution is completely independent of that of the tissues. Under normal conditions the only gas that influences the pH of the solution is CO_2. Thus, CO_2 electrodes provide a pH measurement proportional to the log of the P_{CO_2} in the standard solution. The linear relationship of the pH recording to the log of the P_{CO_2} of the solution can be affected only by the P_{CO_2} of the tissue. Unlike the oxygen electrode which consumes the oxygen in the course of the O_2 measurement, the CO_2 electrode does not consume the CO_2. Detailed discussions concerning the theory and use of CO_2 electrodes as well as a list of suppliers have been published recently (G1, S12). Methods using a combined electrode system have been devised for simultaneous recording of pH, P_{CO_2}, and P_{O_2} from localized areas of tissue (G18).

The electrode systems for *in vivo* gas tension measurements have the advantage of permitting continuous measurement of gas tensions. However, the methods have certain limitations, especially when the electrode is implanted in specific organs or tissues for *in vivo* measurements. The insertion of the electrodes into the tissues will provide a mechanical barrier to gas diffusion; it will distort tissues and capillaries contiguous with it and thereby interfere with local blood flow; even capillaries a short distance away may be influenced by the compression; blood vessels may be ruptured and cause edema; local circulatory reflexes may alter the local blood supply; the oxygen electrode by consuming the oxygen will create new diffusion gradients. The measurement of a local oxygen tension is also dependent upon the proximity of the electrode to the capillaries. Electrodes used today measure only mean gas tensions. There is some question whether the oxygen electrode is measuring absolute oxygen tension or just oxygen availability. Advances in electrode technology seem to be revolving around the production of smaller diameter electrodes, new membrane materials, different metals to replace platinum, and improved circuitry.

Naturally occurring and synthetic radioactive and nonradioactive isotopes of the atmospheric gases have been employed for studies on various circulatory and respiratory problems as well as their interrelationship. Probably the most commonly employed isotope for studying ventilation-perfusion relationships is krypton-85; xenon-133 and oxygen-15 also have been used. Oxygen-15, like the other radioactive gases, can aid in localizing

areas of defective ventilation, but, unlike the other gases, it is metabolized and thereby has the advantage of providing information about regional gas exchange. Because of its short half-life, 2 minutes, oxygen-15 has not been used extensively. Stable isotopes such as oxygen-18 have been used; they suffer the technological disadvantage of requiring more complex instrumentation for measurement than the radioactive gases.

5. Conclusions

Oxygen is considered a drug, and under certain conditions (*vide supra*), like all drugs, it is toxic to man. However, its toxic properties should not deter qualified physicians from utilizing this therapeutic approach. Clinical hyperbaric oxygenation is based upon sound physical and physiological principles. In many instances HPOT has proven of immense value. The disappointing results obtained thus far in other clinical areas (coronary infarction, tetanus) should not be permitted to have a negative influence on the future development of this form of therapy; rather they should be an incentive to spur further research in discovering ways of improving the efficacy of HPOT. This involves a fundamental study of the biology, biochemistry, and physiology of oxygen and the other atmospheric gases.

Hyperbaric oxygenation has opened up new avenues for basic and clinical research. As more is learned about the physiological and biochemical effects of oxygen and other gases under pressure, it is certain that new uses will be found for this latest addition to the medical armamentarium (B39, G17, M2).

One of the basic questions requiring immediate solution for the advancement of hyperbaric oxygenation is concerned with the P_{O_2} of tissues during experimentation or therapy. In order to obtain such information, advances must be made in instantaneous ultramicrotechniques for gas tension measurements as well as for simultaneous monitoring of function, metabolism, and respiration on the cellular level. At present, we do not know to what extent ischemic tissues can derive oxygen by diffusion from neighboring areas having normal blood supply. Advances in hyperbaric oxygen therapy of coronary infarctions, neoplasms, and infectious diseases are being hampered by lack of this type of information. As techniques for local gas tension measurement become refined, new information should be forthcoming on the control of the circulation in localized areas in normal and pathological states as well as on the regulation of the circulation in general. New information will be obtained on the effects of altered gas tensions on oxygen metabolism and metabolism in general. New insights will be obtained concerning the P_{O_2} required to induce oxygen toxicity.

Research into the mechanism of oxygen toxicity must be conducted if

methods are to be devised to prevent its occurrence and thereby eliminate a major obstacle to the full development of hyperbaric oxygenation. Insights obtained from such studies may be useful in determining the causes and treatment of other convulsive disorders.

Whether hyperbaric oxygen is a predisposing factor for the action of other stressors has not been investigated. There is a paucity of knowledge concerning the physiological and biochemical compensatory responses resulting from exposure to high pressure oxygen.

New information concerning the physics and pharmacology of anesthetic agents, with emphasis on the mechanism of anesthesia, should be forthcoming as a result of increasing the effectiveness of hyperbaric oxygen therapy. The pharmacology of old and new drugs must be extended to include studies on drug reaction in high pressure oxygen and other abnormal gaseous environments. The possibility of synergistic action between chemotherapeutic agents and OHP must be explored further. The synergistic effects of antituberculosis drugs and oxygen (G24) and the enhanced effects of oxygen on the action of fumigants on insects (B34) point out the inability to predict the behavior of a drug in an abnormal gaseous environment. The effect of OHP on the host-parasite relationship has been virtually neglected. The possible use of inert gases as respirable breathing mixtures for hyperbaric environments underscores the need for more basic research into the physiological and biochemical effects of the atmospheric inert gases. Due to the potential fire hazard existing in sealed hyperbaric chambers, new developments in the synthesis of fire-resistant fabrics may be desirable. In addition, new concepts in patient monitoring techniques will undoubtedly result from the peculiar requirements of sealed environmental therapy.

The by-product from such studies has wide implications in the fields of space and underwater physiology as well as in clinical and veterinary medicine.

In summary, there appears to be cause for enthusiasm concerning the use of hyperbaric oxygenation as a clinical tool. Whether hyperbaric oxygenation becomes a potent therapeutic regimen or fades as a passing fad (albeit an expensive one) depends on its ability to meet the criteria set forth by the Ad Hoc Committee on Hyperbaric Oxygenation of the National Research Council's Committee on Shock: "It must be uniquely beneficial, having clear-cut advantages over less formidable forms of treatment; it must be fully *practical in its use* in at least a few important conditions; and, in consequence, it must be applicable to a *reasonable number of patients*" (A1).

To help ensure its success, close cooperation will be required between the

basic and clinical research scientists, involved in answering many of the problems discussed above, and the clinician, ultimately concerned with the practical use of high pressure oxygen therapy.

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DETERMINATION OF HEMOGLOBIN AND ITS DERIVATIVES

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1. Introduction

The measurement of the total hemoglobin concentration and the determination of the hemoglobin derivatives oxyhemoglobin (HbO_2), carboxyhemoglobin (HbCO), sulfhemoglobin (SHb), and hemiglobin (Hi) in blood are undoubtedly the most frequent determinations in the clinical chemical laboratory. In contrast to nearly all other determinations, a clinical diagnosis is often made solely on the basis of hemoglobin determinations: anemia when values are too low, polycythemia in cases of abnormally high values.

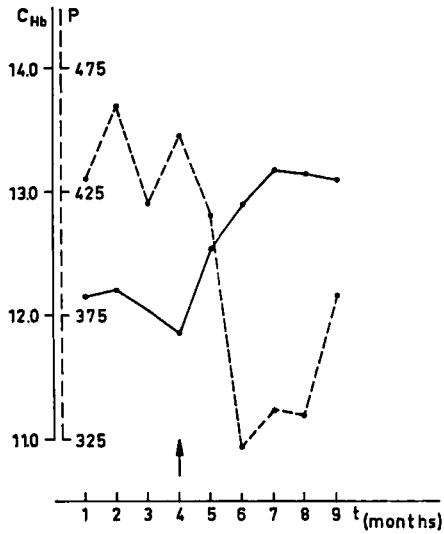

FIG. 1. Relationship between mean monthly hemoglobin concentration (C_{Hb}) and number of pints of blood (P) transfused in a general hospital. Arrow indicates standardization of hemoglobin determination. Based on data from Mann *et al.* (M3).

When significant variations occur in the determination of the hemoglobin concentration, it becomes difficult to give a clear-cut definition of, for example, anemia. The effects of a change in the measurement technique of hemoglobin concentration is clearly illustrated in Fig. 1, where the number of pints of blood transfused in a certain general hospital fell drastically as soon as the hemoglobin determination was standardized. This one example alone indicates that the standardization of hemoglobinometry is absolutely necessary.

1.1. General Requirements for Hb Determination and Standardization

1.1.1. *Method of Choice*

As hemoglobin is itself a colored substance and because a spectrophotometric technique is generally the method of choice in the clinical chemical laboratory, it is logical to prefer a photometric technique for the determination of the Hb concentration.

The first general requirement therefore must be that Lambert-Beer's law is applicable to the method:

$$\log (100/T) = D = \epsilon cl \tag{1}$$

where T = light transmission in %, D = optical density, ϵ = millimolar extinction coefficient, c = concentration in millimoles per liter, and l = layer thickness in cm.

1.1.2. *Dilution Factor*

A second general requirement must be that the dilution factor be so chosen as to ensure an acceptable ratio between blood and reagent. This ratio (e.g., 0.01–0.05 ml of blood, 5.0 ml of reagent) must be acceptable to the clinical chemist while at the same time the extinction must be optimal from the physicochemical view. This requires that the extinction should be found to lie in that region where the random error (dc/c) is smallest.

It follows from Eq. (1) that

$$c = \frac{\log (100/T)}{\epsilon l} \tag{2}$$

and after transposing to natural logarithms

$$\frac{\ln 100 - \ln T}{\ln 10} = \epsilon cl$$

Differentiating T with respect to c gives

$$(1/T)\, dT = -\alpha\, dc \quad \text{or} \quad dc = -(1/\alpha T)\, dT$$

where $\alpha = \epsilon \cdot l \cdot \ln 10$.

Dividing by Eq. (2), after transposing to natural logarithms, gives

$$\frac{dc}{c} = -\frac{1}{T \ln 100/T}\, dT \tag{3}$$

Now $(dc/dT)/c$ is minimal when

$$\frac{d}{dT}\left[-\frac{1}{T \ln 100/T}\right] = 0$$

or

$$\frac{\ln 100 - (T(1/T) + \ln T)}{(T \ln 100/T)^2} = 0$$

thus

$$\ln 100 - 1 - \ln T = 0$$
$$\ln T = 3.6$$
$$T = 36.6\%$$

The most exact spectrophotometric measurements will thus be performed around $T = 36.6\%$, which equals $1/e$, or $D = 0.434$. Figure 2 presents the

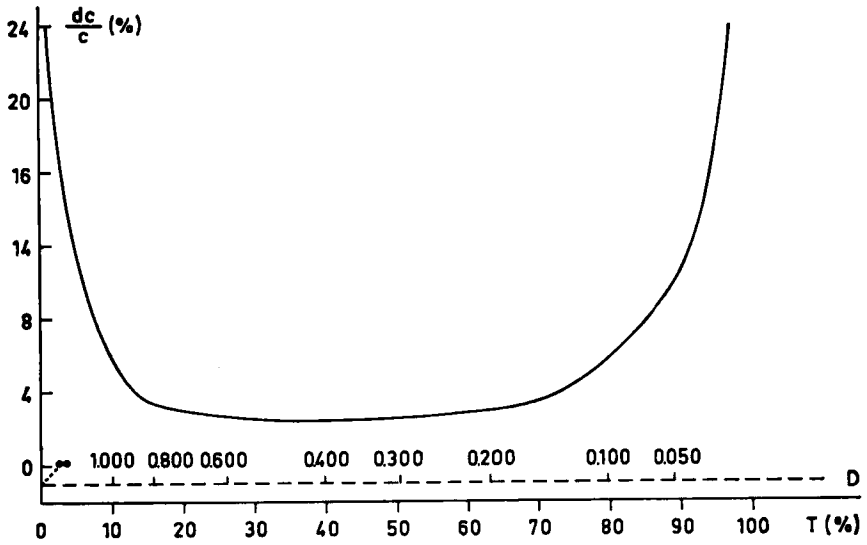

FIG. 2. Relationship between the random error (dc/c) and the light transmission (T). Corresponding optical densities are also indicated (D). Graph based on Eq. (3), taking $dT = 1\%$.

relationship between dc/c (random error in %) and T (%). From this it is clear that the dilution factor must be so chosen as to ensure measurements in the region of $20\% < T < 80\%$ or $0.090 < D < 0.700$.

1.1.3. *Absorption Spectrum*

The light absorption spectrum of the hemoglobin derivative to be chosen for a photometric determination should have a number of favorable properties. It should display a rather flat maximum. If this is not the case, slight alterations in filter or in photometer characteristics will introduce considerable errors. Furthermore, the light absorption maximum should

lie in the spectral region 400 nm $< \lambda <$ 600 nm and be independent of changes in the pH.

Hemiglobincyanide seems to meet perfectly the requirements stated above. The light absorption spectrum of hemiglobincyanide is given in Fig. 3.

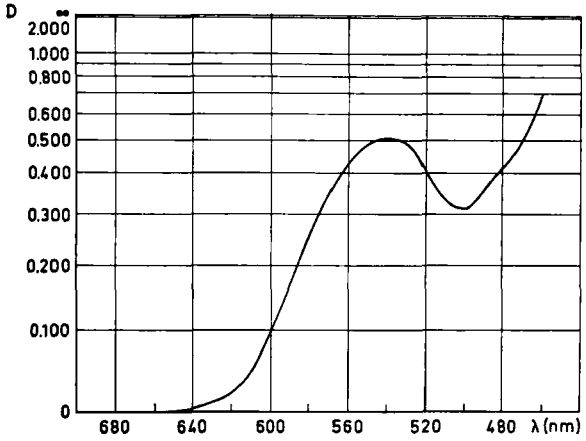

FIG. 3. D/λ curve of hemiglobincyanide (HiCN), prepared as described in Section 2.3. Recorded on a Beckman DB spectrophotometer, layer thickness 1.000 cm.

1.1.4. *Specificity and Stability*

Because measurements dependent upon time always disturb laboratory routine, the hemoglobin derivative to be used for the determination of the Hb concentration in blood must be formed rapidly, all hemoglobins present in the sample must be converted, and the end product formed must be stable. Stability is an essential requisite for the use of standard solutions to calibrate the photometer to be used. The measurement itself should not be disturbed by plasma proteins (globulins) or erythrocyte stromata, either through the introduction of a so-called protein error (C3) or because of turbidity. Only HiCN properly prepared fulfills all requirements mentioned.

1.2. General Aspects of the Determination of Hemoglobin Derivatives

The introduction of modern spectrophotometers, which allow measurements with a small half-intensity band width (1 nm)[1], raised the possibility of applying photometric methods to systems of two or more components. The primary conditions for this are (a) a distinct difference between the

[1] 1 nm = 1 nanometer = 10^{-9} meter.

absorption spectra of the components, and (b) the applicability of Lambert-Beer's law to each of the components. If a solution contains n dissolved substances, complete determination of this n-component system requires measurements at at least n different wavelengths. These measurements provide n equations of the type

$$D^{\lambda} = \epsilon_1^{\lambda} c_1 l + \epsilon_2^{\lambda} c_2 l + \ldots + \epsilon_n^{\lambda} c_n l \tag{4}$$

where D^{λ} = optical density (log I_0/I) at wavelength λ, ϵ_n^{λ} = extinction coefficient of component n at wavelength λ, c_n = concentration of component n, and l = layer thickness.

The values of $c_1, c_2, \ldots, c_n$ can be calculated by solving these equations, if the extinction coefficients (n^2 in number) are known.

Extinction coefficients of a number of hemoglobin derivatives are presented in Section 3. Although these values could be used in the actual analysis of n-component systems, it seems as yet unwise to discontinue using the simple two-wavelength methods which have long been in use with reasonable results. Thus, these established methods are described in Sections 5, 6, and 7, although it now seems probable that future developments will tend to the general use of methods involving the actual extinction coefficients.

2. The Standardized Determination of Hemoglobin

2.1. The Extinction Coefficient of Hemiglobincyanide at λ = 540 nm; ϵ_{HiCN}^{540}

2.1.1. *Introduction*

The fundamental quantity to be used in any standardized spectrophotometric determination of hemoglobin should be the quarter millimolar extinction coefficient because of the four Fe atoms per hemoglobin molecule. This quantity is independent of the molecular weight of hemoglobin. Consequently, a redetermination of the molecular weight will not change the value of the extinction coefficient to be used and will not affect hemoglobin concentrations when these are expressed in quarter millimoles per liter.

When the value of the quarter millimolar extinction coefficient of hemiglobincyanide at λ = 540 nm (ϵ_{HiCN}^{540}) is determined using hemoglobin solutions prepared from washed red cells by toluene hemolysis, the solutions may possibly contain the following substances: (1) hemoglobin (Hb), oxyhemoglobin (HbO_2), carboxyhemoglobin (HbCO), hemiglobin (Hi), and other hemoglobin derivatives ("Hx"), all convertible to HiCN; (2) hemo-

globin degeneration products, other compounds (some of which contain iron), and traces of free iron (non-HiCN).

Spectrophotometry of a hemoglobin solution after conversion to HiCN yields an optical density $D + \Delta D$, where D is caused by HiCN and ΔD by non-HiCN. Iron determinations yield a value of $c + \Delta c$ milligram-atom Fe/liter, where c is hemoglobin iron and Δc belongs to non-HiCN iron. From these values the extinction coefficient of HiCN is calculated ($l = 1.000$ cm):

$$\epsilon = (D + \Delta D)/(c + \Delta c) \tag{5}$$

If we now introduce ϵ', the true extinction coefficient of HiCN, keeping in mind that c milligram-atom Fe/liter corresponds to c quarter millimoles Hb/liter, we may change Eq. (5) to:

$$\epsilon' = \epsilon + (\Delta c/c)\epsilon - (\Delta D/c)$$

The true extinction coefficient thus equals the experimentally obtained value plus a correction for non-HiCN iron and minus a correction for non-HiCN extinction.

Even if it were possible to evaluate the factor $\Delta D/c$ accurately, it would not be advantageous to apply this correction to the experimentally obtained extinction coefficient. In routine hemoglobin photometry some non-HiCN extinction will always be present. Thus, using an extinction coefficient influenced by a similar factor will at least partially eliminate the non-HiCN extinction error from the determination of hemoglobin concentrations.

Theoretically it would be an advantage to apply the correction for non-hemoglobin iron: $(\Delta c/c)\epsilon$. The determination of the oxygen capacity is not suitable for this purpose, since HbCO and Hi will not be determined. However, these hemoglobin derivatives should certainly be included in a (total) hemoglobin determination. The best method presently available to determine the sum of Hb, HbO_2, Hi, and HbCO is the determination of the CO-combining capacity after reduction with $Na_2S_2O_4$. However, as long as the reactions caused by the addition of $Na_2S_2O_4$ and CO to hemoglobin solutions are not fully understood and no common opinion has been established on the significance of the CO capacity in the presence of $Na_2S_2O_4$, it seems unwise to use this method as a basic procedure for evaluating ϵ^{540}_{HiCN}. Consequently, the factor $(\Delta c/c)\epsilon$ being small but not yet accurately known, no correction should be applied and ϵ^{540}_{HiCN} determined on the basis of total iron should be taken as the true extinction coefficient of HiCN at $\lambda = 540$ nm.

It is therefore obviously justified to adopt iron determination as the basic method for the evaluation of ϵ^{540}_{HiCN}, this method being the one with the most solid experimental foundation presently available. The methods

described in Sections 2.1.2, 2.1.3, and 2.1.4 proved to be suitable for an accurate determination of ϵ^{540}_{HiCN} on the basis of iron.

2.1.2. *Spectrophotometric Iron(II) Determination with α,α′-Dipyridyl*

Decomposition. A hemoglobin preparation (1.00 ml) is decomposed using 1 ml nitric acid (65%) and 0.5 ml hydrogen peroxide (30%). To prevent foaming three drops of octyl alcohol are added and the solution is carefully heated. A clear, light yellow solution is obtained, which is evaporated almost to dryness. To remove excess acid, 1–2 ml water is added and evaporated *in vacuo* at 90–100°C; this is repeated once. The contents of the digestion tube are finally transferred to a volumetric flask and made up to 50 ml with water.

Measurement. This solution (2.0 ml) is mixed with 1.0 ml buffered α,α′-dipyridyl solution (0.5 g α,α′-dipyridyl, 3.4 g sodium acetate trihydrate, and 1.41 ml glacial acetic acid, made up to 50 ml with water) and 1.0 ml sodium sulfite solution (2.52 g $Na_2SO_3 \cdot 7H_2O$ made up to 100 ml with water). After adding 3.5 ml water the mixture is heated for 10 min at 100°C in a water bath. During heating the test tubes are covered with glass marbles and the upper end of the tubes is cooled by an air stream. The optical density of the pink solution is then measured at room temperature using a Beckman DU or similar spectrophotometer with water as a blank (λ = 520 nm; slit width = 0.025 mm; l = 1.000 cm). Appropriate corrections are made for traces of iron in reagents and glassware (blank decomposition) and for the faint yellowish background color of the solution obtained by destruction of the hemoglobin.

Calibration. A calibration curve is made using ferric ammonium sulfate, $Fe_2(SO_4)_3 \cdot (NH_4)_2SO_4 \cdot 24H_2O$ (analytical grade, Merck), the iron content of which has repeatedly been checked by gravimetric analysis. The salt is stored in an atmosphere corresponding to the water vapor pressure of the crystals (6 mm Hg). A stock solution is prepared containing 1.7272 g ferric ammonium sulfate per liter, which corresponds to a concentration of 3.582 milligram-atom Fe per liter. After 20-fold dilution of the stock solution, 0.5, 1.0, . . . , and 5.5 ml made up to 7.5 ml with water provide a series of solutions containing 1.194×10^{-2}, 2.388×10^{-2}, . . . , and 13.134×10^{-2} milligram-atom Fe per liter. The ΔD^{520} between two succeeding steps equals 0.103. As the hemoglobin dilution factor in this procedure is 187.5, the iron content of the hemoglobin sample is obtained in milligram-atom per liter by multiplying the value of D^{520} by 21.73.

2.1.3. *Titrimetric Iron(III) Determination with Titanous Chloride*

Decomposition. A hemoglobin preparation (1.00 ml) is decomposed using 1 ml concentrated sulfuric acid and 250 mg potassium sulfate. Mercury or

selenium cannot be used as a digestion catalyst, because they interfere with the titration. After 30 minutes, heating is interrupted for 5 minutes, after which 1 ml concentrated sulfuric acid and 0.5 ml hydrogen peroxide (30%) are added; the addition of hydrogen peroxide is repeated after 1 and 2 hours.

Titration. After cooling, 10 ml water and 1 ml potassium thiocyanate (20%) are added. The titration with titanous chloride is carried out in the digestion tube in a CO_2 atmosphere. The titanous chloride solution (about 0.15%) is frequently standardized against the ferric ammonium sulfate stock solution (mentioned in Section 2.1.2).

2.1.4. *Determination of D^{540} of Hemiglobincyanide*

Preparation of HiCN solution. A hemoglobin preparation (1.00 ml) is pipetted into a 250-ml volumetric flask containing about 50 ml diluting solution (200 mg potassium ferricyanide, 50 mg potassium cyanide, and 1.0 g sodium bicarbonate made up to 1 liter with water). After homogenization the volume is made up to 250 ml with the same diluting solution.

Measurement. The optical density of the HiCN solution is measured after at least 30 minutes using a Beckman DU or similar spectrophotometer with water as a blank (λ = 540 nm; slit width = 0.02 mm; l = 1.000 cm). The optical density of the diluting solution at 540 nm should be zero when measured against water.

2.1.5. *Actual Determination of ϵ^{540}_{HiCN} in 21 Hemoglobin Solutions*

To obtain a hemoglobin solution suitable for the determination of ϵ^{540}_{HiCN}, about 90 ml fresh human blood is collected in a 200-ml conical flask containing 20 ml 3.2% sodium citrate ($Na_3C_6H_5O_7 \cdot 5\frac{1}{2}H_2O$). The erythrocytes are washed twice with 0.9% NaCl solution. An equal volume of twice-distilled water is added to the erythrocytes, and highly purified toluene is added in an amount of 40% of the original erythrocyte volume. After thorough mixing the fluid is stored at 4°C for 12 hours. After centrifugation the mixture displays three clearly distinct layers. The upper layer consists of toluene, the middle one is a turbid suspension of erythrocyte stromata, and the third is a clear oxyhemoglobin solution. The first two layers are sucked off completely. The HbO_2 solution is now filtered through ash-free filter paper and stored at 4°C.

The ϵ^{540}_{HiCN} was determined for 21 hemoglobin solutions thus prepared using the methods described in Sections 2.1.2, 2.1.3, and 2.1.4. All glassware used for the iron determinations was made iron-free as far as possible. Only analytical grade chemicals (Merck) were used. For pipetting hemoglobin solutions, repeatedly calibrated Ostwald pipettes were used. Spectrophotometry was performed with the aid of one Unicam SP-500 and four

Beckman DU spectrophotometers. The results are presented in Table 1. The values given in the second and third columns were obtained independently in two laboratories; the next column shows the total number of D^{540} determinations in the two laboratories. In most cases the values of D^{540} determined in the two laboratories are in good agreement. In a few

TABLE 1

VALUES OF D^{540}_{HiCN} AND OF ϵ^{540}_{HiCN}, BASED ON DIFFERENT DETERMINATIONS OF C_{HiCN}, FOR 21 HEMOGLOBIN PREPARATIONS (Z1)

Preparation	D^{540}			α, α'-Dipyridyl				$TiCl_3$	
				Lab 1		Lab 2		Lab 1	
	Lab 1	Lab 2	n	ϵ^{540}	n	ϵ^{540}	n	ϵ^{540}	n
1	0.419	—	4	—	—	—	—	—	—
2	0.294	0.294	9	10.94	2	11.04	2	—	—
3	0.419	0.420	10	11.30	2	11.15	2	—	—
4	0.362	0.365	7	10.93	5	11.08	1	—	—
5	0.397	0.397[5]	8	11.05	8	—	—	11.07	3
6	0.387	0.390	10	11.13	3	10.83	2	11.11	5
7	0.330	0.331	7	11.10	6	10.92	2	10.95	6
8	0.382	0.394	9	10.95	9	10.94	2	11.07	3
9	0.352	0.357	8	11.10	6	11.06	2	10.93	6
10	0.381	0.353[5]	10	10.98	6	11.10	2	11.23	6
11	0.398	0.399	9	10.89	6	10.92	2	10.97	6
12	0.394	0.396	9	11.04	4	11.12	2	11.01	6
13	0.350	0.351	6	11.05	6	11.01	2	11.19	5
14	0.322	0.322[5]	6	11.04	6	10.81	2	11.20	6
15	0.379	0.375	7	10.97	9	10.82	2	10.95	4
16	0.376	—	5	10.83	6	—	—	11.08	9
17	0.342	0.342[5]	8	10.99	6	10.89	2	11.10	5
18	0.354	0.354	9	10.95	9	10.69	2	11.08	9
19	0.321	0.320	7	10.98	9	10.80	2	11.01	7
20	0.370	0.359	9	10.87	9	10.81	2	10.80	9
21	0.260	0.260	6	11.13	6	11.00	2	11.28	6

cases, however, the difference between the values in the second and third columns exceeds the acceptable random error (e.g., preparations 10 and 20). Nevertheless the resulting ϵ^{540}_{HiCN} values are in good agreement. The explanation of this fact is to be found in corresponding differences in the results of the iron determinations. In a few preparations a significant difference existed between the concentrations of the solutions as analyzed in the two laboratories. The average values of ϵ^{540}_{HiCN} are presented in Table

2, together with the standard deviation and the standard error of the mean. In calculating these quantities, the scatter of the iron determinations as well as that of the spectrophotometric measurements has been taken into account.

2.1.6. *Discussion of* ϵ^{540}_{HiCN} *as Determined by Various Investigators*

In the first determinations of ϵ^{540}_{HiCN}, made by Drabkin and Austin (D3, D4), hemoglobin concentrations were determined by measuring oxygen capacity using Van Slyke's manometric procedure; the optical density of the HiCN solutions was measured with a (visual) König-Martens spectrophotometer. A value of 11.51 was found as the average of 7 determinations. Spectrophotometry of HiCN solutions was repeated on a larger scale yielding $\epsilon^{540}_{HiCN} = 11.53 \pm 0.05 \pm 0.01$ (D1), but it seems that only the spread of the determinations is of significance in this series, since the average constants had been assumed on the basis of the earlier work.

TABLE 2

AVERAGE AND STANDARD DEVIATIONS[a] OF ϵ^{540}_{HiCN}, BASED ON DIFFERENT DETERMINATIONS OF c_{HiCN}

Method for determining c_{HiCN}	ϵ^{540}_{HiCN}	s	$s/n^{1/2}$	n
Fe^{2+} (α, α'-dipyridyl) (lab 1)	10.99	0.126	0.014	123
Fe^{2+} (α, α'-dipyridyl) (lab 2)	10.94	0.153	0.026	35
Fe^{3+} ($TiCl_3$) (lab 1)	11.05	0.156	0.017	101

[a] s = standard deviation; $s/n^{1/2}$ = standard error of the mean; n = number of determinations.

A value of 11.5 for ϵ^{540}_{HiCN}, based on Drabkin's work, has been widely used as a basis for hemoglobin standardization (C1, C2). This value has been accepted rather uncritically, in some instances even in the conviction that it was based on hemoglobin iron determinations. This may be partly due to a paper by Lambertsen *et al.* (L1), where the oxygen capacity measurements underlying $\epsilon^{540}_{HiCN} = 11.5$ are assimilated to hemoglobin iron determinations. In their paper it is stated, "The total pigment was determined independently as cyanmethemoglobin which is equivalent to estimation of hemoglobin iron."

Using a titrimetric iron determination with titanous chloride, Remmer (R3) obtained for ϵ^{540}_{HiCN} a value of 11.09 as an average of 11 determinations. Minkowski (M7) found $\epsilon^{540}_{HiCN} = 11.15$, using a spectrophotometric method involving the use of orthophenanthroline. These values are in fair agreement with our results (see Section 2.1.5 and Table 2). An indirect proof of $\epsilon^{540}_{HiCN} =$

11.0 has been provided by Van Oudheusden *et al.* (V4), who compared the HiCN method based on this value for the quarter millimolar extinction coefficient with a hemoglobin determination involving the evaluation of iron by a modified α,α'-dipyridyl method. The average difference between the two methods was only 0.024 g/100 ml ($n = 17$). Very recently Salvati *et al.* (S1) purified hemoglobin solutions with the aid of a carboxymethyl cellulose (CMC) column, using a phosphate buffer of pH = 6.8 as elution fluid. A modified α,α'-dipyridyl method according to Mason and Adarraga-Elizaran (M5) was then used for the determination of iron. ϵ^{540}_{HiCN} was found to be 10.95 ($n = 46$) with a standard error of 0.03.

Thus there now exists overwhelming experimental evidence that the quarter millimolar extinction coefficient of HiCN at $\lambda = 540$ nm is very near 11.0. This value was therefore proposed by the standardization committee of the European Society of Hematology at its 1963 meeting in Lisbon (B2) and was definitely adopted at the next meeting of this committee (Stockholm, 1964, B3).

2.2. The Hemiglobincyanide Method

2.2.1. *The Diluting Solution*

To achieve a diluent for hemolyzing blood samples as well as for rapid and complete conversion of all hemoglobin derivatives present to HiCN, without the development of turbidity caused by plasma proteins and erythrocyte stromata, the following points should be observed: (1) The absorption spectrum of HiCN, and consequently ϵ^{540}_{HiCN}, is independent of the pH of the solution (V3). (2) The formation of Hi from HbO_2, and to a lesser degree of HiCN from Hi, proceeds more rapidly at low pH and low ionic strength (V3). (3) Therefore, and to promote rapid lysis of the red cells, the total amount of solutes in the diluent should be as low as possible. (4) Dilution of blood samples with a solution of near neutral pH often causes a slight turbidity to develop through precipitation of plasma globulins.

The consequences of (2) and (4) seem rather incompatible. To acquire a high reaction velocity, a diluent with pH 7 is desirable. However, the use of such a diluent is forbidden because of the inevitable occurrence of slight turbidity. Very different diluting solutions have thus been proposed, depending on the direction of the investigator's attention. Chilcote and O'Dea (C3), using a pH of 7.4, obtained stable optical density readings within a few minutes, but their results show a significant influence of proteins, up to 2.6%, dependent on the plasma albumin/globulin ratio. Even the conventional diluting solution (mentioned in Section 2.1.4; pH 8.6), may cause globulin precipitation in the case of pathological sera. This

persuaded Green and Teal (G3) to add 100 mg K_2CO_3, thus increasing the pH to 8.9. Yet the same result could have been attained more simply by diminishing the amount of $NaHCO_3$, the pH of a solution of 200 mg $K_3Fe(CN)_6$ and 50 mg KCN being 9.6.

Using the conventional diluent, stable optical density readings are attained in 15–25 min, which is a distinct disadvantage in clinical practice. Lowering the pH of the diluent to 7.2 yields a stable value for D^{540} within 5 minutes, irrespective of whether the pH is determined by borate, barbiturate, or phosphate buffers (V3). However, as in the observation of Chilcote and O'Dea, the results are 2–3% too high. Addition of 0.5 ml Sterox SE[2] per liter diluting solution appeared to suppress the development of turbidity completely, even at pH 7.

In this concentration, Sterox SE has no influence on the absorption spectrum of HiCN and increases the over-all reaction velocity by its strong

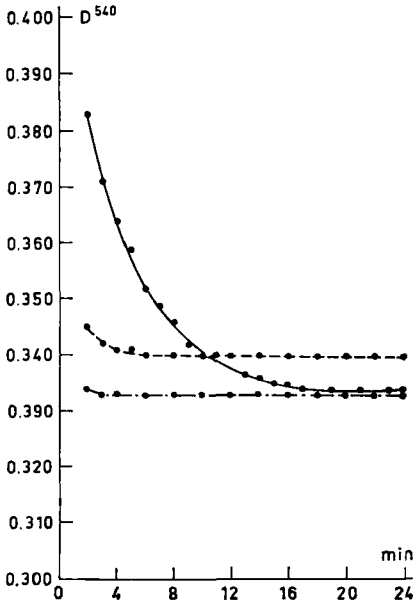

FIG. 4. Formation velocity of HiCN from HbO_2. D^{540} plotted against time. At zero time 0.5 ml blood was mixed with 99.5 ml of different diluting solutions: ——— Diluting solution containing 200 mg $K_3Fe(CN)_6$, 50 mg KCN, and 1 g $NaHCO_3$ per liter, pH 8.6. - - - - - Diluting solution containing 200 mg $K_3Fe(CN)_6$, 50 mg KCN, and 140 mg KH_2PO_4 per liter, pH 7.2. —·—·— Diluting solution containing 200 mg $K_3Fe(CN)_6$, 50 mg KCN, 140 mg KH_2PO_4, and 0.5 ml Sterox SE per liter, pH 7.2.

[2] A polythiol compound manufactured by Hartman-Leddon Company, Philadelphia, Pennsylvania, U.S.

hemolytic action. Thus a diluting solution of the following composition has been introduced: 200 mg $K_3Fe(CN)_6$, 50 mg KCN, 140 mg KH_2PO_4, and 0.5 ml Sterox SE made up to 1 liter with water (V3).

Figure 4 shows the time course of D^{540} when using different diluting solutions. The HiCN solution prepared with the conventional diluting solution reaches its final D^{540} 18 minutes after the moment of mixing blood and diluent. The D^{540} values of the other solutions (pH 7.2) are stable within 3 minutes, the one without Sterox SE giving values slightly too high, caused by turbidity.

2.2.2. *Spectrophotometric Procedure*

A 20-μl homogenized blood sample is added to 5 ml diluting solution. Blood and diluent are mixed well. After 3 minutes the optical density is measured against water (λ = 540 nm; slit width = 0.02 mm; l = 1.000 cm). The hemoglobin concentration is calculated by means of the equation

$$c = (fD^{540})/\epsilon^{540}_{\text{HiCN}} \text{ quarter millimoles/liter} \tag{6}$$

or

$$c = (fD^{540}M \times 10^{-4})/\epsilon^{540}_{\text{HiCN}} \text{ g/100 ml} \tag{7}$$

where f = dilution factor = 251, D^{540} = optical density at λ = 540 nm, $\epsilon^{540}_{\text{HiCN}}$ = quarter millimolar extinction coefficient = 11.0, and M = quarter molecular weight of hemoglobin = 16114. Substituting these values in Eq. (7) gives

$$c = 36.8D^{540} \text{ g/100 ml}$$

2.2.3. *Filter Photometric Procedure*

In this procedure, a filter with a maximum transmission near 540 nm has to be used. An interference filter with a half-intensity band width of about 20 nm proved to be suitable. The hemoglobin concentration is read from a calibration line which is established with the aid of a single HiCN standard solution (Section 2.3). The validity of this procedure is demonstrated in Fig. 5, where it is shown that a calibration line drawn through a point obtained with the standard solution and the origin coincides with a calibration line established using a series of known HiCN solutions.

2.3. Preparation of a Hemiglobincyanide Standard Solution

A partly purified HbO_2 solution is prepared (as described in Section 2.1.5). To make up 1 liter of HiCN standard solution, 5 ml HbO_2 solution is added to the diluting solution (described in Section 2.1.4). The HiCN

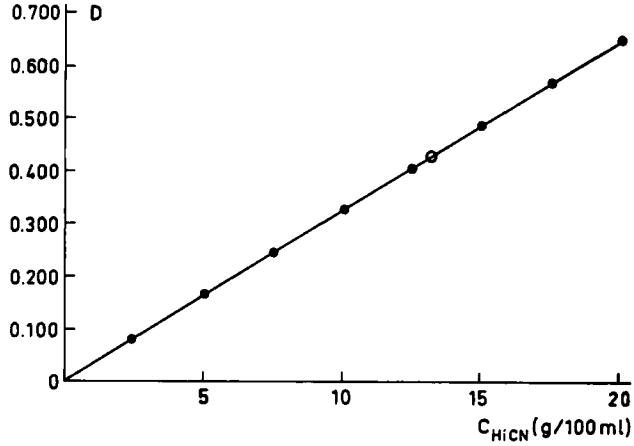

FIG. 5. Calibration line of a Beckman C photometer. D^{540} of a series of HiCN solutions, measured using an interference filter (λ_{max} = 540 nm), has been plotted against the hemoglobin concentration of the undiluted blood; O is the result of the measurement of a single HiCN standard solution.

solution thus obtained is sterilized by Seitz filtration and transferred, under rigid aseptic precautions, to brown glass ampules of 10 ml. The concentration of the HiCN solution in at least 5 ampules, taken at random, is determined spectrophotometrically, using $\epsilon^{540}_{HiCN} = 11.0$ and a quarter molecular weight of 16114. These determinations are a check on the homogeneity of the lot and yield the value to be printed on the ampules (Z2).

Twice 5 ampules, taken at random, are incubated at 22° and 37°C in four different culture media (broth, glucose broth, ascites broth, and an anaerobic medium) at an interval of some weeks. When no growth occurs the lot is considered to be microbiologically sterile.

To check the stability of the preparation, ampules are stored in the dark at 4° and 20°C for at least 1 year. The optical density at λ = 540 and 504 nm is measured every month. During this time D^{540}_{HiCN} should not decrease more than 1%, while the quotient $D^{540}_{HiCN}/D^{504}_{HiCN}$ should remain between 1.62 and 1.58.

3. Absorption Spectra and Extinction Coefficients

When the extinction coefficient of a hemoglobin derivative at a given wavelength is known accurately, any extinction coefficient of any hemoglobin derivative may be determined by using spectrophotometry only. Thus, using $\epsilon^{540}_{HiCN} = 11.0$ (see Section 2.1.5) as a starting point, ϵ/λ curves have been determined for Hb, HbO_2, HbCO, Hi, and HiCN.

3.1. Spectrophotometric Technique

A Beckman DU spectrophotometer was used for all measurements. The slit width was chosen so that a half-intensity band width of 1.6 nm was obtained in the region λ = 650–590 nm, 0.6 nm in the region λ = 590–460 nm, and 0.15 nm in the region of the Soret bands. The wavelength of the instrument had been checked with the aid of the mercury emission spectrum. Measurements at $\lambda \geq 590$ nm were made in a layer thickness of 0.0993 cm, at $\lambda < 590$ nm in a layer thickness of 0.0128 cm. The latter was obtained by inserting a plane parallel glass plate into the 0.0993-cm cuvette (Fig. 13). The layer thickness of the cuvette had been determined spectrophotometrically using HiCN solutions and a 1.000-cm cuvette as a reference.

Assuming Lambert-Beer's law to be valid for all solutions over the entire spectral region, ϵ^λ can be calculated from D^λ for any hemoglobin derivative, when the total hemoglobin concentration of the sample has been determined:

$$\epsilon^\lambda = D^\lambda / cl$$

where ϵ^λ = the quarter millimolar extinction coefficient at wavelength λ, D^λ = optical density at wavelength λ, c = total hemoglobin concentration in quarter millimoles/liter, and l = layer thickness in cm. The total hemoglobin concentration was determined using the spectrophotometric HiCN method described (Section 2.2.2) with the exception that a dilution factor of 100 was used, obtained by diluting 1 ml of the hemoglobin derivative solution to 100 ml with the diluting solution.

3.2. Preparation of Blood Samples

Fresh blood was obtained from nonsmoking individuals by venipuncture, with heparin added to prevent clotting. The erythrocytes were washed 3 times with isotonic saline solution and the packed cells then brought to the original blood volume with 0.7% Sterox SE solution. After mixing thoroughly, the hemolysate was filtered through a folded paper filter and stored in 20-ml all-glass syringes.

3.3. The Absorption Spectrum of Hemoglobin (Hb)

The hemolysate (Section 3.2) was further diluted with 0.7% Sterox SE solution until an optical density of about 0.500, measured in a layer thickness of 0.0128 cm at λ = 577 nm, was reached. The total hemoglobin concentration ($C_{\text{Hb tot.}}$) of the diluted hemolysate was measured (as described in Section 3.1). To the sample, $Na_2S_2O_4$ (4 mg/ml) was added for complete conversion to Hb, and the spectrophotometric measurements

down to $\lambda = 460$ nm were made. The diluted hemolysate was further diluted 10-fold with 0.7% Sterox SE, and $C_{\text{Hb tot.}}$ was determined by adding 90 ml of the diluting solution (described in Section 2.2.1) to 10 ml of the

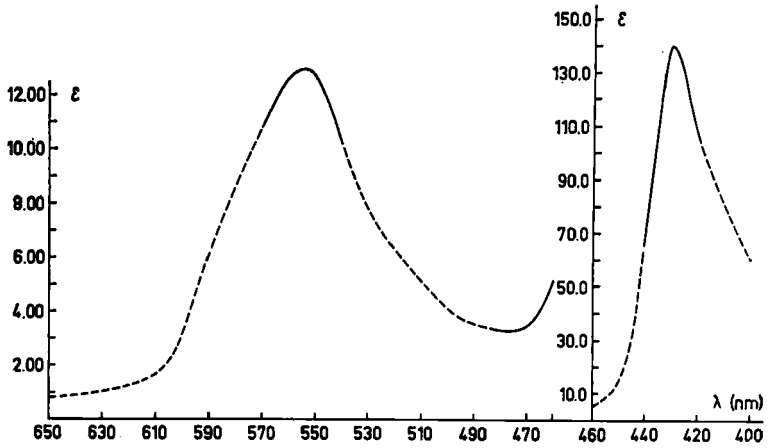

FIG. 6. ϵ/λ curve of hemoglobin (Hb). Quarter millimolar extinction coefficients based on $\epsilon^{540}_{\text{HiCN}} = 11.0$. The *solid part* of the graph represents the mean value resulting from 5 samples, the *dashed part* results from measurements of a single sample. Measurements made with a Beckman DU spectrophotometer, layer thickness 0.0128 cm; 10-fold dilution of samples for measuring below $\lambda = 460$ nm.

sample and measuring at $\lambda = 540$ nm. $Na_2S_2O_4$ (0.4 mg/ml) was then added to the sample, and the measurements down to $\lambda = 400$ nm were carried out. The resulting ϵ/λ curve is shown in Fig. 6.

TABLE 3
ϵ VALUES OF LIGHT ABSORPTION MAXIMA AND MINIMA OF HB

Wavelength (nm)	Mean ϵ ($n = 5$)	Highest value	$\Delta\%$	Lowest value	$\Delta\%$
556–554	13.04	13.26	+1.7	12.86	−1.4
478	3.31	3.45	+4.2	3.17	−4.2
431	140.10	141.70	+1.1	138.44	−1.2

Table 3 presents the extremes of the ϵ values found for the light absorption maxima and minima; in Table 4 some of the isobestic wavelengths and corresponding ϵ values are given. It is interesting to note the agreement of our value for $\epsilon^{431}_{\text{Hb}}$ and that found by Benesch *et al.* (B1), who added $NADH_2$ (formerly called DPNH), methylene blue, and methemoglobin reductase to their hemoglobin solution and arrived at $\epsilon^{431}_{\text{Hb}} = 140.0$ based on $\epsilon^{540}_{\text{HiCN}} = 11.5$.

TABLE 4

ISOBESTIC POINTS (IBP) OF HB WITH HbO_2, HBCO, AND HI; ϵ VALUES OF HB AT THE ISOBESTIC WAVELENGTHS

Hemoglobin derivative	IBP (nm)	ϵ	IBP (nm)	ϵ	IBP (nm)	ϵ	IBP (nm)	ϵ	IBP (nm)	ϵ
HbO_2	586	7.23	569	11.27	548.5	12.46	522	6.42	507	4.81
HbCO	579	8.86	561.5	12.54	547.5	12.37	—	—	—	—
Hi	600	3.20	—	—	—	—	528.5	7.71	—	—

3.4. THE ABSORPTION SPECTRUM OF OXYHEMOGLOBIN (HbO_2)

The hemolysate (Section 3.2) was further diluted with 0.7% Sterox SE solution until an optical density of about 0.400, measured at $\lambda = 577$ nm in a layer thickness of 0.0128 cm, was reached. Of this solution 10 ml was brought into a revolving glass tonometer which was 30-cm long. The tonometer was continuously flushed with pure oxygen (1 liter/min) for

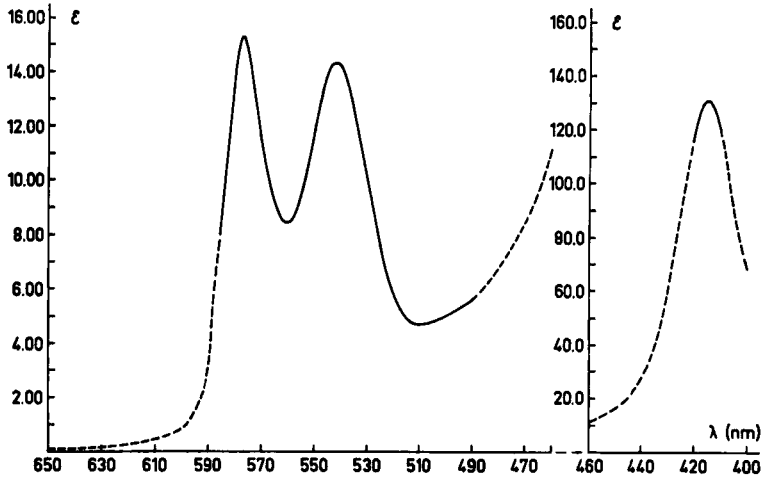

FIG. 7. ϵ/λ curve of oxyhemoglobin (HbO_2). Quarter millimolar extinction coefficients based on $\epsilon^{540}_{HiCN} = 11.0$. The *solid part* of the graph represents the mean value resulting from 5 samples, the *dashed part* results from measurements of a single sample. Measurements made with a Beckman DU spectrophotometer, layer thickness 0.0128 cm; 10-fold dilution of samples for measuring below $\lambda = 460$ nm.

10 minutes, $C_{Hb\ tot.}$ was determined (as described in Section 3.1), and the spectrophotometric measurements down to $\lambda = 460$ nm were made. The HbO_2 solution was next diluted 10-fold with Sterox SE 0.7%, the $C_{Hb\ tot.}$ was redetermined (as described in Section 3.4), and the measurements

TABLE 5

ε VALUES OF LIGHT ABSORPTION MAXIMA AND MINIMA OF HbO_2

Wavelength (nm)	Mean ε (n = 5)	Highest value	Δ%	Lowest value	Δ%
577	15.37	15.46	+0.6	15.31	−0.4
560	8.47	8.51	+0.5	8.40	−0.8
543–541	14.37	14.44	+0.5	14.27	−0.7
510	4.76	4.80	+0.8	4.68	−1.7
415	130.98	133.89	+2.2	127.30	−2.4

TABLE 6

ISOBESTIC POINTS (IBP) OF HbO_2 WITH HB, HBCO, AND HI; ε VALUES OF HbO_2 AT THE ISOBESTIC WAVELENGTHS

Hemoglobin derivative	IBP (nm)	ε	IBP (nm)	ε	IBP (nm)	ε	IBP (nm)	ε	IBP (nm)	ε
Hb	568	7.23	569	11.27	548.5	12.46	522	6.42	507	4.81
HbCO	572.5	13.50	549.3	12.06	540	14.27	—	—	497	5.16
Hi	590.7	3.62	—	—	—	—	525.3	7.72	—	—

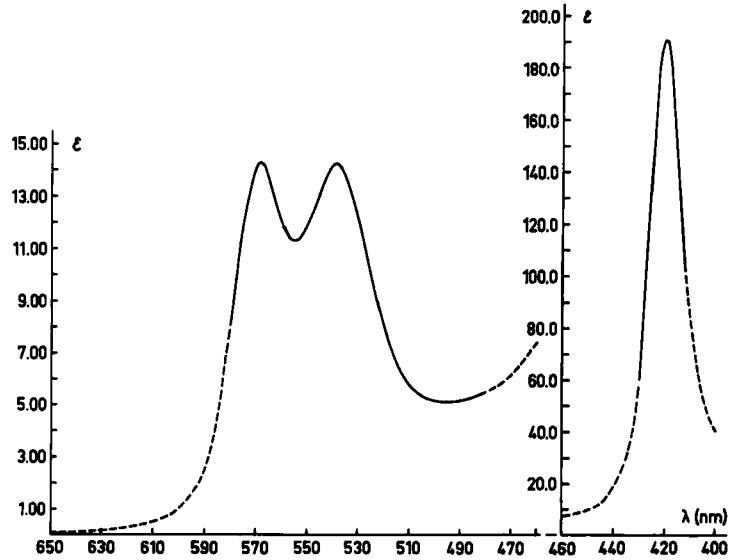

FIG. 8. ε/λ curve of carboxyhemoglobin (HbCO). Quarter millimolar extinction coefficients based on $\epsilon^{540}_{HiCN} = 11.0$. The *solid part* of the graph represents the mean value resulting from 5 samples, the *dashed part* results from measurements of a single sample. Measurements made with a Beckman DU spectrophotometer, layer thickness 0.0128 cm; 10-fold dilution of samples for measuring below λ = 460 nm.

down to $\lambda = 400$ nm were carried out. The resulting ϵ/λ curve is depicted in Fig. 7.

Table 5 presents the extremes of the ϵ values found for the light absorption maxima and minima; in Table 6 some of the isobestic wavelengths and corresponding ϵ values are given. Benesch *et al.* (B1) found $\epsilon^{415}_{HbO_2} = 132$, based on $\epsilon^{540}_{HiCN} = 11.5$.

3.5. The Absorption Spectrum of Carboxyhemoglobin (HbCO)

The HbO_2 hemolysate described in Section 3.4 was used. Tonometry of a 10-ml sample was carried out for 15 minutes while continuously flushing with 99% pure CO, and the $C_{Hb\,tot.}$ was determined (Section 3.1). In this case, measurement of D^{540}_{HiCN} was not made for at least 2 hours, as the conversion of pure HbCO to HiCN proved to take some 90 minutes. The spectrophotometric measurements were then made down to $\lambda = 460$ nm. For the measurements down to $\lambda = 400$ nm, a further 10-fold dilution of the sample and a redetermination of $C_{Hb\,tot.}$ (Section 3.3) were carried out. The resulting ϵ/λ curve is given in Fig. 8.

TABLE 7

ϵ Values of Light Absorption Maxima and Minima of HbCO

Wavelength (nm)	Mean ϵ ($n = 5$)	Highest value	Δ%	Lowest value	Δ%
569–568	14.36	14.43	+0.5	14.32	−0.3
555	11.33	11.45	+1.1	11.25	−0.6
539	14.33	14.37	+0.3	14.30	−0.2
496	5.16	5.27	+2.1	5.07	−1.7
420	191.91	198.52	+3.4	184.32	−4.0

The extremes of the ϵ values found for the light absorption maxima and minima are given in Table 7; some of the isobestic wavelengths with their corresponding ϵ values are given in Table 8.

TABLE 8

Isobestic Points (IBP) of HbCO with Hb, HbO_2, and Hi; ϵ Values of HbCO at the Isobestic Wavelengths

Hemoglobin derivative	IBP (nm)	ϵ	IBP (nm)	ϵ	IBP (nm)	ϵ	IBP (nm)	ϵ	IBP (nm)	ϵ
Hb	579	8.86	561.5	12.54	547.5	12.37	—	—	—	—
HbO_2	572.5	13.50	549.3	12.06	540	14.27	—	—	497	5.16
Hi	585.5	3.79	—	—	—	—	519.7	8.02	—	—

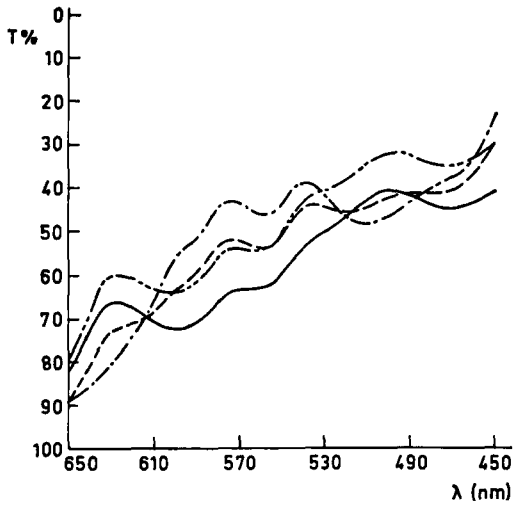

FIG. 9. Light absorption spectra of hemiglobin (Hi) at varying pH. Preparation of Hi from HbO_2 by means of excess $K_3Fe(CN)_6$. Recorded with a Beckman DB spectrophotometer, layer thickness 0.007 cm. —·—· pH 9.12 by means of 0.2 *N* NaOH; - - - - pH 8.06 by means of 0.2 *N* NaOH; —··—·· pH 7.02 by means of 0.5 *N* lactic acid; ——— pH 5.94 by means of 0.5 *N* lactic acid.

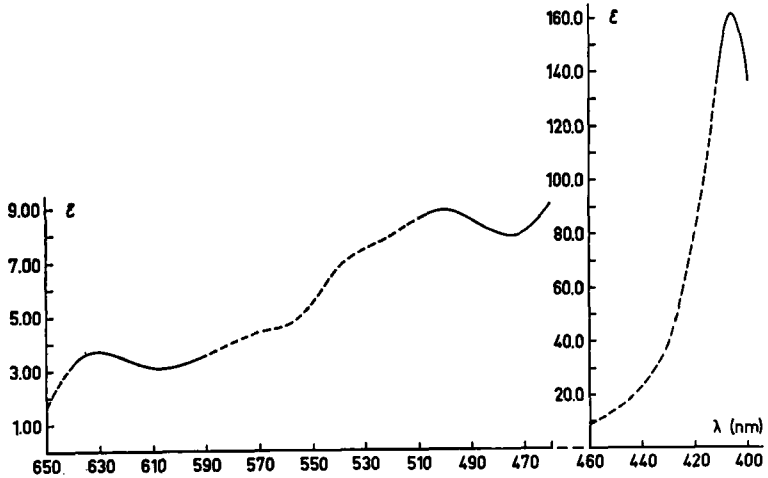

FIG. 10. ϵ/λ curve of hemiglobin (Hi). Quarter millimolar extinction coefficients based on $\epsilon^{540}_{HiCN} = 11.0$. The *solid part* of the graph represents the mean value resulting from 5 samples, the *dashed part* results from measurements of a single sample. Measurements made with a Beckman DU spectrophotometer, layer thickness 0.0128 cm; 10-fold dilution of samples for measuring below $\lambda = 460$ nm.

3.6. The Absorption Spectrum of Hemiglobin (Hi)

Although, as can clearly be seen from Fig. 9, the light absorption spectrum of Hi is greatly dependent upon the pH, no specific measures were taken to ensure all samples having the same pH.

The hemolysate (Section 3.2) was further diluted with an equal volume of 0.7% Sterox SE solution, and 100 mg finely powdered $K_3Fe(CN)_6$ was added to 10 ml of the sample. After some 15 minutes a further 10 mg $K_3Fe(CN)_6$ was added to give an end molar ratio of hemoglobin/$K_3Fe(CN)_6$ of approximately 1:8. The pH was then measured using a radiometer PHM-4C with a capillary glass electrode. Solutions in the pH range 7.0–7.4 were judged suitable and measured spectrophotometrically down to λ = 460 nm after determination of $C_{Hb\ tot.}$ (Section 3.1). A further 10-fold dilution of the sample was made, $C_{Hb\ tot.}$ was redetermined (Section 3.3), and the measurements down to λ = 400 nm were carried out. The resulting ϵ/λ curve is shown in Fig. 10.

In Table 9 extremes of the ϵ values for the light absorption maxima and minima are presented. In this small series no correlation between the

TABLE 9

ϵ Values of Light Absorption Maxima and Minima of Hi

Wavelength (nm)	Mean ϵ (n = 5)	Highest value	Δ%	Lowest value	Δ%
630	3.69	3.85	+4.3	3.44	−6.8
610	3.10	3.22	+3.9	2.95	−4.8
502–500	8.93	9.07	+1.6	8.62	−3.5
476	7.97	8.28	+3.9	7.78	−2.4
406	161.22	167.48	+3.9	153.21	−5.0

pH in this range and the resulting ϵ values could be found. Table 10 gives some of the isobestic wavelengths together with the ϵ values.

TABLE 10

Isobestic Points (IBP) of Hi with Hb, HbO_2, and HbCO; ϵ Values of Hi at the Isobestic Wavelengths

Hemoglobin derivative	IBP (nm)	ϵ	IBP (nm)	ϵ	IBP (nm)	ϵ
Hb	600	3.20	—	—	528.5	7.71
HbO_2	—	—	590.7	3.62	525.3	7.72
HbCO	—	—	585.5	3.79	519.7	8.02

The ϵ values for the Soret band may have been influenced by the presence of porphyrins (see also Section 3.8). Light absorption by $K_3Fe(CN)_6$ in

this region is negligible because of the relatively small amount and the extremely thin layer used.

3.7. The Absorption Spectrum of Hemiglobincyanide (HiCN)

Different HiCN standard solutions, prepared by the Dutch Institute of Public Health (Utrecht, The Netherlands) for the calibration of photometric hemoglobin determinations, were measured in a layer thickness of 1.000 cm. The hemoglobin concentration was calculated from the D^{540} reading. For the measurements in the spectral region $460 > \lambda > 400$ nm, a layer thickness of 0.0993 cm was used.

As a check, a HiCN sample was prepared by diluting the hemolysate of Section 3.2 with 0.7% Sterox SE until an optical density of 0.500 at λ = 577 nm (layer thickness 0.0128 cm) was reached, and then adding 200 mg $K_3Fe(CN)_6$ and 50 mg KCN to 10 ml of this solution. The $C_{Hb\ tot.}$ was determined (as described in Section 3.1) as if the solution contained some unknown hemoglobin sample. The spectrophotometric measurements were then performed in layer thicknesses of 0.0993 and 0.0128 cm down to λ = 600 and 460 nm, respectively. For the measurements down to λ = 400 nm the solution was further diluted 10-fold with 0.7% Sterox SE, and the $C_{Hb\ tot.}$ was redetermined (Section 3.3). The resulting ϵ values did not differ by more than 0.09 when compared with the mean values of the first series of determinations for the spectral region down to λ = 460 nm. The differences at λ = 540 and 504 nm were −0.03 and 0.00, respectively. The differences in the region of the Soret band were less then 4% of the values of the first series, all values of the last sample being lower. This difference may well be due in part to light absorption by $K_3Fe(CN)_6$.

The optical density of all samples in the near infrared was also measured. Whereas the first series shows no absorption in this region, the freshly prepared sample does to a slight degree, undoubtedly caused by incomplete elimination of erythrocyte stromata from the sample. The results are given in Table 11.

TABLE 11
Light Absorption of HiCN Solutions in the Near Infrared

Preparation	ϵ^{1000}	ϵ^{900}	ϵ^{800}	ϵ^{700}	ϵ^{675}
R.I.V.[a] (n = 5)	0.02	0.00	0.00	0.03	0.10
Concentrated sample	0.10	0.05	0.03	0.03	0.12

[a] Rijks Instituut voor de Volksgezondheid (Dutch Institute of Public Health).

The mean ϵ/λ curve from the first series is presented in Fig. 11. The extremes of the ϵ values for the light absorption maxima and minima,

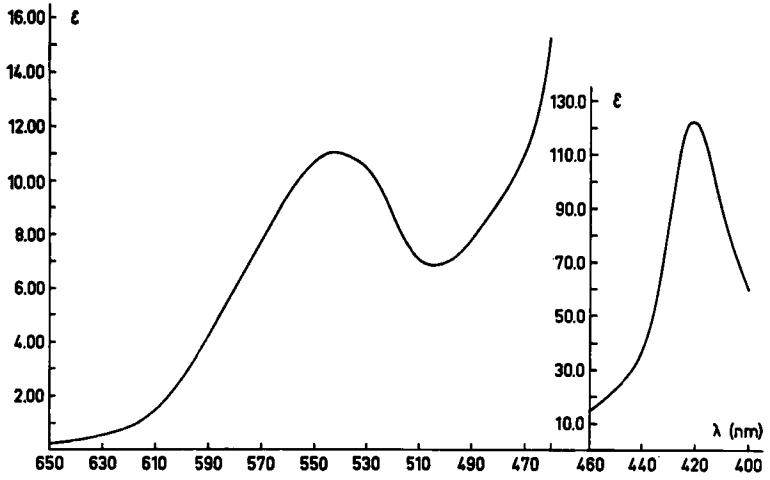

FIG. 11. ϵ/λ curve of hemiglobincyanide (HiCN). Quarter millimolar extinction coefficients based on $\epsilon^{540}_{HiCN} = 11.0$. Results shown are the mean of 5 samples. Measurements made with a Beckman DU spectrophotometer, layer thickness 1.000 cm down to $\lambda = 460$ nm, 0.0993 cm from $\lambda = 460$ nm down to $\lambda = 400$ nm.

together with the value of the quotient $\epsilon^{540}/\epsilon^{504}$ (see Section 2.3), are presented in Table 12.

TABLE 12

ϵ VALUES OF LIGHT ABSORPTION MAXIMA AND MINIMA OF HiCN AND THE VALUE OF $\epsilon^{540}/\epsilon^{504}$

Wavelength (nm)	Mean ϵ ($n = 5$)	Highest value	$\Delta\%$	Lowest value	$\Delta\%$	$\epsilon^{540}/\epsilon^{504}$ High	$\epsilon^{540}/\epsilon^{504}$ Low
542–540	*11.0*	—	—	—	—	1.614	1.606
504	6.83	6.87	+0.6	6.79	−0.6	—	—
421	122.50	125.64	+2.6	120.19	−1.9	—	—

3.8. LIGHT ABSORPTION BY NON-HEMOGLOBIN

In the determination of the quarter millimolar extinction coefficients of hemoglobin derivatives, the following sources of error are present:

(a) *Light absorption by the chemicals used.* The absorption by $Na_2S_2O_4$, KCN, and $K_3Fe(CN)_6$ in the spectral regions studied has been checked. Only $K_3Fe(CN)_6$ proved to have a possibly disturbing effect as it shows an absorption peak at $\lambda = 420$ nm, the millimolar extinction coefficient being 1.01. Because of the quantities used and the layer thickness in which measurements were performed (0.0128 cm), the effect upon the calculated

ϵ values for hemoglobin derivatives is negligible, except in the case of the HiCN standard solutions measured.

(b) *Light absorption by porphyrins.* In the context of the measurements made here the possibility of a disturbing effect through the presence of coproporphyrin III was considered. In Fig. 12 the ϵ/λ curves of the dif-

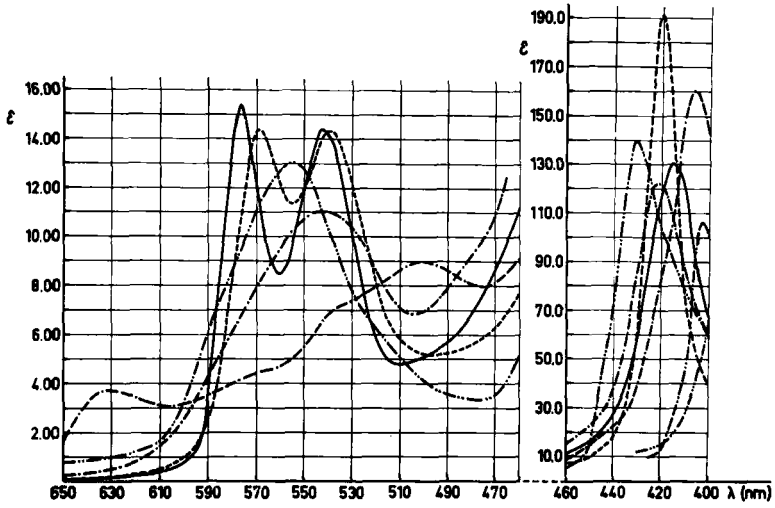

FIG. 12. ϵ/λ curves of hemoglobin derivatives, based on a quarter millimolar extinction coefficient of 11.0 for HiCN at λ = 540 nm. Measurements made with a Beckman DU spectrophotometer, layer thickness 0.0128 cm. For the measurements below λ = 460 nm, 10-fold dilution of the samples. Half-intensity band width 1.6 nm for 650 nm $< \lambda <$ 590 nm, 0.6 nm for 590 nm $< \lambda <$ 460 nm, and 0.15 nm for 460 nm $< \lambda <$ 400 nm. — ·· — ·· = Hb; ——— = HbO_2; - - - - - = HbCO; — — · — — = Hi (pH 7.0–7.4); — · — · — = HiCN; — ··· — ··· = coproporphyrin III, pH 7.0; — — ·· — — = coproporphyrin III, pH 7.7.

ferent hemoglobin derivatives studied are shown together. In addition the millimolar ϵ values found by Zondag and van Kampen (Z4) for coproporphyrin III at pH 7.0 and 7.7 have been drawn in for the spectral region λ = 430–400 nm. It is clear that, if present, a disturbing influence can be expected only for the ϵ values of the Soret band of Hi. As hemolyzed thrice-washed erythrocytes were used in this study, however, it is improbable that any quantity of coproporphyrin was present in the solutions.

4. General Aspects of Two-Wavelength Methods

The determination of the optical density of a two-component system at wavelengths λ_1 and λ_2 provides two equations:

$$D^{\lambda 1} = \epsilon_1^{\lambda 1} c_1 l + \epsilon_2^{\lambda 1} c_2 l$$
$$D^{\lambda 2} = \epsilon_1^{\lambda 2} c_1 l + \epsilon_2^{\lambda 2} c_2 l$$

Dividing $D^{\lambda 1}$ by $D^{\lambda 2}$ yields

$$\frac{D^{\lambda 1}}{D^{\lambda 2}} = \frac{\epsilon_1^{\lambda 1} c_1 + \epsilon_2^{\lambda 1} c_2}{\epsilon_1^{\lambda 2} c_1 + \epsilon_2^{\lambda 2} c_2} \tag{8}$$

The total concentration of light-absorbing substances c is introduced, and $c - c_2$ is substituted for c_1. If, furthermore, λ_2 is an isobestic wavelength, then $\epsilon_1^{\lambda 2} = \epsilon_2^{\lambda 2}$. These conditions being fulfilled, Eq. (8) may be transposed to

$$\frac{D^{\lambda 1}}{D^{\lambda 2}} = \frac{\epsilon_1^{\lambda 1} c + (\epsilon_2^{\lambda 1} - \epsilon_1^{\lambda 1}) c_2}{\epsilon_1^{\lambda 2} c}$$

The concentration of component 2 is now expressed as a fraction of the total pigment concentration, and the equation is solved for c_2/c:

$$\frac{c_2}{c} = \frac{\epsilon_1^{\lambda 2}}{\epsilon_2^{\lambda 1} - \epsilon_1^{\lambda 1}} \frac{D^{\lambda 1}}{D^{\lambda 2}} - \frac{\epsilon_1^{\lambda 1}}{\epsilon_2^{\lambda 1} - \epsilon_1^{\lambda 1}}$$

or

$$c_2/c = a(D^{\lambda 1}/D^{\lambda 2}) - b \tag{9}$$

The constants a and b in Eq. (9) can be calculated from a series of measurements of $D^{\lambda 1}/D^{\lambda 2}$ of solutions containing exclusively component 1 or component 2. This gives the equations

$$0 = a[D^{\lambda 1}/D^{\lambda 2}]_1 - b$$

and

$$1 = a[D^{\lambda 1}/D^{\lambda 2}]_2 - b$$

$[D^{\lambda 1}/D^{\lambda 2}]_1$ and $[D^{\lambda 1}/D^{\lambda 2}]_2$ being determined, a and b follow from these equations.

Equation (9) is of the form $y = Ax + B$, the relationship between c_2/c and $D^{\lambda 1}/D^{\lambda 2}$ thus being *linear*. Knowing two points only, it is therefore possible to construct a calibration line. If, instead of an isobestic wavelength, any other nonisobestic wavelength is chosen, the relationship between c_2/c and $D^{\lambda 1}/D^{\lambda 2}$ is *nonlinear:*

$$\frac{c_2}{c} = \frac{\dfrac{\epsilon_1^{\lambda 1}}{\epsilon_1^{\lambda 2}} - \dfrac{D^{\lambda 1}}{D^{\lambda 2}}}{\dfrac{\epsilon_1^{\lambda 1}}{\epsilon_1^{\lambda 2}} - \dfrac{\epsilon_2^{\lambda 1}}{\epsilon_1^{\lambda 2}} - \dfrac{D^{\lambda 1}}{D^{\lambda 2}}\left(1 - \dfrac{\epsilon_2^{\lambda 2}}{\epsilon_1^{\lambda 2}}\right)} \tag{10}$$

which is of the form

$$y = \frac{A - x}{A - B - (1 - C)x} \tag{11}$$

It is clear that in this case three constants must be determined. If the components 1 and 2 are used only for calibration, extreme care must be taken, when converting 1 to 2, that the total concentration is not altered in any way. If this is not the case, dangerous errors will be made in the determination of the constants B and C, for replacement of ϵ and D in Eq. (10) is allowed only when this requirement has been fulfilled. When use is made of the ϵ values for hemoglobin derivatives (given in Section 3) to overcome this difficulty, one must be certain that measurement of the D values of the sample is performed under exactly the same conditions as those under which the ϵ values were determined.

Any calculation involving Eqs. (9) and (10) presupposes a two-component system. It must be emphasized that this condition is usually fulfilled only as a good approximation. This obviously limits the accuracy of every two-wavelength method.

The necessity of working in a two-component system often complicates the technique insofar as certain measures must be taken to arrive at the desired two components. In the determination of S_{CO}, for example, it is necessary either to saturate the sample with oxygen or to reduce the non-HbCO to Hb by means of $Na_2S_2O_4$. In both cases errors may be introduced (Section 6.2.2).

5. Spectrophotometric Determination of the Oxygen Saturation

Many two-wavelength techniques for the determination of the oxygen saturation (S_{O_2}) have been evolved. As little light is transmitted by Hb and HbO_2 solutions below $\lambda = 600$ nm, methods employing red and near-infrared light are in use (F1, G1, G2, J1). Although excellent results seem to have been procured with these methods, two fundamental objections can be raised. The isobestic wavelength around $\lambda = 800$ nm is quite elusive, not being always found. Also the light absorption of non-hemoglobin in the red and near infrared is relatively large, introducing an important source of error. For this reason many workers have sought ways to overcome the difficulties encountered when measuring in the spectral range $\lambda = 600$–500 nm. As the obvious answer to the problem, diluting the samples, is not permissible in the case of the S_{O_2} determination, special cuvettes with very thin layers have been constructed (D5, H2, K1, N1). Two of these cuvettes have been extensively tested by Refsum and Sveinsson (R2). They compared the results of oxygen saturation determinations using these cuvettes

with those obtained by Van Slyke analysis and found good agreement. In our experience the simple solution of using a 0.1-cm cuvette, into which a plane parallel glass plate of the desired thickness is inserted after filling the cuvette with the sample (Fig. 13), gives excellent results. In this way layers down to 0.005 cm can be procured without difficulty.

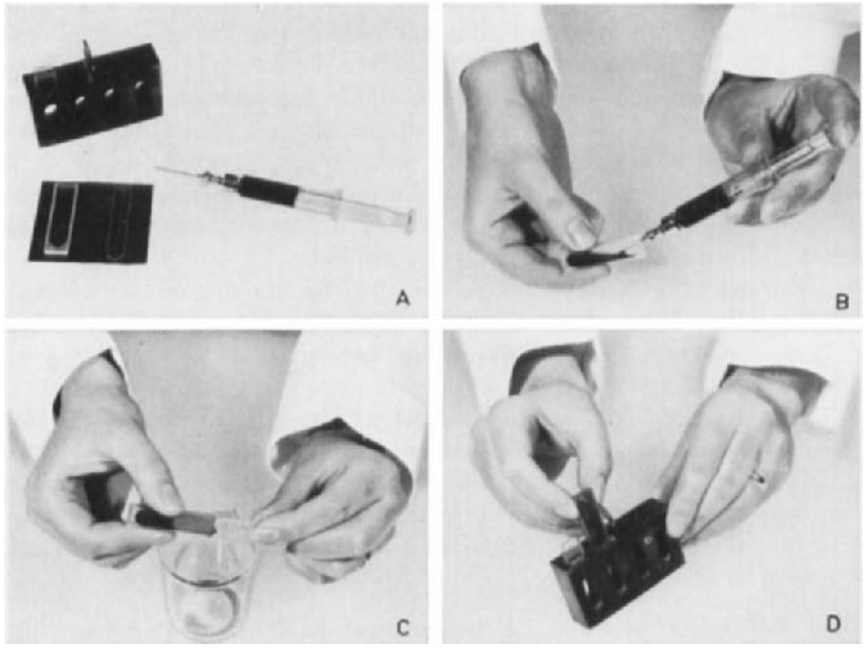

FIG. 13. Filling a 0.10-cm cuvette and inserting a 0.09-cm glass plate. A: Cuvette holder, 0.10-cm cuvette, 0.09-cm glass plate; the syringe contains the blood sample. B: Blood is syringed into the cuvette. C: The glass plate is inserted, leaving a blood layer of 0.01 cm. D: The cuvette is placed in the cuvette holder, which already contains a similar cuvette with distilled water.

Although Refsum (R1) has demonstrated that very good results can be achieved using $\lambda = 576$ and 560 nm (nonisobestic), the methods described (Section 5.1.1) have been chosen because the use of an isobestic wavelength offers distinct advantages (Section 4).

5.1. DETERMINATION OF S_{O_2} MEASURED AT $\lambda = 560$ AND 522 NM, AND $\lambda = 560$ AND 506 NM, RESPECTIVELY; $l = 0.01$ CM

Blood (0.5 ml) is transferred anaerobically from the glass syringe containing the sample to a 2-ml syringe. The syringe has previously been filled with 0.5 ml of an oxygen-free 2% Sterox SE solution and a metal mixing

ring. After thorough mixing and discarding the first three drops, the 0.10-cm cuvette is filled. A 0.09-cm plane parallel glass plate is inserted into the cuvette, and the optical density at 560, 522, and 506 nm is measured. The oxygen saturation is calculated from either

$$S_{O_2}\,(\%) = [3.08 - 1.62(D^{560}/D^{522})]100 \tag{12}$$

or

$$S_{O_2}\,(\%) = [3.13 - 1.22(D^{560}/D^{506})]100 \tag{13}$$

The constants 3.08 and 1.62 have been calculated from a series of 25 measurements of D^{560}/D^{522} for 0% HbO_2 and 100% HbO_2; the constants 3.13 and 1.22 from a series of 17 measurements of D^{560}/D^{506} for 100% HbO_2 and 11 measurements for 0% HbO_2. The series of 17 measurements (100% HbO_2) had a mean value of $D^{560}/D^{506} = 1.753$ with a standard deviation of 0.008; the series of 11 measurements (0% HbO_2) had a mean of $D^{560}/D^{506} = 2.575$ with a standard deviation of 0.012. To get an impression of the accuracy which may be expected when using these constants, 1.753 ± 0.008 and 2.575 ± 0.012 have been substituted in Eq. (13); 1.753 ± 0.008 gives $S_{O_2} = 100 \pm 1.0\%$, and 2.575 ± 0.012 gives $S_{O_2} = 0 \pm 1.5\%$.

5.2. Comparison of the Two Methods

In a series of 30 blood samples sent to the clinical laboratory for routine arterial S_{O_2} determination, the saturation was determined using both the

TABLE 13
Comparison of the Two Methods Described in Section 5.1

S_{O_2} (%)		S_{O_2} (%)	
D^{560}/D^{522}	D^{560}/D^{506}	D^{560}/D^{522}	D^{560}/D^{506}
88	87	62	59
93	93	87	87
85	83	94	95
94	96	95	90
98	99	94	92
95	95	85	83
100	97	94	95
96	95	88	89
98	98	96	96
99	100	95	95
95	96	95	96
95	97	88	92
94	91	96	100
100	100	97	98
96	97	95	95

$\lambda = 560$ and 522 nm and the $\lambda = 560$ and 506 nm method. The results are given in Table 13. In this table each value of S_{O_2} is the mean of a duplicate determination. The standard deviations of the duplicate determinations have been calculated for both methods. Using $\lambda = 560$ and 522 nm a standard deviation of 1.5% saturation was found; in the case of $\lambda = 560$ and 506 nm the standard deviation was 1.4% saturation. The isobestic points were established at $\lambda = 522.2 \pm 0.2$ nm ($n = 100$) and at $\lambda = 506.0 \pm 0.4$ nm ($n = 15$), respectively. Finally, when comparing the two methods, the standard deviation of the difference between the corresponding saturation values (Table 13) was calculated at 1.4% saturation.

It should be borne in mind that establishment of the isobestic point at $\lambda = 522$ nm should be performed extremely carefully, as both the ϵ/λ curves of Hb and of HbO_2 are quite steep in this region (Fig. 12). Accurate wavelength calibration of the spectrophotometer used is thus an absolute requirement.

5.3. Comparison of S_{O_2} Measured Spectrophotometrically at $\lambda = 560$ and 522 nm and Determined by Van Slyke's Manometric Method

In a series of 30 blood samples taken during heart catheterization the S_{O_2} was determined using Van Slyke's manometric technique and the two-wavelength method using $\lambda = 560$ and 522 nm. The results are given in Table 14. The mean difference between the spectrophotometric and manometric methods was +0.57% saturation with a standard deviation of 2.8% saturation.

6. Spectrophotometric Determination of Carboxyhemoglobin

As long as carbon compounds are used for the production of energy and in heating, carbon monoxide intoxication will remain a danger to man. Besides the fact that hemoglobin has a strong affinity for CO (about 200 times the affinity for O_2), the presence of HbCO in blood also impairs oxygen transport by causing a significant shift to the left of the oxygen dissociation curve. This phenomenon has been described as partial molecular poisoning (D2), since the underlying cause is an increase in the oxygen affinity of the remaining iron atoms when a single Fe-CO bond is present in the Hb_4 molecule. This fully explains why a relatively low HbCO concentration is accompanied by severe symptoms, 25–30% sufficing to cause unconsciousness. Through the resulting anoxia, HbCO intoxication can give widespread disorders of the nervous system (polyneuritis), even demyelinization within the central nervous system having been found.

TABLE 14
COMPARISON OF S_{O_2} AS MEASURED BY VAN SLYKE ANALYSIS AND BY SPECTROPHOTOMETRIC MEASUREMENT AT λ = 560 AND 522 NM

S_{O_2} (%)		
Van Slyke	D^{560}/D^{522}	ΔS_{O_2} (%)
61.6	59.7	−1.9
83.5	81.1	−2.4
73.3	72.4	−0.9
92.3	88.7	−3.6
72.5	74.4	+1.9
72.8	76.1	+3.3
88.8	93.3	+4.5
88.0	87.3	−0.7
99.1	98.8	−0.3
75.9	78.3	+2.4
91.4	95.6	+4.2
83.2	83.0	−0.2
94.8	94.9	+0.1
53.0	53.1	+0.1
73.5	78.6	+5.1
42.3	41.3	−1.0
59.6	58.4	−1.2
67.8	73.1	+5.3
33.9	34.9	+1.0
68.8	71.2	+2.4
89.5	91.5	+2.0
79.6	82.9	+3.0
93.7	96.0	+2.3
57.2	55.2	−2.0
73.2	73.0	−0.2
84.0	89.0	+5.0
54.8	49.1	−5.7
71.4	70.5	−0.9
67.8	64.6	−3.2
89.4	88.1	−1.3

In normal blood 0.2–0.5% HbCO is present, due to CO formation from the α-methene groups of the hemoglobin molecule during physiological decomposition (M2, S2). In smokers up to 3% HbCO has been found.

The frequent occurrence of carbon monoxide poisoning necessitates the availability of rapid and accurate methods for the determination of HbCO in human blood samples. These methods may also be of value in evaluating the results of the usual oxygen therapy in severely poisoned patients, and in the detection of cases of chronic poisoning of moderate degree. A review

of various, essentially spectrophotometric methods is given in references (V1) and (V2).

In this section, 2 two-wavelength methods making use of an isobestic point, which have proven to be quite suitable in our laboratories for many years, are described. The general requirements dealt with in Section 4 are also valid for these techniques.

6.1. Determination of S_{CO} in a Mixture Containing Hb and HbCO, Measured at λ = 538 and 578 nm; l = 1.000 cm

6.1.1. *Procedure*

Blood (0.1 ml) is mixed with 20 ml 0.1% ammonia solution, and 20 mg sodium dithionite ($Na_2S_2O_4$) is added to convert HbO_2 to Hb. The optical density is measured at λ = 538 and 578 nm (isobestic wavelength); l = 1.000 cm; slit width = 0.02 mm. The measurements should be carried out within 10 minutes of the addition of $Na_2S_2O_4$. The CO saturation is calculated from the equation

$$S_{CO}\ (\%) = [(2.44D^{538}/D^{578}) - 2.68]100 \qquad (14)$$

The constants 2.44 and 2.68 have been calculated from a series of 30 measurements of D^{538}/D^{578} for 0% HbCO (100% Hb) and 100% HbCO. The isobestic point was established at λ = 578 ± 0.5 nm (n = 100).

6.1.2. *Calibration*

Normal blood (0.1 ml) is mixed with 20 ml 0.1% ammonia solution and divided into two equal lots. To each lot 20 mg $Na_2S_2O_4$ is added to arrive at 100% Hb. Through one of the lots pure CO, formed by heating HCOOH and H_2SO_4, is allowed to bubble slowly for 2 minutes to give a 100% HbCO

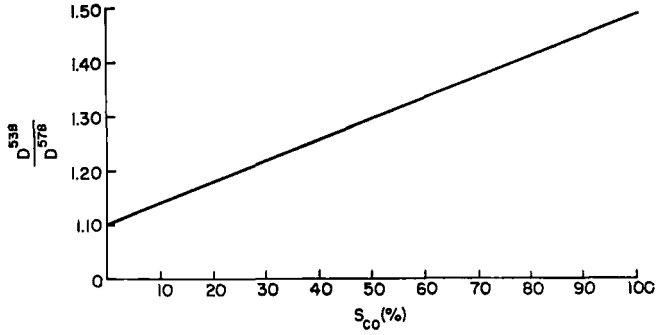

Fig. 14. Calibration line for the determination of S_{CO}, based on Eq. (14).

solution. Both samples are then measured at λ = 538 and 578 nm; l = 1.000 cm; slit width = 0.02 mm. The measurements should be carried out within 10 minutes of the addition of $Na_2S_2O_4$.

In a series of 30 determinations, $[D^{538}/D^{578}]_{Hb}$ equaled 1.10 ± 0.02 and $[D^{538}/D^{578}]_{HbCO}$ 1.50 ± 0.03. In practice it proved advantageous to make use of a calibration line. In Fig. 14, D^{538}/D^{578} has been plotted against S_{CO}. The HbCO saturation of any blood sample can be easily read from this graph.

6.1.3. *Discussion*

It has been stated (P1) that a certain amount of CO dissociates from HbCO when the sample is highly diluted. This view is based on the finding that a change in photometric reading is observed when CO is allowed to run through a diluted HbCO solution containing $Na_2S_2O_4$. As the method described (Section 6.1.1) involves a dilution factor of 200, the values of S_{CO} should therefore be too low. It has, however, been shown (V1) that a change in photometric reading also occurs when using hydrogen instead of CO, or when a dilute HbCO solution is simply allowed to stand for 30–50 minutes in the presence of $Na_2S_2O_4$. This has led to the supposition that compounds

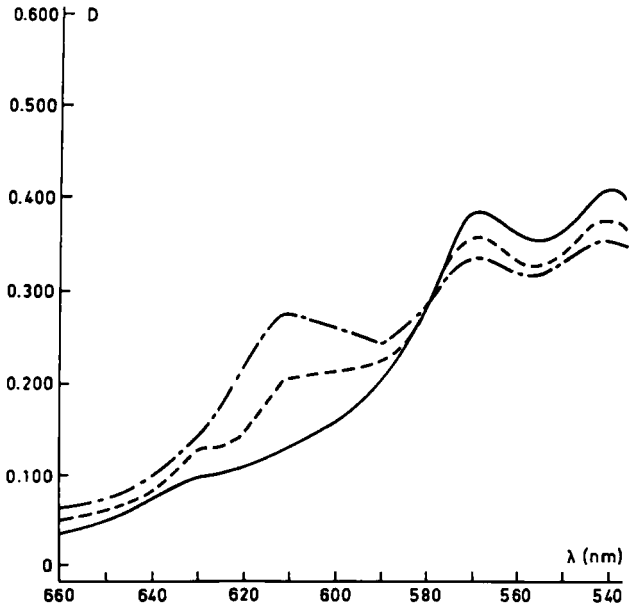

FIG. 15. Effect of running CO for 2, 6, and 10 min through a dilute HbCO solution containing $Na_2S_2O_4$. ——— 2 min CO; - - - - 6 min CO; — · — · 10 min CO.

other than HbCO are formed under these conditions. An extensive spectrophotometric investigation (V2) revealed that, after dispersing CO in a Hb solution containing $Na_2S_2O_4$, an increasing amount of SHbCO, sulfcarboxyhemoglobin, may be formed. In Fig. 15 are shown the absorption spectra of a HbCO solution after 2, 6, and 10 minutes of bubbling CO through the solution. In the absorption spectrum the appearance of a peak with a maximum at $\lambda = 614$ nm, characteristic for SHbCO (N2), can be observed.

The formation of SHbCO is a distinct disadvantage of the procedure described in Section 6.1.1. When a HbCO sample is prepared in the calibration procedure by dispersing CO in a dilute Hb solution containing $Na_2S_2O_4$, formation of small amounts of SHbCO is almost inevitable. From the results presented (Section 6.3) it may, however, be concluded that only slight errors are thus introduced.

6.2. Determination of S_{CO} in a Mixture Containing HbO_2 and HbCO, Measured at $\lambda = 562$ and 540 nm; $l = 0.01$ cm

6.2.1. *Procedure*

To convert Hb to HbO_2, a heparinized blood sample is rotated for 5 minutes in a small tonometer filled with room air. The CO saturation of the sample is not influenced measurably by this procedure. The sample is then diluted with an equal amount of 2% Sterox SE solution in a syringe containing a metal mixing ring. After thorough mixing and discarding the first three drops, the 0.10-cm cuvette is filled. A 0.09-cm plane parallel glass plate is inserted into the cuvette, leaving a blood layer of 0.01 cm (Fig. 13).

The optical density is measured at $\lambda = 562$ and 540 nm (isobestic wavelength). The CO saturation is calculated from the equation

$$S_{CO}\,(\%) = [(3.26D^{562}/D^{540}) - 1.98]100 \qquad (15)$$

The constants 3.26 and 1.98 have been calculated from a series of 10 measurements of D^{562}/D^{540} for 0% HbCO (100% HbO_2) and 100% HbCO.

6.2.2. *Discussion*

When measuring in the system HbO_2/HbCO, the use of a layer thickness of 1.000 cm is impossible without diluting the blood sample some 100 times. In such circumstances, however, a conversion of HbCO to HbO_2 would take place because of the oxygen-rich environment. The result of dilution of a sample containing HbCO and HbO_2 is clearly demonstrated in Table 15.

TABLE 15
EFFECT ON S_{CO} WHEN DILUTING A BLOOD SAMPLE CONTAINING HbCO AND HbO_2

Dilution factor	Layer thickness (cm)	S_{CO} (%)
Undiluted	0.013	34
2.5	0.013	33
10	0.100	32
100	1.000	30.5
200	1.000	29.5

It is therefore necessary to use cuvettes with very thin layers; although this may seem complicated, these cuvettes (Z3) are in fact very easy to handle.

When measuring in thin layers with a dilution factor 1, little or no conversion of HbCO to HbO_2 is encountered. To demonstrate this, three samples containing HbCO were measured as follows:

(a) system HbCO/Hb, addition of $Na_2S_2O_4$ (method 6.1.1)
(b) system HbCO/Hb, saturation with O_2 then addition of $Na_2S_2O_4$ (method 6.1.1)
(c) system HbCO/HbO_2, saturation with O_2 (method 6.2.1)

The results are presented in Table 16.

TABLE 16
EFFECT OF SATURATION WITH OXYGEN ON S_{CO} AS DETERMINED WITH THE METHODS DESCRIBED IN SECTIONS 6.1.1 AND 6.2.1

System	Method	Saturation with O_2	S_{CO} (%)
HbCO/Hb	6.1.1	−	32
HbCO/Hb	6.1.1	+	32
HbCO/HbO_2	6.2.1	+	32
HbCO/Hb	6.1.1	−	19
HbCO/Hb	6.1.1	+	21
HbCO/HbO_2	6.2.1	+	20
HbCO/Hb	6.1.1	−	18
HbCO/Hb	6.1.1	+	20
HbCO/HbO_2	6.2.1	+	18

The method given in Section 6.2.1 may be regarded as the most suitable spectrophotometric method for the determination of S_{CO}. If, however, proper measures are taken against errors caused by the formation of SHbCO, the procedures 6.1.1 and 6.2.1 are of the same order of accuracy.

In the calibration experiments of procedure 6.2.1, the average value of $[D^{562}/D^{540}]_{HbO_2}$ was 0.609 with a standard deviation of 0.002. For a group

of 16 nonsmokers, however, $[D^{562}/D^{540}]_{HbO_2}$ was found to be 0.603 with a standard deviation of 0.004. Use of Eq. (15) may therefore well give slightly negative results in a few cases.

6.3. Comparison of the Two Methods

Fresh normal heparinized blood (25 ml) was hemolyzed with 3 ml 7% Sterox SE solution; 10 ml of the hemolysate was rotated for 3 minutes in a small tonometer filled with oxygen to procure a solution containing 100% HbO_2 ($D^{562}/D^{540} = 0.609$). The Hi content of this solution was estimated to be near zero with the method described in Section 7.3. This solution was designated A. Another 10 ml of the hemolysate was rotated for 5 minutes in a small tonometer with pure CO to procure a solution containing 100% HbCO ($D^{562}/D^{540} = 0.915$). This solution was designated B.

From A and B, mixtures were made containing approximately 40, 35, 30, 25, 20, 15, 10, 5, and 2% HbCO, respectively. The HbCO concentrations were determined according to the two methods described. The results, given in Table 17, show very good agreement.

TABLE 17

S_{CO} Measured by Methods 6.1.1 and 6.2.1 in Mixtures of Solutions Containing 100% HbO_2 and 100% HbCO, Respectively

S_{CO} (%)		
Calculated	Method 6.2.1	Method 6.1.1
100	100	—
40	40	40
35	35	37
30	29	32
25	22	25
20	18	17
15	13	13
10	10	8
5	4	5
2	1	2
0	1	0.5

The reliability of the two methods was further checked by determining the S_{CO} of a series of blood samples brought into contact with varying amounts of CO (Table 18).

7. Spectrophotometric Determination of Hemiglobin

Hemiglobin (Hi) is the hemoglobin derivative in which the ferrous porphyrin complex is converted to the ferric form by oxidation. This trans-

TABLE 18
COMPARISON OF S_{CO} DETERMINED USING METHODS 6.1.1 AND 6.2.1

Sample	S_{CO} (%)	
	Method 6.1.1	Method 6.2.1
1	0	0
2	100	102
3	100	100
4	91	91
5	98	96
6	0	0
7	9	9
8	30	27
9	47	42
10	12	14

formation can take place in different ways. When encountered clinically, it is usually due to toxic agents. These act either by direct oxidation (NO_2^-, NO_3^- after reduction in the intestinal tract to NO_2^-, Fe^{+++}, etc.), or by indirect oxidation (phenacetin, sulfonamides, salicylazosulfapyridine, aniline and derivatives, nitrobenzene, hydroxylamines, etc.).

Besides intoxications, congenital and hereditary hemiglobinemias have been shown to exist. In many of these cases, hemiglobinemia is the result of a deficiency of the hemiglobin reductase system prevalent in the normal red cell. Another type of congenital hemiglobinemia is due to the presence of an abnormal hemoglobin (HbM), which has an increased sensitivity to oxidation.

The stepwise oxidation of the four heme groups to Hi normally goes on continuously *in vivo;* this process, however, is kept at an equilibrium by the enzymatic reduction of Hi to Hb. Due to this equilibrium up to 0.5% Hi can usually be demonstrated in normal blood. If, for any reason, Hi formation exceeds reduction to Hb, the Hi content of the blood increases, and cyanosis may appear. The determination of hemiglobin in blood is therefore important in clinical medicine.

7.1. PRINCIPLES

Hemiglobin can be estimated in the presence of HbO_2, using a two-wavelength method (Section 4). A suitable isobestic point is situated at $\lambda = 523$ nm, while a large difference between ϵ_{Hi} and ϵ_{HbO_2} is found at $\lambda = 558$ nm. Other possibilities are referred to by Henry (H1).

Another approach to the determination of Hi is to make use of the change in the light absorption spectrum occurring on the addition of CN^-. At

neutral pH, Hi has a maximum at $\lambda = 630$ nm which disappears on the addition of CN^-. Calculations can be made from D^{630} measured before and after addition of CN^-, as has been done by Evelyn and Malloy (E1) and Marti (M4). By subsequently converting all hemoglobin present to Hi, using $K_3Fe(CN)_6$, the percentage Hi initially present can be determined easily. Although the principle on which these methods are based is attractive, in practice they are disturbed by the occurrence of turbidity. In the modification described in Section 7.3, this difficulty has been overcome by the use of Sterox SE.

A third possibility, and an important one from a clinical point of view, is a spectrophotometric screening method. Using this method it is possible to establish within a few minutes whether or not hemiglobin is present in a 0.1-ml blood sample. At the same time it is possible to estimate S_{Hi} from the recorded spectrum (Section 7.4).

7.2. Determination of S_{Hi} Measured at $\lambda = 558$ and 523 nm; $l = 0.01$ cm

Blood (2.0 ml) is oxygenated for 5 minutes in a tonometer; 0.5 ml oxygenated blood is drawn into a 2-ml glass syringe containing 0.5 ml 2% Sterox SE solution and a metal mixing ring. After thorough mixing and discarding the first three drops, the 0.10-cm cuvette is filled. A 0.09-cm plane parallel glass plate is inserted into the cuvette, leaving a blood layer of 0.01 cm (Fig. 13). The optical density is measured at $\lambda = 558$ and 523 nm (isobestic wavelength). The Hi concentration is calculated from the equation

$$S_{Hi}\,(\%) = [1.94 - 1.56(D^{558}/D^{523})]100 \qquad (16)$$

The constants 1.94 and 1.56 have been calculated from a series of 25 measurements of D^{558}/D^{523} for 0% Hi (100% HbO_2) and 100% Hi.

7.3. Determination of S_{Hi} by Converting Hi to HiCN and Measuring at $\lambda = 630$ nm

Blood (0.2 ml) is hemolyzed in a solution containing 4 ml phosphate buffer, 0.1 *M* (pH 6.8), and 6 ml 1% Sterox SE. The Sörensen buffer contains 34.6 g KH_2PO_4 and 45.24 g $Na_2HPO_4 \cdot 2H_2O$ per 250 ml. This stock solution must be diluted 20-fold before use. The hemolysate is divided into two equal parts, designated A and B. A is measured at $\lambda = 630$ nm; slit width = 0.03 mm; $l = 1.000$ cm. This measurement yields D_1. Next one drop of a 5% KCN solution is added, and after mixing D^{630} is measured again, yielding D_2.

To B one drop of 5% $K_3Fe(CN)_6$ solution is added, and after 5 minutes

D^{630} is measured. This yields D_3. Next one drop of the 5% KCN solution is added, and after mixing D^{630} is measured again, yielding D_4.

All measurements should be made against a blank containing phosphate buffer solution and Sterox SE in the same proportion as present in the sample. The Hi concentration is calculated from the equation

$$S_{\mathrm{Hi}}\,(\%) = [(D_1 - D_2)/(D_3 - D_4)]100 \tag{17}$$

7.4. Estimation of S_{Hi} by a Screening Method

Blood (0.1 ml) is mixed with 10 ml of 2% Sterox SE solution. The absorption spectrum of this clear solution is recorded in a Beckman DB or similar spectrophotometer from $\lambda = 650$ nm down to 560 nm; $l = 1.000$ cm. The 2% Sterox SE solution is used as a blank.

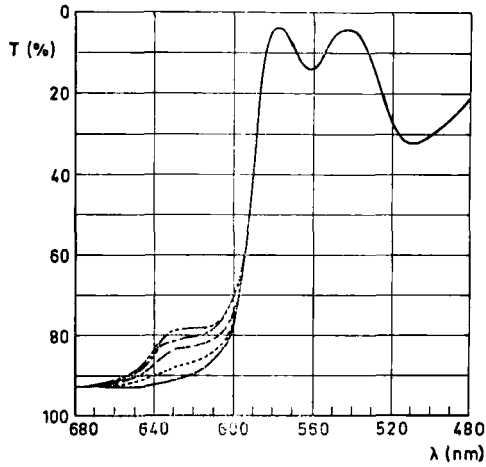

Fig. 16. $T(\%)/\lambda$ curves of blood samples containing: ——— HbO_2; - - - - - - 5% Hi (95% HbO_2); — — — 10% Hi (90% HbO_2); — - — - 15% Hi (85% HbO_2); — - - — 20% Hi (80% HbO_2); the last four down to $\lambda = 590$ nm.

In Fig. 16, the absorption spectrum from $\lambda = 690$ down to 590 nm of blood samples containing 5, 10, 15, and 20% Hi is presented. The spectrum of HbO_2 down to $\lambda = 480$ nm has been included. From this figure an estimation of S_{Hi} can be made as follows. Suppose that a sample contains $aC_{\mathrm{Hb\ tot.}}$ quarter millimoles Hi per liter and $(1 - a)C_{\mathrm{Hb\ tot.}}$ quarter millimoles HbO_2 per liter. As Lambert-Beer's law is obeyed, $\epsilon = D/cl$. We know from Sections 3.4 and 3.6 that $\epsilon^{630}_{\mathrm{HbO_2}} = 0.18$ and $\epsilon^{630}_{\mathrm{Hi}} = 3.69$. Thus

$$3.69 = D^{630}_{\mathrm{Hi}}/(aC_{\mathrm{Hb\ tot.}}l) \tag{18}$$

and

$$0.18 = D^{630}_{HbO_2}/[(1 - a)C_{Hb\ tot.}l] \tag{19}$$

Dividing Eq. (18) by Eq. (19) gives

$$(D^{630}_{Hi}/D^{630}_{HbO_2})[(1 - a)/a] \approx 20$$

or

$$a/(1 - a) \approx 0.05(D^{630}_{Hi}/D^{630}_{HbO_2})$$

And, for small values of a, as a first approximation

$$S_{Hi}\ (\%) = 100a = 5(D^{630}_{Hi}/D^{630}_{HbO_2}) \tag{20}$$

For a blood sample containing some Hi, D^{630} measured equals the sum of D^{630}_{Hi} and $D^{630}_{HbO_2}$. A fair approximation of $D^{630}_{HbO_2}$ is obtained by measuring D^{578} of the sample and dividing this value by 45. This factor has been determined experimentally from 10 HbO_2 absorption spectra recorded using this method and is valid for this method only. Thus after reading D^{630} and D^{578} from the recorded spectrum, $D^{630}_{HbO_2}$ and D^{630}_{Hi} are calculated, and S_{Hi} is determined using Eq. (20).

This screening method proved to be of great value in clinical emergencies as is demonstrated by the following case report:

A 40-year-old fruit sprayer was brought to the hospital one evening with severe cyanosis. During the day he had sprayed an orchard without a mask;

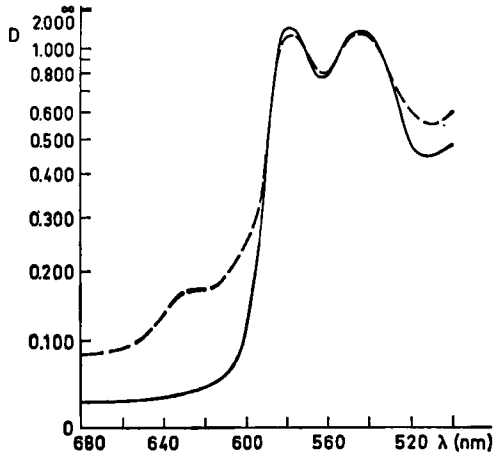

FIG. 17. D/λ curves of blood samples taken from a patient with nitrobenzene poisoning: - - - - on admittance to the hospital; ——— 12 hours after treatment.

using nitrobenzene. The absorption spectrum of a blood sample recorded on admission is shown in Fig. 17. From this spectrum the Hi concentration was estimated to be near 20%. A prompt improvement followed intravenous injection of 1 g sodium ascorbate and 1 g methylene blue. The next day an absorption spectrum of a second blood sample was recorded. Hi could no longer be demonstrated (Fig. 17).

7.5. Comparison of the Three Methods

Fresh normal human blood (50 ml) was hemolyzed with 5 ml 7% Sterox SE and oxygenated by tonometry for 20 minutes. The total hemoglobin concentration (determined by the method described in Section 2.2.2) was 9.5 quarter millimoles per liter. It can be calculated that the addition of 1.6 mg $K_3Fe(CN)_6$ to 10 ml of this hemolysate will result in a Hi concentration of 5%. In this way samples containing approximately 0, 5, 10, 15, and

TABLE 19

S_{Hi} of 5 Blood Samples as Determined by the Three Methods Described

S_{Hi} (%)			
Calculated	Method 7.3	Method 7.2	Method 7.4
0	0.1	0.5	—
5	3	4	3
10	8	9	8
15	13	14	12
20	17	18	15

20% Hi were made and subsequently analyzed (using the methods described in Sections 7.2, 7.3, and 7.4). The results are given in Table 19. The agreement between the three methods is reasonable.

In a second experiment, a solution containing 100% Hi was made by the addition of excess $K_3Fe(CN)_6$ to a hemolyzed blood sample. The excess

TABLE 20

S_{Hi} of 4 Blood Samples as Determined by the Methods Described in Sections 7.2 and 7.3

S_{Hi} (%)		
Calculated	Method 7.2	Method 7.3
10	10.5	10
14	15	14
15.5	18.5	15
18	—	20

$K_3Fe(CN)_6$ was then removed by dialysis overnight, and $C_{Hb\ tot.}$ was determined (using the method described in Section 2.2.2). Four samples were then made by mixing this solution with a solution containing 100% HbO_2 with an equivalent total hemoglobin concentration. The S_{Hi} was then determined (using the methods described in Sections 7.2 and 7.3). The results, which are in fair agreement, are shown in Table 20.

8. Spectrophotometric Determination of Sulfhemoglobin

The chemical structure of sulfhemoglobin (SHb), a green hemoglobin derivative, has not yet been completely elucidated. The central iron atom is in the ferrous state. Combination of SHb with CO to SHbCO has been encountered (V2). The structure proposed by Nijveld (N2) for the α-methene group in the porphyrin ring,

breaking the conjugated structure, has not yet been confirmed. As pure SHb solutions have not yet been made, the extinction coefficients of SHb have not been determined accurately.

The actual agent in the formation of sulfhemoglobinemia is hydrogen sulfide (H_2S) produced in the intestine by the action of bacteria on food residues and absorbed in the large intestine. The H_2S normally is excreted from the lungs or destroyed, but if present in excess, or in the presence of substances promoting its action on hemoglobin, SHb is produced (L3). Drugs known to favor the formation of SHb are phenacetin, acetanilide, sulfonamides, salicylazosulfapyridine, nitrites, and nitroglycerin. In some of these cases there seems to be a synergetic action on reductase systems in the intestine leading to the formation of $S^=$ from $SO_4^=$. In some cases sulfhemoglobinemia has been reported to be accompanied by an increased glutathione concentration in the erythrocytes (M1). Finally, the occurrence of congenital sulfhemoglobinemia has been described (M6).

SHb cannot be converted to active hemoglobin, either *in vivo* or *in vitro*. Thus the disappearance of SHb, once formed in the blood, is determined solely by the life span of the erythrocytes concerned.

The light absorption spectrum of SHb shows an absorption maximum at λ = 618–622 nm (Fig. 18), which can be used for determination purposes. The addition of CN^- does not affect this peak, while the Hi maximum at λ = 630 nm disappears completely through the formation of HiCN (Section 7.1). Use of this property serves to distinguish between these two hemoglobin derivatives.

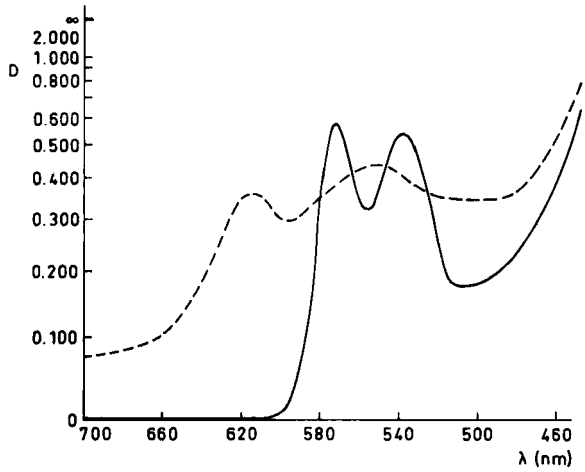

FIG. 18. D/λ curves of sulfhemoglobin (*dashed line*) and oxyhemoglobin (*solid line*). Recorded with a Beckman DB spectrophotometer; l = 0.005 cm.

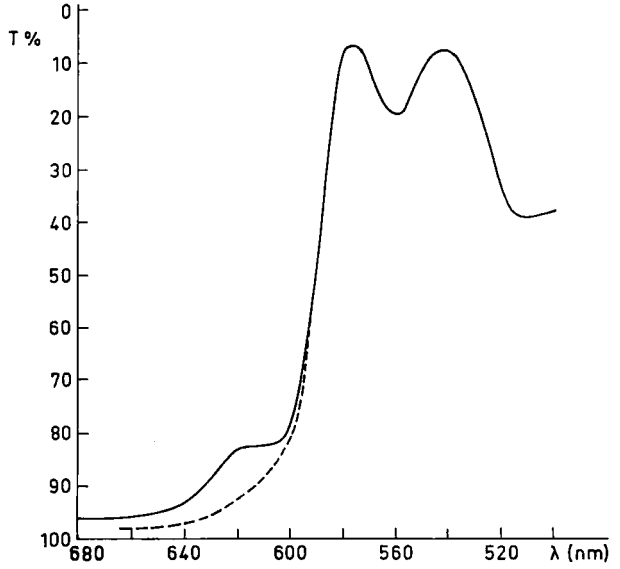

FIG. 19. $T\%/\lambda$ curve of a blood sample taken from a patient who took 10 g phenacetin in 2 days (*solid line*). The *dashed part* of the curve denotes the shape if only HbO_2 had been present in the sample.

8.1. Estimation of S_{SHb} by a Screening Method

Blood (0.1 ml) is mixed with 10 ml of 2% Sterox SE solution, and one drop of 5% KCN solution is added. The absorption spectrum of this clear solution is recorded in a Beckman DB or similar spectrophotometer from $\lambda = 650$ nm down to 560 nm; $l = 1.000$ cm. The 2% Sterox SE solution is used as a blank.

Figure 19 presents the absorption spectrum of a blood sample containing SHb. From this figure an estimation of S_{SHb} can be made as follows. Suppose that a sample contains $aC_{Hb\ tot.}$ quarter millimoles SHb per liter and $(1 - a)C_{Hb\ tot.}$ quarter millimoles HbO_2 per liter. As Lambert-Beer's law is obeyed, $\epsilon = D/cl$. We know from Section 3.4 that $\epsilon^{620}_{HbO_2} = 0.27$; for ϵ^{620}_{SHb} we take 13.0 as calculated by E. M. Jope (in L2). Thus

$$13.0 = D^{620}_{SHb}/(aC_{Hb\ tot.}l) \tag{21}$$

and

$$0.27 = D^{620}_{HbO_2}/[(1 - a)C_{Hb\ tot.}l] \tag{22}$$

Dividing Eq. (21) by Eq. (22) gives

$$(D^{620}_{SHb}/D^{620}_{HbO_2})[(1 - a)/a] \approx 50$$

or

$$a/(1 - a) \approx 0.02(D^{620}_{SHb}/D^{620}_{HbO_2})$$

and, for small values of a, as a first approximation

$$S_{SHb}\ (\%) = 100a = 2(D^{620}_{SHb}/D^{620}_{HbO_2}) \tag{23}$$

For a blood sample containing some SHb, D^{620} measured equals the sum of D^{620}_{SHb} and $D^{620}_{HbO_2}$. A fair approximation of $D^{620}_{HbO_2}$ is obtained by measuring D^{578} of the sample and dividing this value by 35. This factor has been determined experimentally from several HbO_2 absorption spectra recorded using this method and is valid for this method only. Thus after reading D^{620} and D^{578} from the recorded spectrum, $D^{620}_{HbO_2}$ and D^{620}_{SHb} are calculated, and S_{SHb} is determined using Eq. (23).

The absorption spectrum shown in Fig. 19 was recorded from a blood sample of a patient who had taken 10 g phenacetin in 2 days. Using the method described above, S_{SHb} was calculated to be approximately 3%. In this case the sample did not contain Hi.

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BLOOD-COAGULATION FACTOR VIII: GENETICS, PHYSIOLOGICAL CONTROL, AND BIOASSAY

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1. Introduction

The numeral VIII has been agreed upon internationally to designate the clotting factor which is missing from the blood of hemophiliacs. Other names include antihemophilic globulin (AHG), in common British usage, and antihemophilic factor (AHF), more commonly used in the United States; it is also known as antihemophilic factor A, because the bleeding disorder now properly called "hemophilia" is sometimes distinguished from allied conditions as hemophilia A. Factor VIII is believed to be an α_2-globulin (B1) and to have a high molecular weight (W1). In the sequence of reactions taking place when blood clots, the "resting" plasma form of factor VIII is thought to be acted upon by "activated" factor IX (M1) and, when

thus "activated," reacts with factor X. Phospholipid probably also participates in the "activation" of factor VIII. The meaning of "activation" in terms of structural molecular changes has been discussed by Vroman (V1).

The rate of growth in our knowledge of blood coagulation is becoming increasingly rapid, and it is a sign of the times that the section of this volume devoted to clotting can be wholly occupied by a review of three virtually distinct fields of work related to a single clotting factor. Indeed, the aspect of factor VIII perhaps receiving most attention at the time of writing is its role in the coagulation sequence; but, during the present intensive phase of this work, a review would become so quickly out of date that it has seemed better to concentrate on fields where research is momentarily less active.

While factor VIII has been chosen by the present reviewer because it is his main interest, other writers, even if less prolix, could have filled as many pages with a corresponding discussion of almost any other clotting factor. Factor VIII provides, however, an appropriate subject for the "Clinical Chemistry" series because the work to be described springs largely from clinical observations on hemophiliacs; and although the techniques employed are seldom those of classical chemistry, they have provided much basic knowledge of the biological activities of the factor VIII molecule.

Only in recent times have investigators of blood coagulation felt able to refer to "concentrations" of plasma coagulation factors. They have long retained a deep-seated suspicion that many of the factors described would eventually prove not to be distinct chemical substances but only "activities," or even more ephemeral experimental phenomena (as indeed happened with factor VI, now omitted from the international list). It seems fair now to accept factor VIII as a real entity, however, even though its recognition still depends entirely on biological activity and is at times uncertain (see Section 3.4, below). Although something of the old *caveat* is therefore still justifiable, the term "concentration" will be freely used for factor VIII in this review.

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2. Genetics

2.1. Synopsis

Classical studies of the recessive, sex-linked disorder hemophilia provided evidence that a gene concerned with the synthesis of factor VIII must be situated on the X-chromosome. When it was discovered that a reduction in factor VIII was commonly present also in von Willebrand's disease, with a somatic dominant inheritance, it became clear that another gene, on an autosome, must also be involved. Cross-transfusion experiments between patients suffering from hemophilia and von Willebrand's disease showed that hemophilic blood would stimulate factor VIII synthesis in von Willebrand's disease, but not vice versa. These data have made it possible to construct a number of alternative genetic models now being submitted to critical experimentation.

Some progress has also been made in mapping the position of the factor VIII locus on the X-chromosome.

2.2. The Growth of Data

2.2.1. *Hemophilia and von Willebrand's Disease*

Otto (O1) of Philadelphia, credited with the first clear account of hemophilia (B5), described a lifelong bleeding disorder affecting males and transmitted by unaffected females. The classical studies of Bulloch and Fildes (B7) definitively associated the name "hemophilia" with this pattern of inheritance, called sex-linked recessive, and were later confirmed in hemophilic dogs by obtaining the expected progenies from the various possible crosses of affected and unaffected males with normal and carrier females (B6, F1, H2). Thirteen years ago Aggeler *et al.* (A3) and Biggs *et al.* (B3) distinguished a closely allied disorder, plasma thromboplastic component (PTC) deficiency or Christmas disease, having a similar mode of inheritance but due to deficiency of a different clotting factor (factor IX). It was thus apparent that at least two genes on the normal X-chromosome must be concerned in the synthesis of clotting factors, one being factor VIII, but little development of genetic theory could be undertaken with only these facts available.

Another lifelong, hereditary bleeding disorder had been described by von Willebrand (V1, V2) among the inhabitants of the Aaland Islands, in the mouth of the Gulf of Bothnia. This condition affects both sexes but is

transmitted only by affected persons; the bleeding symptoms also differ somewhat from those of hemophilia. The disorder is further distinguished by a prolonged bleeding time but a normal or near-normal whole-blood clotting time, whereas characteristically in hemophilia the bleeding time is normal, but the clotting time greatly prolonged, at least in the severe, so-called classical hemophilia, as Bulloch and Fildes (B7) had realized. It was therefore a quite unexpected discovery (A4, J2, L1, N2) that the plasma concentration of factor VIII might be distinctly reduced in cases of von Willebrand's disease, although not usually to the very low levels encountered in severe hemophilia. Thus it became clear that one of the autosomes (the somatic chromosomes) must also carry a gene concerned in factor VIII synthesis, because of the different mode of inheritance of this disease.

A small series of cases implying yet a third gene controlling factor VIII synthesis has been described in recent years. In these cases factors V and VIII are reduced together and the defect seems to be transmitted as an autosomal recessive disorder (J1). It also appears from studies of normal populations that the factor VIII concentration of an individual is correlated with the concentrations of both his parents; this has been taken to imply the effects of (several?) autosomal genes (P1). Furthermore, persons with blood group A tend to have higher concentrations than group O subjects (P2). These findings, although suggesting that more than one autosomal gene is involved, are not yet sufficiently clear to be usefully included in genetic models, and will not be further discussed here.

2.2.2. *The Bleeding-Time Factor*

The long bleeding time in von Willebrand's disease was mentioned above. This was at first thought to be due to an abnormality of the platelets (V3), but was not confirmed, and so a capillary abnormality was postulated and became generally accepted, some workers feeling confident that morphological abnormalities of the small vessels could be observed (B4). It was thus surprising to find (N3) that an infusion of normal plasma would temporarily shorten the bleeding time in von Willebrand's disease, a phenomenon now amply confirmed. Although the prolonged bleeding time is distinct from the deficiency of factor VIII, it is mentioned here because it has been natural to attempt to account for both features of the disorder when constructing genetic models, and will be referred to below. In fact, the principle in normal (or hemophilic) plasma having the ability to shorten the bleeding time in von Willebrand's disease is known as the "von Willebrand factor" or "bleeding-time factor" (B1), and is now known to be a necessary component in the mechanism whereby adenosine diphosphate

causes the clumping of platelets (H1, S1), and presumably their adhesion to the edges of a rent in a damaged blood vessel.

2.2.3. *Stimulated Synthesis of Factor VIII in von Willebrand's Disease*

To return to the concentration of factor VIII in von Willebrand's disease: at about the time when the corrective value of normal plasma on the bleeding time was discovered, it was also found that an infusion of a fraction of normal plasma led to a prolonged rise in the factor VIII concentration in the patient's blood (N2). In contrast, the effect of infusing normal plasma in classical hemophilia was already familiar; it was known that after a single infusion the rise in factor VIII in the patient's plasma bore a reasonably predictable relationship to the patient's plasma volume and to the factor VIII content of the infused material, and that the concentration then immediately began to fall at more than 60% per day. The effect in von Willebrand's disease was strikingly different; the initial rise continued far beyond that anticipated from the factor VIII content of the plasma infused, and the high concentration was maintained for about a day, eventually returning to the original low level some 36–48 hours from the time of the infusion. This seemed to mean that, following the infusion, the patient had been enabled to synthesize a quantity of factor VIII considerably in excess of the amount infused, assuming that the rate of disappearance of factor VIII was the same in von Willebrand's disease as in hemophilia. It was even more striking to find that a similar response could be obtained by infusing hemophilic plasma (C1, C2, N1), without detectable factor VIII activity, for which no explanation other than "new synthesis" seemed possible. In contrast, infusing von Willebrand plasma into hemophiliacs produced no more rise in the patient's factor VIII than would be expected from the factor VIII content of the infusate (B1, C1). The most economical hypothesis at first was to suppose that the bleeding-time factor and the component stimulating factor VIII synthesis were the same substance, but it was shown at more than one center that a certain factor VIII concentrate made in Great Britain could stimulate synthesis in von Willebrand's disease, but did not affect the bleeding time. Also, Egeberg (E1) showed that serum prepared from an exercised donor could shorten the bleeding time in a case of von Willebrand's disease without raising the factor VIII concentration [although a factor VIII response following serum infusions has been reported by Biggs and Matthews (B2)]. Therefore it now seems necessary to consider the possibility of two separate components. A similar "new synthesis" of factor VIII has been observed in von Willebrand's disease in pigs following the infusion of plasma and serum from normal pigs (M4).

2.2.4. *Summary of Findings*

The main facts now available on which to build a genetic hypothesis are as follows: first, that factor VIII synthesis requires the activity of at least two distinct genes, one on the X-chromosome and the other on an autosome; and that mutation of the X-chromosome gene might lead to complete failure to synthesize any biologically active product (the situation in classical hemophilia, with zero factor VIII activity in the plasma), but that mutation of *one* autosome gene usually leads to only partial reduction (note the implication that cases of von Willebrand's disease are assumed to be heterozygotes, because of the apparent rarity of the disorder in most populations); second, that cross-transfusion experiments show a component to be present in normal and in hemophilic blood which can lead to rapid synthesis of factor VIII in von Willebrand's disease, but that von Willebrand blood has no such effect in hemophilia. This implies that factor VIII is reduced in the plasma in von Willebrand's disease because there is failure adequately to synthesize some component being made normally in hemophilia.

2.3. Genetic Models

In the foregoing discussion of hemophilia, the emphasis has been on the "classical" disorder, in which no factor VIII can be detected in the patient's plasma. There are, however, many cases of hemophilia in which the symptoms are relatively mild (the patients being free of the well-known spontaneous bleeding and suffering only from abnormally prolonged post-traumatic bleeding) and in which the plasma factor VIII concentration is less seriously reduced. It seems reasonable to postulate that many different mutations may occur in the sex-linked (X-chromosome) gene, and that in the milder cases the gene product retains some biological activity, whereas in the classical cases the product either cannot be formed at all or is functionless in the clotting system. Therefore, no special difficulty exists in dealing with variations in the severity of hemophilia, where the hemophiliac, being a male, has only one X-chromosome. It is thus convenient to construct models in terms of classical hemophilia with total lack of factor VIII activity in the plasma, and then to apply them to mild cases with the appropriate modification. With this understanding, the remainder of the discussion will be in terms of the severe disorder.

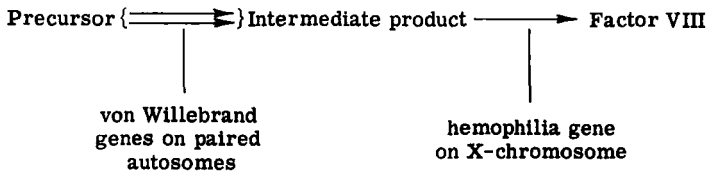
The position of the heterozygous (carrier) female can now be considered. She has the same complement of one normal X-chromosome as the normal male, which appears to account for her normal phenotype. A complication arises if the Lyon hypothesis (L3) is considered to apply: this states that only half the female complement of X-chromosomes are functional, so that

the female carrier would operate only half the number of normal X-chromosomes active in the normal male. Compensatory mechanisms apart, this leads to the expectation of factor VIII concentrations in heterozygous women (carriers of the severe disorder) of around half those in normal women, which a number of authors have in fact reported as an average finding (M3, P3) although many individual heterozygotes have a normal concentration. (A woman must be heterozygous for hemophilia if she is the daughter of a hemophiliac and her mother is homozygous normal; also, she may be presumed to be heterozygous if she is the mother of more than one hemophilic son, or has a hemophilic grandson by more than one daughter, or one of each, or has one hemophilic son or grandson and also a hemophilic brother or maternal uncle or a hemophilic cousin who is the son of a maternal aunt.) Even if heterozygous women normally have factor VIII concentrations of about half that of normal females, their lack of symptoms is not difficult to accept, because the normal range in the population lies between about half and twice the mean factor VIII concentration (P2), and hemostasis is virtually normal even if the plasma concentration is as low as 20–30% of the mean. Carriers of the milder forms of the disorder would be correspondingly even less likely to have symptoms, although some such carriers have been found with considerable expression of the abnormality (G3).

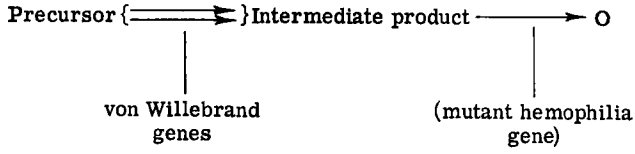
2.3.1. *A Simple Model*

On the lines just discussed, it is possible to construct a simple model of the genetics of factor VIII synthesis in von Willebrand's disease and hemophilia. This model will be discarded because it will not explain all the facts, but it may be easier to accept the greater complexities of models proposed by J. B. Graham and outlined below when the defects of a simpler model have been grasped.

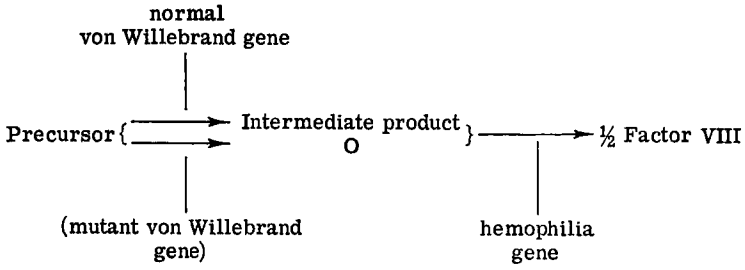
The simplest explanation of the results of cross-transfusion experiments seems to be to suppose that factor VIII is synthesized in two stages: that the "von-Willebrand" genes normally create an intermediate product which the "hemophilia" gene then converts to the final product, factor VIII. This may be written



Thus the situation in hemophilia could be written



and that in von Willebrand's disease



The model would explain the results of cross-transfusion experiments by supposing that hemophilic blood contains a normal complement of intermediate product, which could be converted to factor VIII after infusion into the von Willebrand's patient. This model, however, has two defects. First, it assumes a serial process of factor VIII synthesis, which is not how proteins are currently thought to be formed (D2). Second, it predicts a factor VIII concentration of at least 50% in von Willebrand's disease. The illustration shows a mutant yielding no active intermediate; with a mutant synthesizing a product with some activity, more than 50% of factor VIII would be predicted. In fact, the factor VIII concentration may be much lower than 50% in von Willebrand's disease, and to explain this on the suggested model it would be necessary to postulate, for instance, that the inactive product of the mutant could variably block the handling of its normal counterpart by the product of the hemophilia gene, and this might presuppose the hemophilia gene product to have a greater affinity for the abnormal than the normal intermediate, or that more abnormal than normal intermediate was formed. Furthermore, the model does not explain how a mutation leading to a diminished output of final product can be dominantly inherited, as is von Willebrand's disease.

2.3.2. *The "Regulatory" Model*

To avoid these difficulties, J. B. Graham (G2, G4) proposed the model illustrated in Fig. 1. Analogies can be found in protein synthesis in other

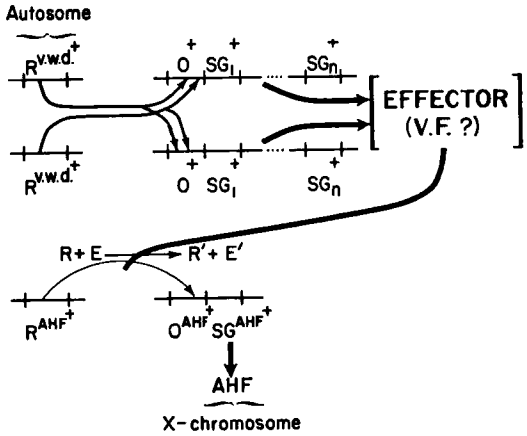


FIG. 1. Graham's (G4) "regulatory" hypothesis for normal synthesis of factor VIII: + indicates the normal or "wild-type" gene constitution. $R^{v.w.d.+}$ and O^+ SG_1^+ . . . SG_n^+ are the autosomal regulators and operons (operator, with structural genes 1 through n) concerned in von Willebrand's disease. R^{AHF+} and O^{AHF+} SG^{AHF+} are the regulator and operon concerned in hemophilia; while the A.H.F. operon must be on the X-chromosome, the regulator could be on any chromosome, and could thus be single if on the X-chromosome, or paired if autosomal.

Regulators are conceived of as acting by repression, so that full operation of the R genes would result in total suppression of the output of the corresponding structural genes, and a deficit of repressor would lead to more than the normal output of final product.

The crux of this model is the concept that the product of the autosomal genes (von Willebrand operons), marked "effector," blocks the activity of R^{AHF+} (indicated by the heavy black line and by the expression $R + E \rightarrow R' + E'$), and thus permits activity in O^{AHF+} SG^{AHF+}, and thus synthesis of factor VIII (AHF).

(V.F.?) is intended to suggest that the effector might also be the von Willebrand factor or bleeding-time factor; this feature of the model was proposed before it became apparent that factor VIII synthesis and the shortening of the bleeding time are mediated through distinct components.

Note that the repressor product of each $R^{v.w.d.+}$ acts indifferently on each target operator gene, and that the effector substance produced by each structural gene series is indistinguishable in its ability to block the repressor activity of R^{AHF+}.

organisms although none is yet known in man (E2). The particular virtue of this model is that it allows for any degree of factor VIII deficiency in either hemophilia or von Willebrand's disease. Hemophilia can be represented as before by the reasonable hypothesis of a family of possible mutations in the hemophilia operon on the X-chromosome, some yielding a product with reduced activity, others either no product or a product which is biologically quite inactive. The state of affairs in von Willebrand's disease is illustrated in Fig. 2, where the von Willebrand mutation is located

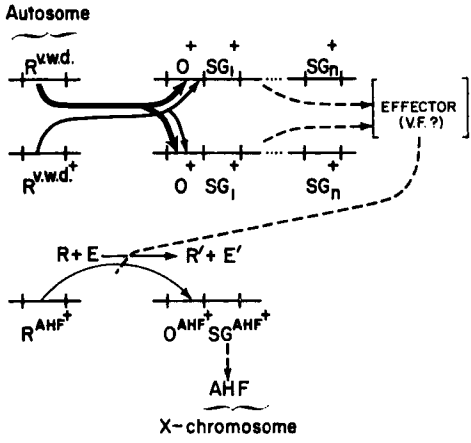


FIG. 2. Graham's (G4) "regulatory" hypothesis for heterozygous von Willebrand's disease. The mutant gene is shown as $R^{v.w.d.}$ and the corresponding normal gene $R^{v.w.d.+}$ as before. The mutation of von Willebrand's disease is thus allocated to the regulator. It is then necessary to postulate that the abnormal product is a "superrepressor" (indicated by the heavy black line), that is, that it has a greater repressor effect upon its target operon than the normal product. This means that the effector product of the operon is less than normal (indicated by the word "effector" being written more lightly than in Fig. 1), so that it in turn has less than the normal inhibitory effect on the hemophilia-gene repressor (indicated by the long dotted line replacing the heavy black line of Fig. 1). Thus the X-chromosome operon is excessively repressed and less factor VIII than normal is produced, although what is produced is of normal structure.

in the regulator rather than the structural gene group. The detailed consequences of this proposal are explained in the legend to the figure. Any degree of factor VIII deficiency can be produced by imagining a family of mutations in the von Willebrand regulator, producing a series of superrepressors of varying degrees of activity, causing interference of varying degrees in the output of effector. Furthermore, this model explains how a gene product (i.e., the effector) less active than the normal can be inherited as a "dominant" characteristic, if its normal function is to block a repressor.

According to this model, the infusion of hemophilic plasma into a patient with von Willebrand's disease would increase the plasma concentration of effector, which in turn would block the action of the hemophilia-gene repressor toward a normal level of activity, and thus increase the synthesis of factor VIII by the X-chromosome operon. A crucial implication is that the transfusion effect should operate in the same way whether the von Willebrand patient is heterozygous or homozygous, for in either case his (or her) normal X-chromosome operon should synthesize normal factor

VIII when the hemophilia-gene repressor is blocked by the infused effector. Another phenomenon explicable by this model is discussed below (Section 3.5.2).

2.3.3. *The "Combining Subunit" Model*

A critical test of the "regulatory" hypothesis would therefore be to transfuse a patient homozygous for von Willebrand's disease with hemophilic plasma and see whether or not his factor VIII level rose. If it failed to rise in the same manner as in heterozygous von Willebrand's disease, the model would be seriously called in question. The problem is to identify the homozygotes, who might be expected among the affected families in the Aaland Islands but might otherwise be extremely rare.

Graham (B8), working in North Carolina, however, has located one kindred in which two severely affected members may be homozygous for von Willebrand's disease. They are double third cousins, one male and one female, each the offspring of a second-cousin marriage, such that four of their eight grandparents are themselves grandchildren of one marriage. In one case, that of the girl, it is likely that both parents are mildly affected. The hematological findings in the parents and daughter are shown in Table 1. The striking features are the expected "new synthesis" of factor VIII

TABLE 1
GRAHAM'S "M" KINDRED[a,b]

Subject	Bleeding time (Ivy's method) (min)	Plasma factor VIII concentration (% of average normal)	
		Before infusion	After infusion
Daughter	>15	<5	13
Mother	11	61	240
Father	8	63	120
Normal range	>6	50–200	—

[a] From Barrow *et al.* (B8).

[b] Bleeding time, and plasma factor VIII concentrations, before and after infusion of hemophilic plasma, in subjects V/1, IV/1, and IV/2 in the "M" kindred.

in the parents on infusion of hemophilic plasma, and the very low response in the daughter. A similar effect was observed when the experiment was repeated on the severely affected boy, although it is less clear on the laboratory evidence that his parents are both mildly affected, so his status as a homozygote is more doubtful. This evidence, however, has seriously questioned the validity of the "regulatory" model. Graham's "combining

	Normal male		Hemophilia (male)		von Willebrand's Disease			
					Heterozygous male		Homozygous male	
Loci:	Autosomal	X	Autosomal	X	Autosomal	X	Autosomal	X
Genes:	+ / +	+	+ / +	h	+ / v	+	v / v	+
	↓ ↓	↓	↓ ↓	↓	↓ ↓	↓	↓ ↓	↓
Product:	A^+ A^+	B^+	A^+ A^+	B^h	A^+ A^v	B^+	A^v A^v	B^+
Combination of A- and B-chains:	$A^+ + B^+$ = 100% factor VIII activity		$A^+ + B^h$ = 0% factor VIII activity		$\frac{1}{2}A^+ + \frac{1}{2}B^+$ / $\frac{1}{2}A^v + \frac{1}{2}B^+$ = 50% factor VIII activity		$A^v + B^+$ = 0% factor VIII activity	
Free A-chains:	A^+ in excess		A^+ in excess		$\frac{1}{2}A^+$ / $\frac{1}{2}A^v$ in excess		A^v in excess	

FIG. 3. Graham's (G4) "Combining Subunit" hypothesis for synthesis of factor VIII in the normal subject, in hemophilia, and in von Willebrand's disease: $^+$ indicates the normal or "wild-type" gene constitution; ^{v} and ^{h} indicate the von Willebrand and hemophilic mutants, respectively. (For comparison with hemophilia, only male subjects are represented.)

It is supposed that each autosomal locus (the von Willebrand genes) and the X-chromosome locus (the hemophilia gene) create products at the same rate, molecule for molecule, and that these are polypeptide chains (designated A and B, respectively), which subsequently combine (in the cytoplasm of the synthesizing cells?) to form factor VIII. Since two A-chains are formed for each B-chain, an excess of free A-chains remains. In hemophilia all the B-chains are abnormal (B^h), and thus the completed molecules ($A^+ + B^h$) are all abnormal, despite the normal A^+ component; the excess consists entirely of normal A-chains (A^+). In von Willebrand's disease some (heterozygote) or all (homozygote) of the completed molecules are abnormal because they contain abnormal A-chains (A^v). The excess will contain free A^v-chains.

Note that by this model, abnormal factor VIII is predicted in von Willebrand's disease ($A^v + B^+$) as well as in hemophilia ($A^+ + B^h$).

subunit" model therefore makes an attractive alternative (Fig. 3). By this hypothesis, the infusion of hemophilic plasma into a case of von Willebrand's disease introduces a quantity of normal A-chains, which then enter cells (because the same effect cannot be obtained by mixing plasmas *in vitro*) and there combine with the normal B-chains to form normal factor VIII. It must probably be supposed that the B-chains have a greater affinity for the normal introduced chains than for the endogenous abnormal chains, to account for the very considerable synthesis of factor VIII often observed after infusions. This creates difficulty in explaining the great variation in factor VIII concentrations observed in von Willebrand's disease, particularly the low levels, because this model, like the first considered (Section 2.3.1), predicts 50%. An increased affinity of B-chains for abnormal A-chains (McLester and Graham, M1a) cannot be invoked, for the opposite relationship has just been posited. One possible explanation is that more subjects are homozygous for von Willebrand's disease than is supposed, and that the spectrum of factor VIII concentrations encountered is due to the existence of a family of possible mutations in the autosomal loci which may thus give rise to A-chains with various degrees of partial activity. Barrow and Graham (B1) have pointed out that von Willebrand's disease shows great variability of expression of the mutant gene, and have published a kindred of some 60 members illustrating this. Therefore it is possible that family studies have failed to detect the inheritance of the condition from both parents in a number of homozygotes.

2.4. Antibody Studies

It has been mentioned that zero factor VIII activity in the plasma might be due either to no product or to a biologically inert molecule being produced. These alternatives are of course often met in studying the products of mutant genes, and it may be possible to choose between them by applying an immunological test. This depends on the concept that an abnormal product, devoid of its ordinary biological functions, may yet be sufficiently normal in structure to react ("cross-react") with an antibody to the normal product. If these maneuvers are followed and neutralization of the antibody can be demonstrated, this is taken to indicate the presence of "cross-reacting material" (CRM), and the extract tested is said to be CRM-positive. If neutralization of the antibody does not occur, the extract is said to be CRM-negative, and it is concluded that the mutant gene forms a product so abnormal as to lack even the antigenic sites, or else no product whatever.

To apply this test satisfactorily a sufficiently specific antiserum is necessary. In dealing with factor VIII it is difficult to obtain a pure sample of

antigen with which to prepare the antiserum, for this factor is identifiable only by its biological activity. While the factor can be concentrated several thousandfold, there is always the possibility that inert but antigenic material may be still present, which would confuse the results of the experiment.

However, factor VIII-deficient patients have been encountered from time to time whose blood has possessed the ability to destroy added factor VIII from normal plasma. Although fortunately not very common (these patients present a serious clinical problem because the hemostatic mechanism fails to respond to infusions of material containing factor VIII), the condition is now well recognized, and extensive tests in a number of such cases have shown that the inactivating material reacts with no other known clotting factor. Therefore it seems reasonable to regard the abnormal constituent as a specific anti-VIII antibody, despite a frequent failure to demonstrate convincing precipitin lines in agar diffusion.

Two such cases, St. and De., were studied by Goudemand *et al.* (G1), and in these instances rather indefinite precipitin lines were obtained on Ouchterlony plates. The authors then attempted to use the serum of patient De. for CRM testing of plasma and serum from other patients. The procedure was immensely complicated, for the only specific test of antibody neutralization was the loss of ability to neutralize normal factor VIII, and this involved the following steps: first, incubating De.'s serum with the material to be tested; second, adding a fixed quantity of normal plasma (as a source of normal factor VIII) and continuing the incubation; and, finally, adding this mixture to a blood-coagulation test to assay the residual factor VIII remaining in the normal plasma added at the second step. It was necessary not only to adjust the quantities at each step so as to achieve a final factor VIII concentration appropriate to the dose-response curve of the assay system, but also to include sufficient blank runs adequately to control the effect on the assay system of the numerous other components present in the mixture of test material and De.'s serum. While paying tribute to the ingenuity and patience of the authors, a certain reserve is inevitable in considering their findings. Some relatively clear-cut results are shown in Table 2. The data are interpreted as showing complete neutralization of the anti-VIII antibody by normal serum, partial neutralization by hemophilic plasma, and none by von Willebrand plasma. From this the authors deduce that both normal serum and hemophilic plasma contain factor VIII in an inactive form, but that no such material is present in von Willebrand's disease.

The work of Goudemand *et al.* (G1) received some support from the studies of Adelson *et al.* (A2), who prepared in rabbits an anti-factor VIII

TABLE 2
NEUTRALIZATION TESTS OF ANTI-FACTOR-VIII BY HEMOPHILIC AND VON WILLEBRAND PLASMAS[a,b]

Test material	Mean residual anti-factor-VIII (units)		
	Buffer control (full residual activity)	Normal plasma control ("maximum consumption" of antibody activity)	Test material
Normal serum (29 tests)	3.38	1.76	1.82
Hemophilic plasma (13 tests)	3.21	1.35	2.14
von Willebrand plasma (14 tests)	3.45	1.70	3.33

[a] From Goudemand *et al.* (G1).

[b] The data are interpreted as showing complete neutralization of antibody by normal serum, partial neutralization by hemophilic plasma, but no neutralization by von Willebrand plasma.

antiserum and used it to follow the disappearance of labeled factor VIII infused into normal human recipients, and obtained a weighted mean half-survival time of 2.9 days in five subjects. This is considerably greater than the numerous values, all below 20 hours (A1), which have been reported by various observers for the rate of disappearance of factor VIII infused into hemophilic subjects, as measured by biological assays. Unfortunately parallel studies in hemophilic subjects by the antibody and biological assay technique have not yet been reported, so the discrepancy cannot be closely examined; but if there is a real discrepancy between the results of the two methods, it might be due to the presence in normal plasma of material antigenically indistinguishable from factor VIII, yet lacking its biological activity. This might be a precursor, with a longer biological survival in the plasma. At least both these lines of work (A2, G1) are compatible with the hypothesis of a CRM-positive but biologically inactive form of factor VIII in normal serum (G1), and perhaps in normal plasma (A2). The findings of Goudemand *et al.* (G1) further suggest that classical hemophilia is CRM-positive but that von Willebrand's disease is CRM-negative. This finding would seem to support Graham's "regulatory" hypothesis (Fig. 2) rather than his "combining subunit" model (Fig. 3), for the regulatory hypothesis suggests that in von Willebrand's disease the operon on the X-chromosome may be completely suppressed.

McLester and Wagner (M1b) have since reported that the plasma from several hemophilic dogs was CRM-negative when tested with a rabbit antiserum prepared with purified canine factor VIII. They also suggest that CRM-positive reactions of the type reported by Goudemand *et al.* (G1) might be due to the previous immunization of the hemophiliacs with isoantigens other than factor VIII, in plasmas with which they had previously been infused for the treatment of bleeding episodes (the patients St. and De. had in fact both received numerous transfusions). This appears to be an important criticism, but to explain the findings of Table 2 it would be necessary to suppose that the patients with von Willebrand's disease had lacked some isoantigen other than "antigenic VIII" present in the hemophiliacs and also in the normal subjects, to which De. had become immunized.

Barrow and Graham (B1) feel that the experimental evidence tends to favor the combining subunit model, and that the failure to identify other genetic systems in man like their regulatory model makes this latter hypothesis rather less likely. For what the immunological evidence is worth it appears to favor the regulatory model, but more data are clearly required. A model analogous to Graham's combining subunits has been proposed for hemoglobin synthesis (R1).

2.5. The Factor VIII Locus on the X-Chromosome

Information available about the human X-chromosome was reviewed by Stewart at the end of 1962 (S2).

The recognition of the X-linked blood group system Xg (M2) had stimulated a number of workers to investigate the Xg group in families transmitting abnormalities due to mutations of X-chromosome genes. A large combined study (D1) covered 35 hemophilic families in which the bloods of 245 members were tested with anti-Xg, and useful information about linkage was obtained from 10 families. On the basis of this and other work, the order of genes which can be topographically related to hemophilia, with estimates of the distances in centimorgans which separate them, has been given (K1) as Xg-27-G.6P.D[1]-5-deutan color vision-12-hemophilia. The gene for X-linked ichthyosis has been estimated as lying 23 centimorgans from Xg (K1). A tentative map of the X-chromosome has recently been published by Ledinot and Frézal (L2), who suggest that the Xg and deutan loci are in the short arm and that the loci for G.6P.D, protan color vision, and factors VIII and IX are all in the long arm of the chromosome, in that order, but the order quoted above is probably better founded (M1).

[1] G.6P.D = glucose-6-phosphate-dehydrogenase deficiency.

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3. Physiological Control

3.1. Synopsis

Although little is known of the regulatory mechanisms maintaining the plasma concentrations of the clotting factors at their normal levels in a given individual, various circumstances have been recorded in which their levels are observed to rise. For factor VIII, some involve relatively long-term changes, such as pregnancy, inflammatory states, and hyperthyroidism, while very sudden but short-lived increases follow hard muscular exercise or the injection of adrenaline. These changes are now being extensively investigated, and attempts have been made to elucidate their mechanism.

3.2. Long-Term Changes

Under this heading it is possible to do little more as yet than record the situations in which a maintained rise in plasma factor VIII concentration has been observed. It seems worthwhile to do this because these facts will

form part of the corpus of data for which future hypotheses must be able to account and against which they will be tested.

3.2.1. *Pregnancy*

Factor VIII levels up to 4 times normal have been reported at term (P5, S3, T3). Some other clotting factors also rise in concentration during pregnancy, for instance fibrinogen (I6, M3, P5), factor VII (L1, N1), and factor X (P1), but apparently not prothrombin (D1). The significance of this pattern of changes is not known.

3.2.2. *Chronic Inflammatory States*

O. Egeberg has estimated factor VIII concentrations in a number of clinical conditions. Among those that may be loosely grouped together as "chronic inflammatory," he has found an increase in factor VIII concentration in patients with atherosclerotic heart disease (E5), diabetes (E15), and miscellaneous chronic disorders (E3). In each study concomitant increases in factor VIII and fibrinogen were found, and in the second and third there was also some increase in factor V among the patients studied. An increase in the bleeding-time factor has been described in diabetes (O2), in atherosclerotic disease (O3) and in multiple myeloma (P6).

3.2.3. *Hyperthyroidism*

Egeberg (E9, E17) found that the plasma concentration of factor VIII was correlated with the basal metabolic rate (BMR) over the range of values obtained from 4 hypothyroid and 11 hyperthyroid patients, lying between 2 and 4 times the average normal concentration in the latter group. The rise in concentration closely paralleled the rise in BMR when hypothyroid patients were treated with thyroxine. It was most interesting that the bleeding time was prolonged in hypothyroid patients, and fell to normal on thyroxine treatment, in view of the association of a long bleeding time with a low factor VIII concentration in von Willebrand's disease (see Section 2.2.1, above), and the opposite association in atherosclerosis and diabetes.

3.2.4. *Oral Anticoagulants*

A moderate rise in factor VIII concentration occurs in patients treated with the oral anticoagulants, which may continue until slightly after the end of treatment (E4, P3).

3.2.5. *Dietary Changes in Animals*

Davidson *et al.* (D2, D3) fed an atherogenic or a thrombogenic diet to groups of rats for periods of a month or more, in each case with and without

added phenindione (2-phenylindane-1,3-dione). In all four groups the mean factor VIII concentration rose by comparison with the rats on a control diet: with the atherogenic diet, containing arachis oil, the concentration rose about 2-fold, and with the thrombogenic diet, containing butter fat, the increase was 3–4-fold; the presence of phenindione did not affect the factor VIII response. Similar results were obtained by Merskey and Wohl (M5) with a diet enriched with beef fat.

3.3. Short-Term Changes

3.3.1. *Effect of Adrenaline*

In 1903, Vosburgh and Richards were studying the effect of adrenaline injections on the blood sugar concentration in anesthetized animals, and noted that blood samples withdrawn from animals which had received adrenaline tended to clot in the syringe before they could be ejected into test tubes. After further investigation, they reported (V2) a definite shortening of the clotting time following adrenaline administration. Ten years later, their work stimulated W. B. Cannon to undertake further studies in anesthetized or decerebrate cats, hares, and rabbits, for which he and Mendenhall (C2) constructed a special coagulometer to measure and record the clotting time of arterial blood samples. In a series of eight papers over 3 years, Cannon and his associates reported most detailed investigations into the effects on blood clotting of adrenaline injection and of various procedures calculated to liberate adrenaline in the body (C1, C3, C4, D4, G3, G4, M4, S2). Although their work was left unfinished, they established that the intravenous injection of adrenaline at 10^{-6} g/kg, as well as hemorrhage and splanchnic stimulation, led to a shortening of the clotting time of whole blood. During the next 40 years this work was referred to from time to time and, as new clotting tests were developed, various workers attempted to elucidate the mechanism of this response to adrenaline (F3, M6, P2, R2, T1, T2, U1, W2) but with rather inconclusive results; this and other work has been summarized by Forwell (F2).

In 1958, Christie *et al.* (C5) made a series of daily assays by the Biggs method (B1) of factor VIII concentrations in the blood of a hemophiliac being treated with animal antihemophilic material during the period of wound healing following abdominoperineal resection of the rectum for adenocarcinoma. The hemophilia was of only moderate severity, and the usual factor VIII concentration was about 10% of average normal. At the operation there had been some difficulty in securing a blood vessel high in the perineal wound, and after considerable blood loss the wound had been packed. The only bleeding during the postoperative period occurred on two occasions when the packing was removed, and then sudden, serious hemor-

rhage was provoked. Christie *et al.* noted that on the day of operation and on these two other days the responses to the antihemophilic treatment were unusually high. It seemed possible that either the bleeding itself, or the blood transfusion it had necessitated, had somehow induced an increase in the patient's own factor VIII, since it became clear on the subsequent days of treatment that the relatively small variations in the amounts of antihemophilic material given could not have been responsible for the differences in the responses observed. It was thought that if hemorrhage had been the stimulus responsible, its effect might have been mediated through adrenaline release (G5) and the Cannon effect. Accordingly, another hemophiliac of comparable severity, subject Bat. of Ingram (I4), received an intravenous infusion of adrenaline over 15 minutes, after which the factor VIII concentration was found to have risen from 14% to 40%. Studies were then made on other hemophiliacs of varied severity, and on normal subjects, infusing adrenaline at the rate of $10–16 \times 10^{-6}$ g/min for 10–15 minutes. Three severely affected hemophiliacs, with no detectable factor VIII in the plasma, showed none after adrenaline; but, of five other less severely affected patients, four showed a rise as had subject Bat., and a comparable result was obtained in normal males, the mean rise in 13 subjects being to 176% of the initial level (I4). The effect appeared to last for a few hours. These results were confirmed by Egeberg (E16).

3.3.2. *Effect of Exercise*

Kesseler and Egli (K1) had shown that the clotting time of whole blood was shortened following muscular exercise. At about the time when the above studies were being made with adrenaline, but quite independently, Rizza (R1) extended this finding by noting that samples of blood taken from normal subjects following severe exercise yielded higher factor VIII concentrations on assay than samples taken from other subjects. He found that a relatively short period of brisk muscular exercise roughly doubled the plasma factor VIII concentration, and thus obtained a very similar effect to that following adrenaline infusion, both in normal subjects and in mildly affected hemophiliacs. This work was confirmed by Iatridis and Ferguson (I1) and by Egeberg (E10) using a different assay system; Egeberg observed immediate increases in factor VIII concentration of 2–3.5-fold following strenuous exercise of only 3-minute duration. The effect began to pass off immediately and was completely lost by the following day. Parallel assays or determinations were made for fibrinogen, prothrombin, and factors V, VII, IX, XI, and XII, but no comparable change was observed in any. It was particularly interesting that the rise in factor VIII was not here accompanied by a corresponding rise in fibrinogen concentra-

tion, as had been observed under other circumstances (see Section 3.2, above). Egeberg also demonstrated a rise in factor VIII concentration in patients with von Willebrand's disease, together with a shortening of the bleeding time (E14), and in one patient with thrombasthenia (E16), who were exercised in a similar manner.

3.3.3. *Other Acute States*

Egeberg studied a number of acute states and found that a rise of factor VIII was associated with the induction of nonspecific fever (E6, E10), intravenous infusion of serum (E7), intramuscular injection of blood (E8) or intravenous infusion of hemolyzed blood (E2), drug-induced diuresis in a patient with cardiac edema (E11), and surgical operations (E1). In the very acute changes, factor VIII alone rose in concentration, but where the process occupied several days there were normally associated increases in fibrinogen and in factor V. Other clotting factors remained at their previous levels. These investigations were all made in persons with normal hemostasis.

3.4. Investigations into the Nature of the Acute Rise in Factor VIII Concentration

Experimental study of the rise in factor VIII concentrations, now known to occur in so many situations, has been almost entirely confined for practical reasons to a study of the effects of exercise or adrenaline administration. These situations, it will be recalled, are those in which an isolated rise in factor VIII can be observed, unaccompanied by changes in fibrinogen or factor V. It is thus uncertain to what extent the results may be applied to the more protracted, combined increase in fibrinogen and factors V and VIII.

First, it should be remembered that the basic observation, a rise in plasma factor VIII concentration, depends entirely upon a biological assay, in which the corrective effect of the test plasma is compared to that of a control plasma when both are added to plasma obtained from a severely affected hemophiliac, or to an artificial system containing necessary clotting factors other than factor VIII. In acute experiments it has been usual to assay the subject's pretreatment plasma as well as the plasma obtained after the experiment, or to use the pretreatment plasma as the "standard" (nominally 100%) for the assay. The second procedure eliminates errors due to differences between subjects, but uncertainty still remains regarding the effects of the experimental treatment upon the assay system, apart from a possible true increase in factor VIII concentration. A number of experiments have therefore been directed to testing the validity of the

assay system under the conditions of experiment—i.e., when plasma from exercised or adrenaline-treated subjects is added to it. In other work, the mode of action of the stimulus has been studied in the tested subjects; other tests have been carried out on the plasma obtained from them. A special investigation was made of a local increase in factor VIII concentration in congested veins.

3.4.1. *Validity of the Assay System under These Experimental Conditions*

The most obvious source of biological invalidity in the assay would be a direct effect upon the system of either adrenaline itself or some component other than factor VIII, of which the blood concentration rose as a result of adrenaline treatment or exercise. These possibilities have been fairly fully investigated and it has not proved possible to explain the effects in this way. Adrenaline itself has been found to accelerate blood coagulation *in vitro* (F3, I4, W2), but only at concentrations of an order of magnitude—one dex (A3)—higher than those produced *in vivo*. Noradrenaline, glucose, fructose, insulin, histamine, and lactate were found to have no effect (F3, I4). An especially important component to investigate has been plasmin, the proteolytic enzyme known to appear in the blood after adrenaline administration or exercise (B2, K2). The plasmin response passes off much more quickly than the factor VIII response (R1, V1) and, even in the initial phase, shows no correlation in magnitude with the rise in factor VIII (I4), so it seems clear that an explanation in terms of plasmin may be discounted.

Another possibility is that some other clotting factor is increased or activated in such a way that the assay system responds fortuitously to it in a way indistinguishable from the usual response to factor VIII, e.g., factors XI and XII; factor XII is known to rise on exercise (I1). That this might occur over a limited range of the dose-response curve in the thromboplastin generation test system was shown by experiments in which the addition of "activation product" (W1) simulated an increased factor VIII concentration (author's unpublished observations, 1960; F1), although statistical invalidity would probably be detectable over a series of experiments if this were the explanation. This also was looked for, but was not found (I4). It is interesting that, in a patient with severe factor VIII deficiency and partial factor XI deficiency (S1), adrenaline infusion was followed by a marked rise in factor XI concentration and the appearance of a trace of factor VIII (K. Schulz, personal communication, 1964). Furthermore, the confusion that arose some years ago over factor IX assay now seems to have been due to activation of the contact factors (P4), hence

there is good reason to investigate the same possibility in the assay of factor VIII.

However, the exercise effect has been demonstrated with assays involving (supposedly maximal) activation of factors XI and XII with kaolin (I3), and a system to which activation product was added (V1) duly registered the adrenaline effect. Furthermore, a rise in factor VIII concentration has now been produced by exercise in patients with severe deficiencies of factors XI and XII. Egeberg (E13) tested two patients with gross deficiencies of factor XI, and Goudemand *et al.* (G2) and A. Parquet-Gernez (personal communication, 1964) tested factor XII-deficient patients.

Finally, it might be that some component not recognized or even detectable as a clotting factor, yet able to reinforce factor VIII concentration in the assay, might be released by adrenaline. If this occurred, it would be reasonable to expect it to be unconnected with hemophilia, and it might therefore be released as well in hemophiliacs as in normal subjects, although of course in severely affected patients it would not normally be detectable. To investigate this possibility, two severely affected hemophiliacs were treated with adrenaline, blood samples being taken before and afterward. The treated and untreated hemophilic plasmas were then used to make a series of dilutions of normal untreated plasma, and the dilutions in the treated hemophiliac plasma assayed against those in the untreated one, but no difference in factor VIII concentration was found (the results in one hemophiliac subject have been reported (I4); the experiment has since been repeated in another hemophiliac). Another experiment (I4), repeated twice in the same hemophiliac, was to precede the adrenaline infusion with an infusion of normal plasma; in neither instance did the adrenaline treatment further increase the factor VIII concentration in the subject's blood.

Thus none of these experiments has produced evidence that the assayed rise in factor VIII can be discounted as spurious. Nevertheless this possibility should not be forgotten, for it may simply be that the correct experiments to demonstrate this have not yet been contrived. The ultimate test of an increase in factor VIII activity is the demonstration of greater hemostatic value in hemophilic bleeding; this is unfortunately a very difficult test to apply.

3.4.2. *Other Observations on Tested Subjects*

A number of interesting observations have been carried out in tested subjects. Egeberg (E13, E16) repeated the exercise test on 6 successive days in three normal subjects, and showed that successively lower responses were obtained, with a high response again after a subsequent interval of

4 days without exercise. Similarly, six normal subjects were exercised on 5 successive days and treated with adrenaline on the sixth day; the average adrenaline response was considerably lower than would otherwise have been expected and also lower than the initial response to exercise (E16). Egeberg was able, however, to obtain a marked response to exercise in two normal subjects who were already showing a moderate response to artificially induced fever, although the total response was not more than might have been obtained by a single stimulus alone (E10). These results suggest that the mechanism, whatever it is, can be exhausted fairly readily.

Goudemand *et al.* (G1) considered the possibility that a quantity of factor VIII might be released from the spleen in response to adrenaline administration or release. Therefore they tested a series of previously splenectomized subjects; in seven of them a rise in factor VIII after exercise could not be demonstrated, but adrenaline infusion produced a mean 2-fold increase in 11.

Vaughan Jones *et al.* (V1) studied the effect of adrenaline-blocking drugs. Ahlquist (A2) had suggested that the action of adrenaline might be divided into two groups, the α-effects which include vasoconstriction and other excitatory functions, and the β-effects of vasodilation and cardiac stimulation. The α-effects may be blocked by phentolamine [2-*N*-(3-hydroxyphenyl)-*p*-toluidinomethyl-2-imidazoline] and the β-effects by pronethalol [2-isopropylamino-1-(2-naphthyl)ethanol]. Vaughan Jones *et al.* (V1) gave adrenaline infusions to a small series of subjects, with and without these blocking drugs. The factor VIII response appeared to be blocked by pronethalol but not by phentolamine, which suggests that it was a β-effect.

3.4.3. *Tests on Plasma from Treated Subjects*

Various experiments have been concerned with transfusing plasma from exercised donors into factor VIII-deficient subjects. Rizza (R1) gave plasma from exercised donors to two hemophiliacs, and reported a more prolonged survival than would be expected after an ordinary plasma infusion; Egeberg (E14) noted a similar phenomenon when transfusing a patient with moderately severe von Willebrand's disease. Egeberg also gave whole blood from an exercised donor to a similar patient, and reported a more pronounced and longer-lasting effect on the bleeding time than had previously been obtained with ordinary transfusions, or by exercising the patient. He also showed that this effect resided in the plasma and not the platelets of blood from exercised donors, and that it could also be demonstrated in serum (E16). In other, more severely affected von Willebrand patients, however, the bleeding-time response was not obtained, and Egeberg

raises the possibility that these may have been homozygotes; in one for whom the data are given, it seems that the patient did not synthesize factor VIII following the infusion (see Section 2.2.3, above). These observations suggest that plasma does not itself contain the bleeding-time factor, but stimulates at least the von Willebrand heterozygote to form it, and that this ability is heightened by exercise. It would now be possible to test this hypothesis *in vitro* with critical concentrations of adenosine diphosphate (ADP) (O1, O2). However this may be, all the foregoing observations suggest that the increased biological activities recorded as raised concentrations of factor VIII and bleeding-time factor in exercised normal subjects and von Willebrand patients, respectively, can still be recognized after plasma from a treated subject is transfused into factor VIII-deficient subjects. "Passage" through another subject in this way might be expected to remove extraneous material, so that what is "recovered" as factor VIII activity after passage is more credibly acceptable as such. The same argument may be applied rather more forcefully to the bleeding-time factor, for here the test (the bleeding time) is a genuine measure of hemostatic competence.

Other evidence, however, makes this conclusion more doubtful. Egeberg (E10, E16) dialyzed plasma samples from exercised persons, and showed that the high factor VIII concentration survived this treatment without loss. A quantity of plasma taken after exercise was then divided into two portions, one of which was dialyzed and the other maintained under similar conditions undialyzed. The factor VIII concentrations in the subsamples remained closely similar, but, on transfusing them into a patient with von Willebrand's disease, the dialyzed sample (given first) produced no change in the patient's factor VIII concentration or bleeding time, whereas the undialyzed sample, given 2 hours later, was rapidly followed by a rise in factor VIII and a shortening of bleeding time.

This important experiment clearly requires confirmation, because it casts serious doubt on the assumption that biological assay dependably identifies "true" factor VIII activity in these experimental conditions. Independent work calling the same assumption in question has been reported by Goudemand *et al.* (G2). It will be recalled that these authors employed a naturally occurring anti-VIII antibody to detect material in plasma and serum having the antigenic characteristics of factor VIII even if lacking its biological activity (Section 2.4, above). This group (G1) have since applied their test to the plasma of 11 normal subjects before and after exercise. While ordinary bioassays yielded a mean increase of 237% in factor VIII concentrations, neutralization of the antibody increased by only 117%, a figure the authors do not consider to represent a significant

rise. The extreme complexity of these experiments must be remembered, but on their face value the results suggest that the rise in biological activity did not indicate an absolute increase in factor VIII concentration.

3.4.4. *Effect of Venous Stasis*

Egeberg (E12) applied a blood-pressure cuff inflated to 90 mm Hg for 10 minutes to one upper arm in normal subjects, taking blood samples from antecubital veins in both arms. Observations on the hematocrit and plasma protein concentrations suggested that a definite hemoconcentration had been produced, but with loss of some plasma protein presumably by diffusion from the vascular bed. Parallel assays of a number of clotting factors were undertaken in the paired samples. There was a general increase in concentration comparable to the effect on total plasma protein, but a much larger increase was observed uniquely (at least when silicone-coated glassware was used) in factor VIII. Egeberg suggested that his findings were due to tissue material entering the blood from the congested arm. Goudemand *et al.* (G1) have also put forward this idea to explain the adrenaline response, believing that adrenaline causes tissue material to enter the circulation (S4) and that this then activates a factor VIII precursor.

3.5. Possible Mechanisms for the Rise in Assayable Factor VIII

Readers who have followed the writer thus far will realize that this discussion now suffers from almost too much evidence. It is therefore important to try to set down the principal findings in order to summarize the present position and suggest the most useful lines for further observation. The facts of the long-term increase in factor VIII concentration are the less confused and, as before, will be taken first.

3.5.1. *Long-Term Changes*

In pregnancy, diabetes, and "chronic inflammatory" states there is a sustained rise in factor VIII concentration, accompanied by increases in other clotting factors, of which fibrinogen is the most constant and probably the most closely correlated quantitatively. In diabetes and atherosclerotic disease there is also an increase in the bleeding-time factor. Similar effects on the clotting factors can be produced by applying "stresses" which operate over a few days, such as the intramuscular injection of the subject's own blood. The rate of increase in factor VIII concentration indicated by serial assays is not more rapid than may be seen in patients with von Willebrand's disease after infusion of hemophilic plasma, and may therefore be reasonably ascribed to an increased rate of synthesis, since there is

no reason for the rate of removal from the plasma ("utilization") to decrease. The determination of fibrinogen is relatively straightforward, and there is no reason to doubt that the plasma concentration of this protein rises about as rapidly as is postulated for factor VIII, or to question that this implies an increased rate of synthesis of fibrinogen.

3.5.2. *Short-Term Changes*

There are two special characteristics of the experimental short-term rise in factor VIII concentration, its extreme rapidity (e.g., following only 3 minutes of severe exercise) (E16), and that it is unaccompanied by an increase in any other hemostatic component except the bleeding-time factor. Hypotheses must therefore account for an apparent increase in plasma concentration up to about 6-fold in a matter of minutes in this clotting factor alone. Could increased rate of synthesis account for an effect of this magnitude? McLester and Graham (M2), discussing their "regulatory" model for the genetic control of factor VIII synthesis (Section 2.3.2, above), suggested that a substance of small molecular weight like adrenaline might act as an inducer, which should normally inactivate the repressor of the autosomal operon (although the abnormal repressor formed in von Willebrand's disease would not be inhibited); but even with this possible mechanism in view, the synthetic effort required to meet the experimental findings seems truly phenomenal and would require the support of strong direct evidence before serious acceptance.

It also seems difficult to envisage a sufficiently rapid mechanism for flooding the circulation with factor VIII from some tissue reserve, and there is no apparent reason for so great a store to be maintained. Nour-Eldin (N2) believed that the tissues contain much factor VIII, but, if his work is relied upon here, it is then necessary to explain why factor VIII does not appear in the blood of severely affected hemophiliacs after adrenaline infusion, for hemophilic tissue extracts will clot hemophilic blood as rapidly as extracts from normal tissue (B3), and so presumably contain the same clotting factor(s). Goudemand *et al.* (G1) inquire with reason, *"Comment pourrait être stocké en permanence une quantité aussi énorme de G.A.H.* [i.e., factor VIII] *telle que le taux dans le sang puisse être multiplié par 2 à 6 fois?"*[2]

The only other explanation in view at present is that the acute response consists of a potentiation or activation of circulating factor VIII without true increase in quantity, and that bioassay systems fail to distinguish between the two postulated forms of the factor. There is evidence that

[2] "How could a reserve of factor VIII be maintained sufficient to provide for a 2- to 6-fold rise in blood concentration?"

factor VIII can be "activated" by thrombin (R3) and by activated factor IX (B4, L2, M1), to give approximately the necessary increase in activity, but that activated factor VIII is unstable, whereas the activity generated by adrenaline or exercise survives incubation at 37°C for 30 minutes (I4) or storage at −20°C for a month with two cycles of freezing and thawing (E10). The antibody experiments described in Section 3.4.3 (A1, G1) support a potentiation hypothesis, but it requires to be supported also by the demonstration that factor VIII may be potentiated without loss of stability. The possibility of a tissue "activator" entering the circulation (E12, G1) requires further study. Egeberg's (E16) experiment showing loss of effect *in vivo* after dialysis, despite unchanged *in vitro* activity, should be repeated, for this evidence suggests an alteration in the molecule involving a small (dialyzable) component.

Evidence was given above (Section 3.4.1) that the exercise or adrenaline effect was demonstrated by assay systems in which precautions had been taken to provide full surface activation. The comparative data of both Ikkala (I2, I3) and Ingram (I5) show, however, that the effects were more marked when these precautions were not taken, and Ikkala (I2) also showed that the effect was more marked when phospholipid was not added to the system, although it was still clearly seen in its presence. The reduction in the effect either by surface contact or by phospholipid, or both (I2), raises the suspicion that if even more efficient agents had been added to the assay the effect would have been eliminated altogether, but so far this has not been achieved.

3.5.3. *Conclusion*

It seems likely that the relatively slow rise in factor VIII concentration in response to subacute or chronic "stress" represents an increased synthesis, and is comparable in rate to the increased synthesis seen in von Willebrand's disease after infusing normal or hemophilic plasma. The very rapid increase which can be produced by exercise or adrenaline infusion is more likely to be due to potentiation of factor VIII in the blood stream, but a type of potentiation satisfying the experimental data does not yet appear to have been demonstrated.

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4. Bioassay

4.1. Synopsis

The errors and uncertainties of bioassay are becoming a more serious obstacle to progress as work extends (S2). Until biological assay can be dispensed with, it is therefore necessary to refine it as much as possible. A

number of the biological problems involved have arisen in the previous sections of this review. The present section therefore deals with certain statistical aspects of bioassay, which it may be helpful to discuss together, and provides tables for rapidly computing assay results when it is desired to avoid the sometimes rather arbitrary procedure of a graphical solution.

4.2. Some Principles of the Bioassay of Factor VIII

Although they are now fairly widely known, it may be helpful to summarize some general principles of the bioassay of a clotting factor.

4.2.1. *Basis of Potency Assay*

The essence of the procedure consists in adding a series of dilutions of the test material to a clotting system deficient only in the factor to be assayed, and then comparing the corrections of clotting time so obtained with those following the addition of a similar series of dilutions of a standard (or control) material. It is essential to test more than one dilution of both the test and standard materials (e.g., plasmas) because it must be verified that the clotting system is responding similarly to both materials, by producing parallel lines when clotting time ("response") is plotted as y against dilution ("dose") as x. These lines are referred to as "dose-response curves." The curves would not be parallel, and the assay consequently invalid, if, for instance, the test and standard dilution series differed in respect to more than one clotting factor, especially in different proportions, say factor V as well as factor VIII (M1). Biological considerations will determine the steps to be taken to ensure that all relevant factors, except that being assayed, are identical for tests containing the two materials under comparison; for factor VIII, there are particular problems here when assaying concentrates against normal plasma (K1), which are not yet properly understood.

4.2.2. *Features Desirable in an Assay*

Besides the biological characteristic of *specificity*, with its statistical counterpart in the validity check provided by parallelism of dose-response curves, discussed above, an assay should be sufficiently sensitive and discriminating for the purpose in hand. By *sensitivity* is meant the lowest concentration of factor VIII that the assay can effectively measure; *discrimination* means the ability of the assay to distinguish between different concentrations of factor VIII within its useful range. Like specificity, sensitivity is a biological characteristic of the system. For instance, in assays employing hemophilic plasma as the diluent, full sensitivity will depend on obtaining this reagent from a severely affected patient with no

detectable factor VIII in the plasma. Discrimination, however, depends on the balance between two features: the accuracy with which the dose-response curves can be plotted, and their mean slope. The effect of observational error is obvious; the influence of the slope of the curves can also be readily appreciated, for if the assay has a very "flat" slope, it will require a relatively big increase in dose to cause an unequivocal rise in response.

4.2.3. *The Shape of the Dose-Response Curve*

Normal plasma does not clot instantaneously, and even in plasma from severely affected hemophiliacs the clotting time is not infinite; hence the possible range of responses lies between definite limits. This produces the characteristic reversed-S shape of dose-response curves in clotting-factor assays, most clearly seen when clotting time is plotted against *log dose*. In this form, there is usually a fairly long central portion which is more or less linear, or it may be more obvious if *log clotting time* is plotted. Many workers employing factor VIII assays have found this log transformation convenient, and there has been the impression that the *log dose:log response* relationship is of fundamental significance (M1). This is not so, for the fundamental form is the reversed-S shape, although one limb of the S may be more pronounced than the other, or the curved portions at the ends (i.e., where the curve approaches its natural limits) may be relatively insignificant.

The term "parallel" when applied to S-shaped curves has the somewhat restricted meaning that the two lines are at all points equidistant when measured on the dose axis. This is the essential criterion of validity mentioned above. When dealing with the relatively short pieces of the curves ordinarily used for actual assays, it is obviously easier to apply this criterion to a pair of straight lines than to a pair of curves fitted to experimental points. If the data happen to lie in a very curved region, it is therefore helpful to apply a transform which has the effect of reducing curvature in this region. Any transform may be used, since no such manipulation applied equally to paired dose-response curves can alter the horizontal distance between them. In the lower portion of the curve, corresponding to fairly high doses, the log transform is often convenient, as mentioned above. Toward the upper end, squares or cubes of the data may be taken. Transforms of this type are very simple to apply, because the transformed data can be taken directly from tables, or, in the case of the log transform and some others, graph paper can be obtained in the appropriate ruling. Transforms applicable to data at any level are those specifically related to S-shaped curves, namely, probits and logits. These transforms were developed for assay systems in which the response would naturally be

expressed as a proportion of a maximum, such as the proportion of a group of animals surviving a given dose of a drug. If tolerance to the drug is normally distributed among these animals, then the graph of the proportion surviving vs. log dose is S-shaped. The probit is a transform of the proportion which exactly linearizes this relationship. It can be shown that the logit transform has about the same effect and is mathematically simpler to manipulate, the logit of proportion p being defined as $\log_e (p/1 - p)$. In the present context the maximum possible response is the response of the clotting system to zero dose, which is the "blank" clotting time obtained when the plasma dilutions are replaced by the diluent alone (e.g., hemophilic plasma); but the error of determination is high, and the mean of several replicates should be used, which should be obtained at various times during the performance of the assay. Probit or logit transforms are read from tables, by entering with the ratio (observed value/"blank") for each reading.

Having applied a suitable transform when necessary, the readings are then examined for satisfactory parallelism. Clearly, nonparallelism will be most obvious, if present, when the longest possible stretches of dose-response curve are examined; each assay should therefore employ as much of the useful range as possible. This may be done by testing numerous different dilutions, or, less laboriously, by choosing a wide dilution step, e.g., 4-fold, 6-fold, or 10-fold, instead of the more usual 2-fold. At least three dilutions of each plasma should ordinarily be tested.

4.3. Sources of Error in Clotting-Factor Assays

It is important to distinguish between systematic and random errors. Systematic errors must be foreseen and eliminated by taking biological precautions in the choice of reagents and methods or by experimental design, for errors of this type are extremely difficult to eliminate from the data afterward. Random errors can at least be measured statistically and expressed in various indices showing the precision of the potency estimate obtained in a given assay.

4.3.1. *Systematic Error Due to Temporal "Drift"*

Despite such precautions as keeping the reagents on ice during an assay, repeated clotting times often show a steady drift over a working period. Unlike the strictly biological systematic errors (due to the use of inappropriate reagents and so on), the effect of drift can be eliminated by suitable assay design. The normal procedure in biological assay is to randomize the order of obtaining readings. In clotting assays, however, this may prove

very confusing and, by thus making the work more time-consuming, is to some extent self-defeating. A reasonable compromise is to obtain the data in a symmetrically balanced order, as 123, 321 for two replicates on each of three samples, or 123, 321, 321, 123 for four replicates. The properties of such arrangements have been studied by Cox (C2); when means are taken, the first arrangement, for two replicates, eliminates the effect of a linear time trend, and the second, for four, also eliminates quadratic effects. So simple a refinement seems worth adopting as a routine, and has the practical advantage that the extent of any time trend is at once apparent from the trend of differences between paired values.

4.3.2. *Random Error*

It is clearly apparent that the error of the determination of clotting times will be reflected in the accuracy of the final result of the assay—the potency ratio. Manual methods of determining the end point in plasma tests have errors, expressed as coefficients of variation, around 5% (A1, M2, P1); it is said that the value may be reduced to about 1% by mechanical methods (M1). However, as this basic error is reduced, other errors become relatively more important, and these have sometimes not received the consideration they deserve. For instance, the writer finds that fluctuations of dose means about the dose-response curve are of about the same magnitude as differences within doses, i.e., between replicates (obtained manually). Little improvement in ultimate accuracy is gained by reducing the within-doses error much below the magnitude of the between-doses component.

4.4. Calculating the Potency Ratio

4.4.1. *Graphical Solution*

To judge from the literature, most people working on coagulation obtain the results of their assays graphically. Parallel dose-response curves are fitted by eye to the responses plotted against log dose, and the horizontal distance between them measured as a ratio of concentrations. Thus, a straight line through the patient's points (patient's plasma assumed of lower activity than the standard) is produced to cut the ordinate of the log unit representing 100% activity; a horizontal line at this level is next drawn to cut the parallel straight line through the points obtained from the standard, and the ordinate intersected at this point represents the potency of the patient's plasma. These maneuvers are most conveniently carried out with the plasma dilutions plotted on the logarithmic ruling of special

graph paper, for the actual dilutions tested can then be directly entered as percentages.

Graphical solutions are usually quick and simple to carry out, and for most purposes quite satisfactory. However, it may sometimes be difficult to know where to draw lines between rather scattered points, or responses from two or more plasmas may overlap. In these and other circumstances it may be preferable to obtain the results by calculation.

4.4.2. *Computed Solutions*

It has been seen that the potency estimation is based on the distance apart of the patient and the control lines, and, while for graphical solutions it is convenient to make the measurement horizontally, it is obvious that if parallel straight lines are used a vertical measurement could be used to express the same thing, since such lines may be superimposed by a shift in any direction. In effect, parallel regression lines are fitted to the two series of readings. The estimate of potency is derived from the vertical distance between them, calculated as the difference between the sums of the control and the patient readings. (If unequal numbers of readings are obtained from the two plasmas or if these were tested at different concentrations, an appropriate correction may be applied, but the arithmetic is much simpler if equal numbers of readings are obtained on each of the plasmas tested in a given assay, and at dilutions separated by the same log interval throughout, e.g., all 2-fold or all 6-fold.)

The calculations are done in whichever transformation is necessary to allow parallel straight lines to be fitted. The estimation of potency from the vertical distance between the line requires also an index of slope for the portion of the curve being employed, which is given by the difference between the high and low responses of the two lines, divided by the log distance between the dilutions tested. This distance is the log of the dilution factor when serial dilutions are employed, e.g., log 2 for a series of 2-fold dilutions, log 4 for a 4-fold series. The estimate of potency is obtained from the ratio of the difference between patient and control readings to the slope of the lines (see below).

A number of single readings may be taken at different concentrations of each plasma, in order to obtain detailed information over a fairly long stretch of the dose-response curve. Alternatively, a certain technical advantage may be gained by obtaining replicate readings at a few concentrations only. Since both methods are in use, worked examples are provided for each.

By either method, approximately the same number of readings should be

taken on the patient and the control plasmas, since in this way the greatest accuracy is obtained by a given total number of readings. Unfortunately, accuracy increases only as the square root of the actual number of readings obtained; hence to double the accuracy, 4 times the number of readings must be taken, and so on. This means that it is hardly worthwhile to increase the number of readings from each plasma by any practicable number above, say, 6 or 8.

By computation, an estimate of the accuracy of the potency determination may also be obtained from the data. This problem is discussed in general terms by Coward (C1), Emmens (E1), Gaddum (G1, G2), and Finney (F1), Finney's being the definitive book. Examples of how these principles may be applied to factor VIII assay are given by Savage (S1), Morati-Schmitt *et al.* (M3), Hardisty and Macpherson (H1), and Baumgarten *et al.* (B1).

In making the calculations, the first step should be to determine the slope indices of the lines and to confirm that they are parallel. As a guide, the slopes of the lines may be regarded as sufficiently the same if the lower value is not less than 0.8 the greater, and, where several plasmas are assayed together against one control, this should be true of each patient's plasma separately with respect to the control. If any difference is greater than this, a transformation should be applied, but this is valid only if it is clear that the curves are essentially parallel in the special sense explained (Section 4.2.2, above).

Worked Example for Single Readings at a Number of Different Dilutions

In this procedure, standard methods are used for fitting linear regressions. The logic is not difficult to follow, although the arithmetic is a little cumbersome.

In a one-stage factor VIII assay employing serial 2-fold dilutions the following clotting times were obtained.

	Clotting time (seconds) at plasma dilutions of				
	1/10	1/20	1/40	1/80	1/160
Patient	107	118	130	132	—
Control	88	100	106	118	126

The first step is to rewrite the plasma dilutions in logs, which is conveniently done in wholly negative logs by writing the logs of the denominators preceded by minus signs (thus, $\log 1/20 = -(\log 20) = -1.301$). The arithmetic may be lightened if the clotting times are converted to smaller working units; since the calculations depend on *differences*,

the results are unaffected if a constant is first subtracted from each observed value. The most efficient constant to use is the mean of all clotting times taken to the nearest whole number, which in this case is 114 seconds. This maneuver is optional, but if employed the working data become:

	Clotting times[a] at log plasma dilutions (x) of				
	−1.00	−1.30	−1.60	−1.90	−2.20
Patient (y_p)	−7	4	16	18	—
Control (y_c)	−26	−14	−8	4	12

[a] Working units = clotting times minus 114.

Provided the lines are not significantly nonparallel, the patient's activity can be expressed in terms of the control as a potency ratio by the formula

$$M = \bar{x}_c - \bar{x}_p - \left[\frac{\bar{y}_c - \bar{y}_p}{B}\right]$$

where M is the log potency ratio (the potency being given by antilog M), $\bar{x}_p$ and $\bar{x}_c$ are the mean log concentrations tested, and $\bar{y}_p$ and $\bar{y}_c$ are the mean clotting times for the patient and the control plasma, respectively; B is the common slope, given by

$$\frac{[x_c y_c] + [x_p y_p]}{[x_c^2] + [x_p^2]}$$

If 2-fold dilutions are used, the quantities $\bar{x}_p$, $\bar{x}_c$, $[x_p^2]$, and $[x_c^2]$ may be obtained from Table 3. The products $[xy]$ are calculated as $Sxy - (SxSy/n)$, where Sxy indicates the sum of the products of each log plasma dilution with its corresponding clotting time in working units, and $SxSy/n$ is the "correction for the mean" and is the product of the sum of the log dilutions (from Table 3) and the sum of the clotting times; n is the number of plasma dilutions tested.

Calculation: (1) The following quantities are obtained from Table 3:

Patient's plasma: $Sx_p = -5.80$; $\bar{x}_p = -1.45$; $[x_p^2] = 0.453$

Control plasma: $Sx_c = -8.00$; $\bar{x}_c = -1.60$; $[x_c^2] = 0.906$

(2) The mean clotting times in working units are

Patient's plasma: $\bar{y}_p = \frac{1}{4}(-7 + 4 + 16 + 18) = \frac{31}{4} = 7.75$

Control plasma: $\bar{y}_c = \frac{1}{5}(-26 - 14 - 8 + 4 + 12) = -\frac{32}{5} = -6.40$

TABLE 3

QUANTITIES REQUIRED IN THE CALCULATION OF POTENCY RATIOS FROM CLOTTING-FACTOR ASSAYS BASED ON SINGLE READINGS ON DIFFERING NUMBERS OF 2-FOLD DILUTIONS OF PATIENT AND CONTROL PLASMAS[a]

Plasma dilutions:	1/5	1/10	1/20	1/40	1/80	1/160	1/320				
Dilutions in negative logs:	−0.699	−1.00	−1.30	−1.60	−1.90	−2.20	−2.50	Sx	$\bar{x}$	$[x^2]$	$1/[x^2]$
Number of dilutions (n):											
3	*	*	*					−3.00	−1.00	0.181	5.525
		*	*	*				−3.90	−1.30		
			*	*	*			−4.80	−1.60		
				*	*	*		−5.70	−1.90		
					*	*	*	−6.61	−2.20		
4	*	*	*	*				−4.60	−1.15	0.453	2.208
		*	*	*	*			−5.80	−1.45		
			*	*	*	*		−7.00	−1.75		
				*	*	*	*	−8.21	−2.05		
5	*	*	*	*	*			−6.50	−1.30	0.906	1.104
		*	*	*	*	*		−8.00	−1.60		
			*	*	*	*	*	−9.51	−1.90		
6	*	*	*	*	*	*		−8.70	−1.45	1.586	0.630
		*	*	*	*	*	*	−10.51	−1.75		
7	*	*	*	*	*	*	*	−11.21	−1.60	2.537	0.394

[a] Asterisk indicates that a reading has been taken at this dilution.

(3) The common slope, B, is obtained from the expression

$$B = \frac{[x_c y_c] + [x_p y_p]}{[x_c^2] + [x_p^2]} = \frac{\left(Sx_c y_c - \frac{Sx_c Sy_c}{n_c}\right) + \left(Sx_p y_p - \frac{Sx_p Sy_p}{n_p}\right)}{[x_c^2] + [x_p^2]}$$

$$= \frac{(-1.00)(-26) + \ldots + (-2.20)(12) - \frac{(-8.00)(-32)}{5}}{0.906 + 0.453}$$

$$+ \frac{(-1.00)(-7) + \ldots + (-1.90)(18) - \frac{(-5.80)(31)}{4}}{0.906 + 0.453}$$

$$= \frac{(23.00 - 51.20) + (-58.00 + 44.95)}{0.906 + 0.453} = \frac{-41.25}{1.359} = -30.3$$

Note that B is negative, because clotting time is *inversely* related to factor VIII concentration, and that if more than two plasmas are assayed together the common slope for all is obtained by including all the $[x_p y_p]/[x_p^2]$ terms in the formula.

(4) Hence,

$$M = -1.60 + 1.45 - \frac{(-6.40 - 7.75)}{(-30.3)}$$

$$= -0.15 - 0.47 = -0.62$$

Note that this, again, is a wholly negative logarithm. Thus antilog M = 1/antilog 0.62 = 1/4.2 = 0.24, or 24%.

(5) Note that the individual slopes for the two curves are given by

$$b_p = [x_p y_p]/[x_p^2] \quad \text{and} \quad b_c = [x_c y_c]/[x_c^2]$$

which enter into the calculation of the common slope in (3) above. Thus

$$b_p = \frac{-13.05}{0.453} = -28.8 \quad \text{and} \quad b_c = \frac{-28.20}{0.906} = -31.3$$

which are sufficiently parallel. A method for testing the significance of nonparallelism in doubtful cases, and which conveniently follows the above arithmetic, is given by Hardisty and Macpherson (H1).

Worked Example for Two Replicate Readings at Each of Three Dilutions of Each Plasma

In principle there can be any number of replicates, any number of dilutions, and any number of plasmas; but the same number of dilutions must be tested for each plasma, separated by the same dilution factor, and the same number of readings must be taken at each dilution. The fewer the dilutions, the more widely they should be spaced, and the greater the number of replicates should be, to obtain comparable accuracy. The suggested design, of two replicates at each of three 6-fold dilutions for each plasma, has been found to be a reasonable compromise.

TABLE 4

FORMULAS REQUIRED IN OBTAINING SLOPE INDICES AND POTENCY RATIOS FOR ASSAYS IN WHICH ALL PLASMAS ARE TESTED AT THE SAME NUMBERS OF DILUTIONS[a]

Dilutions tested				Number of dilutions tested	Values of Q for various numbers of plasmas and different dilution factors						
					Number of plasmas in assay (n), including standard	Dilution factor (i)					
No.	Designations	Slope index	Q			2	3	4	5	6	10
2	*(1)*, *(2)*	$b' = (2) - (1)$	$\frac{n(\log i)}{2}$	2	2	0.301	0.477	0.602	0.699	0.778	1.00
					3	0.452	0.716	0.903	1.05	1.17	1.50
					4	0.602	0.954	1.20	1.40	1.56	2.00
					5	0.753	1.19	1.51	1.75	1.95	2.50
3	*(1)*, *(2)*, *(3)*	$b' = (3) - (1)$	$\frac{2n(\log i)}{3}$	3	2	0.401	0.636	0.803	0.932	1.04	1.33
					3	0.602	0.954	1.20	1.40	1.56	2.00
					4	0.803	1.27	1.61	1.86	2.08	2.67
					5	1.00	1.59	2.01	2.33	2.59	3.33
4	*(1)*, *(2)*, *(3)*, *(4)*	$b' = 3 \times (4) + (3) - (2) - 3 \times (1)$	$\frac{5n(\log i)}{2}$	4	2	1.51	2.39	3.01	3.50	3.89	5.00
					3	2.26	3.58	4.52	5.24	5.84	7.50
					4	3.01	4.77	6.02	6.99	7.78	10.0
					5	3.76	5.96	7.53	8.74	9.73	12.5

[a] *Left-hand part of table:* the numbers in brackets in the second column refer to plasma dilutions so that (1) is always the highest concentration tested; when italicized in the third column, these numbers refer to the sums of replicate clotting times given by the corresponding dilutions; b' is the slope index for an individual plasma.

Right-hand part of table: for any given assay design, Q is a constant and its value can be precomputed; this part of the table gives three tabulations of Q for various numbers of plasmas and different dilution factors; for other dilution factors Q can readily be obtained from the formulas given; it is unlikely that more than five plasmas will be assayed together because serious alterations in reagents might occur over the long time-span of the experiment.

If the readings are arranged in this sort of way it allows certain short cuts to be taken in the arithmetic. In consequence the logic is somewhat more difficult to follow than in the first procedure, but the working may be done by rule and with the tabulated constants provided (Tables 4 and 5). The underlying principles are naturally the same as those of the first procedure, but the method is much easier; it is used habitually by the writer.

TABLE 5
VALUES OF Q FOR $i = 6$ AND $i = 10$[a]

Dilution factor (i)	Number of plasmas in assay (including standard) (n)	Number of dilutions of each plasma tested		
		2	3	4
6	2	0.778	1.04	3.89
	3	1.17	1.56	5.84
	4	1.56	2.08	7.78
10	2	1.00	1.33	5.00
	3	1.50	2.00	7.50
	4	2.00	2.67	10.00

[a] Often working at 6-fold or 10-fold dilutions, the author finds this summary table convenient for quick reference.

The minimum requirement is to test only two dilutions, but testing more than two dilutions allows for the possibility that one may not have correctly judged the approximate potency of the patient's plasma, causing some points to fall beyond the linear part of the dose-response curve; and even if the whole of the data cannot be used in a transformation because points at one end or the other do not fall on the straight line, these and an equal number of points from the other plasma data can be discarded and the potency calculated from the remainder. Taking two replicates at each dilution allows the testing easily to be arranged in a balanced order to eliminate the bias introduced by a time trend (Section 4.3.1). Random differences between replicates are a measure of experimental error.

To calculate the potency of a patient's plasma in terms of the control, take the following four steps.

First, calculate an index of slope, b', for each plasma by the appropriate formula from column 3 of Table 4. It will be seen that, where two dilutions of a plasma are tested, b' is obtained as the difference between the sums of the replicate clotting times at these dilutions. Where three dilutions are tested, b' is calculated as the difference between the sums of the replicate clotting times at the highest and lowest dilutions. Responses at the middle dilution do not affect the slope (although they contribute to the potency estimate) because this value acts (to use a mechanical analogy) as the "point of rotation" for the line. Where there are four dilutions, each set of clotting times contributes to the slope, but the two extreme values contribute proportionately more than those which are intermediate; an analogy would be the principle of moments acting on a pivoted beam. In calculating b', the appropriate weighting of the highest and lowest values is achieved

by multiplying them by a factor of 3, as is shown in the third formula for b' given in Table 4.

Note that by this method values of b' take positive signs since, in effect, the signs of T_c and T_p (see below; cf. $\bar{y}_c$ and $\bar{y}_p$ of the previous method) have been reversed. This is done to make the working easier and is allowed for subsequently. Check the several values of b' for parallelism by the rule given above, and apply a transformation if necessary.

Second, add together the individual slope indices to obtain a common index B', and divide this into the appropriate value of Q obtained from Table 4, to obtain Q/B'.

Third, total the readings from each plasma and obtain for each test plasma the difference $d = T_c - T_p$, where T_c is the total of the readings on the standard plasma, and T_p the total for a given test plasma. Note that d may be positive or negative.

Fourth, obtain the log potency ratio, M, for each test plasma from the expression $M = d(Q/B')$. Then, antilog M gives the potency ratio, and 100 (antilog M) the percentage activity of the patient's plasma in terms of the control. M may be either positive or negative, according to the sign of d, and the method of handling a negative value is shown in the example in Table 6. Also shown in Table 6 are the results obtained in a one-stage factor VIII assay.

TABLE 6

RESULTS OBTAINED IN A FACTOR VIII ASSAY

Dilutions tested	Replicate readings[a] (seconds) 1st	2nd	Totals of replicates	Slope indices (3) − (1)	Plasma totals (1) + (2) + (3)
Control plasma	①				
1 in 20	50	49 ↑	99 (1)		
1 in 120	65	69	134 (2)	70	402
1 in 720	85	84	169 (3)		
Patient's plasma					
1 in 5	70	69	139 (1)		
1 in 30	92	99	191 (2)	111	580
1 in 180	135 ↓	115	250 (3)		
		②			

[a] The readings were obtained in the order shown by the arrows. The reagents alone gave a "blank" clotting time of 140 seconds.

Here the plasma slope indices are not sufficiently similar (ratio 70/111 = 0.64), and it may be seen that the higher index is associated with the higher plasma total. A log transformation would therefore be likely to make the fitted lines more parallel. The data are therefore rewritten in logs, as shown in Table 7, and the calculation repeated. Note that since the final calculations depend entirely upon differences (slope indices, and control total minus patient total), the characteristic can be reduced by 1 to lighten the manipulations.

By this maneuver, the ratio between the individual slope indices has been raised to 0.92 so that the lines are now sufficiently parallel. Notice also the reduction in the

TABLE 7
LOG TRANSFORM OF THE RESULTS SHOWN IN TABLE 6

Dilutions tested	Log replicate readings: 1st	Log replicate readings: 2nd	Totals of log replicates	Slope indices (3) − (1)	Plasma totals (1) + (2) + (3)
Control plasma					
1 in 20	0.70	0.69	1.39 (1)		
1 in 120	0.81	0.84	1.65 (2)	0.46	4.89
1 in 720	0.93	0.92	1.85 (3)		
Patient's plasma					
1 in 5	0.85	0.84	1.69 (1)		
1 in 30	0.96	1.00	1.96 (2)	0.50	5.84
1 in 180	1.13	1.06	2.19 (3)		
Common slope index (B')				0.96	
Control total minus patient's total (d)					−0.95

relative magnitude of the error between the two replicates of the third dilution of the patient's plasma.

From the right-hand part of Table 4, for testing three dilutions of two plasmas at a 6-fold step interval ($n = 2$, $i = 6$), $Q = 1.04$. From Table 7 above, $d = 4.89 - 5.84 = -0.95$. Then $M = d(Q/B') = -0.95(1.04/0.96) = -1.026$. Now the antilog of -1.026 = 1/antilog 1.026 = 1/10.62 = 0.0942, or 9.42% as the figures stand, but the patient's plasma was tested at 4 times the concentrations of the control plasma; therefore the potency of the patient's plasma is (9.42/4)% = 2.36, about 2.4%.

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ALBUMIN AND "TOTAL GLOBULIN" FRACTIONS OF BLOOD

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1. Introduction

Albumin has been more extensively studied than any other plasma protein. Among the many reasons for this are the natural abundance of blood plasma, the relatively high stability of albumin, and the early clinical interest in the low plasma albumin levels found in chronic diseases of the kidneys and liver (E5, M32). Prominence was at one time given to the role of albumin as a regulator of tissue fluid balance and an inhibitor of edema

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(S12, S42), but it is now known that albumin is not the governing factor, nor is it the most important factor to be considered in the accumulation of abnormal collections of body fluids (H8). Furthermore, it is apparent from the existence of relatively healthy adults with no albumin in the blood (B5, B11) that the protein is not essential for life. Notwithstanding these lately appreciated facts, an important if not unique physiological role of albumin is its capacity to act as a vehicular substance for a wide variety of ions with different structures. In addition to its ability to bind and transport fatty acids, certain hormones, steroids, peptides, and amino acids, it can hold and effectively isolate the ions of many different substances that may be undesirable or dangerous to cellular life, e.g., phenols, phenolic acids, poisonous metals, bilirubin.

While albumin[2] is becoming more clearly characterized as a single plasma protein, globulin reveals itself as an increasingly complex mixture of glyco- and lipoproteins. In the present review an account is given of the clinical chemistry of the two entities that comprise the A/G ratio, a ratio introduced by Epstein in 1912 (E5) and subsequently used widely as an activity index of certain disease states.

2. Separation of Albumin from Globulins

2.1. Separation with Concentrated Salt Solutions

Toward the end of the nineteenth century, European pharmacologists demonstrated the heterogeneity of the protein (albumen) in blood serum. Concentrated salt solutions were employed to precipitate the globulins. After the work of Kander (K2) and Lewith (L10) the serum protein, which remained in solution after mixing diluted serum with an equal volume of ammonium sulfate solution saturated at 25°C (final concentration 2.05 M), was designated "albumin."

New interest in the nature of the constituent serum proteins resulted from the introduction of the sodium sulfate fractionation scheme by Howe (H24, H25). This was a technical advance because, unlike ammonium sulfate, sodium sulfate need not be removed prior to nitrogen analysis, the only accurate available means at that time of determining protein. Howe (H24) observed three ranges of sodium sulfate concentration at which an increase in molarity of 0.05 in a 1:30 dilution of calf serum failed to produce the expected increase in precipitation. One of these "critical" concentrations occurred at 1.45–1.50 M and was thought to indicate complete globulin precipitation.

[2] This name, unqualified, will be used to mean albumin obtainable from human blood plasma.

The concept of proteins as heterogeneous colloidal systems rather than as discrete molecules tended to discourage the development of new separation processes. Protein separation with other salts, e.g., magnesium sulfate and mixtures of the hydrogen phosphates of sodium and potassium, were investigated, but in the studies undertaken the salt concentrations in the protein mixtures rarely exceeded half-saturation.

Majoor restudied the "salting out" of serum proteins with sodium sulfate in concentrations up to 400 g/liter (M7–M9). In the curves obtained by plotting insoluble protein nitrogen against salt concentration, small horizontal parts were seen to occur. The position of the latter varied slightly with different sera, an observation paralleled by Derrien and colleagues (D9, S49), who worked with concentrated phosphate buffers under controlled conditions. From a careful examination of the discontinuities in the protein curves, Majoor concluded that albumin precipitation from diluted serum did not begin to occur until a concentration of 260 g/liter (1.80 *M*) sodium sulfate was exceeded, a conclusion also reached by Milne (M24) and subsequently confirmed by other techniques.

Both free electrophoresis (K16, L8) and zone electrophoresis followed by dye binding (Y4) have been shown to give measures of albumin close to the albumin nitrogen values obtained when 1.80 *M* (260 g/liter) sodium sulfate is used for albumin/globulin separation. Furthermore, since free electrophoresis had early shown the presence of α-globulins in the serum "albumin" as determined by Howe's method (P4), it is surprising that this inadequate separation method has for so long been entrenched in many clinical chemistry manuals. Use of the Howe method has led to cases of congenital analbuminemia being considered as hypoalbuminemia (G13). Its continued use also delayed appreciation of the fact that many metabolic diseases and infective and neoplastic disorders are associated with slightly elevated α-globulin and slightly decreased albumin in the blood (S56).

The amounts of plasma protein nitrogen precipitated in 1.5 *M* sodium sulfate and in 2.05 *M* (half-saturation) ammonium sulfate were, for the same serum, shown to be approximately equal (H24), and this observation, although denied by some workers (F5, S43), now seems reliable (T14, W9). Although the albumin soluble in 2.05 *M* ammonium sulfate was reported (T10) to be contaminated with 25% of the globulins, this amount of unprecipitated serum globulin can be considerably reduced by allowing the salt-protein mixture to reach equilibrium before separation. From the precipitation curves of Herken and Remner (H12), it is apparent that 2.45 *M* ammonium sulfate will effect the sharpest separation of the serum globulins from albumin, and the use of ammonium sulfate at this final concentration gives analytical results in close agreement with those ob-

tained by 1.8 *M* sodium sulfate fractionation (W9). However, it must be admitted that the use of a fixed salt concentration to precipitate globulins in plasma suffers from an inherent lack of precision, since the concentration of salt required to precipitate the globulins completely is to some extent dependent upon the concentration of those globulins (D11).

Although as many as eight distinct proteins have been demonstrated immunologically in the soluble fraction obtained at 65% ammonium sulfate saturation (2.6 *M*), the total amount of these antigens represents a very small part (about 3%) of the total albumin fraction (O6).

2.1.1. *Sodium Sulfate and Sulfite Technique*

The time allowed by Howe for the precipitation of serum globulins in 1.5 *M* sodium sulfate (H24) was 12 hours. Most workers who have studied this separation procedure have used from 1 to 4 hours at 37°C (H17, K16, K19, L8, R16). Fine (F5) used a precipitation time of 15 minutes and more recently Rappaport and Loew (R4) found 5 minutes sufficient.

Believing that 12 hours was the optimal time for this purpose, Campbell and Hanna (C3) suggested replacing the sulfate by sodium sulfite which they found required 2 minutes for globulin separation. A higher molarity of sulfite is required to effect globulin precipitation, and a 2.1 *M* sulfite solution containing serum at 1% dilution has a pH of 8.9. Variation in pH between 5 and 9 for sodium sulfate fractionation has been shown to be without influence on the precipitation of globulin (B4, C20, W31).

The temperature for "salting out" need be only as high as is required to prevent crystallization of the salt (R16). For 1.8 *M* sodium sulfate and 2.1 *M* sodium sulfite the minimum working temperature is 25°C. A mixture of sulfate and sulfite has been proposed (R7), but solubility is not appreciably increased and the mixture still requires storing in a warm place, i.e., above 25°C, to prevent crystallization. In practice, sulfite holds no advantage over sulfate when a 37°C incubator for storing the concentrated solution is available. Warm sulfite solutions also deteriorate and must be made fresh every week (L8). Furthermore, sulfite increases the danger of albumin denaturation by ether, while sulfate has a protective influence (B31, S4).

Separation of albumin from globulin, originally done by filtration, was greatly facilitated by Kingsley's introduction of the ether technique (K20). Ether is added to the salt-protein mixture, which is then repeatedly mixed by inversion for 20 seconds and subsequently centrifuged. Flotation of the globulins ensues, and the albumin fraction separates as a subnatant solution.

Shaking with ether must not be done too vigorously or longer than 20

seconds, or loss of albumin will occur. The latter is said to be due to absorption of denatured albumin on the globulin precipitate (C12, C13). Restriction of the air space is also advisable (S4). Addition of a surface-active reagent to the ether (W26) has little advantage for sodium sulfate (or sulfite) separations beyond reducing the shaking time required for effective separation (S4).

2.2. Solution in Organic Solvents

2.2.1. *Cold "Neutral" Alcohol*

It has long been known that the addition of organic solvents to aqueous solutions of proteins frequently causes their precipitation (M18). Among the first to achieve fractionation of serum protein with alcohol at low temperature were Liu and Wu (L13). Under controlled conditions, methanol can be made to precipitate all the globulins and leave the albumin in solution.

Pillemer and Hutchinson (P10) developed an improved technique in which methanol is added with stirring to a mixture of serum and acetate buffer, molarity 0.08, pH 6.7. The alcohol is prediluted to a 60% aqueous solution. This decreases the heat of dilution evolved on adding the organic solvent to the diluted serum, and lessens the likelihood of the albumin becoming denatured owing to high local concentrations during its addition. The mixture stands at 0°C for 30 minutes, and the soluble remaining protein is separated by cold filtration or centrifugation.

Owing to the low temperature requirement, this type of method has not been extensively used or investigated for quantitative microanalysis. It should be noted, however, that the albumin separation is satisfactory even when the temperature during filtration rises as high as 7°C (P10). Careful control at temperatures below zero is demanded for the ethanol fractionation scheme of Cohn *et al.* (C14). The higher dielectric constant of methanol undoubtedly renders the earlier method less sensitive to small changes in the many variables—temperature, molarity, pH of buffer, concentration of solvent and serum—and less likely to be adversely affected by albumin "denaturation."

Using this method, some investigators have obtained albumin yields which compare well with those obtained by 1.8 *M* sodium sulfate fractionation and by separation by free electrophoresis (M13). However, several other reports suggest that difficulties may be encountered with the procedure of Pillemer and Hutchinson, which probably arise from variable and uncontrollable factors owing to the high concentration of serum in the

mixture. Micromethods more suitable for clinical use have been devised (C10, E3). In one, 7 ml cold 60% methanol is added to 67 μl serum diluted in 3 ml acetate buffer (molarity 0.05, pH 5.5).

2.2.2. *Acid and Organic Solvent*

One of the earlier European methods commonly used for determining proteins in serum involved their precipitation with trichloroacetic acid (TCA) followed by filtration, drying, and weighing. Alcohol and ether were used in the drying process. This frequently led to unrecognized errors (L18) varying in extent according to the technique used. In particular, the amount of water contained in the packed TCA-albumin precipitate contributes markedly to its solubility in organic solvents.

In 1932, Race discovered that plasma albumin after precipitation with TCA was soluble in alcohol and, further, that horse, human, sheep, and ox albumin were completely soluble, while their globulins were totally insoluble, in a solution containing 20 g TCA, 80 ml acetone, and 15 ml water. At the same time Race (R1) devised a method for determining the albumin in plasma by first extracting it from TCA-precipitated proteins with 80% aqueous acetone containing 2–3 ml 20% TCA. Albumin nitrogen was then determined in the extract. With this acid-acetone fractionation technique, plasma from patients with rheumatoid arthritis gave lower albumin levels than those found by Howe's method, and, indeed, these were probably the first really accurate albumin assays on blood to be reported.

The observation that albumin is soluble in acid alcohol and acid acetone seems to have remained unrecognized for more than 20 years (C11, L18) until it was rediscovered in 1954 by Delaville *et al.* (D7, D8). Improved methods on a microscale, based on this property, have been devised (D5, D7, D8, W8). The phenomenon under discussion is probably due to the formation of the unexpanded F form of albumin at a pH between 3 and 4 when COOH ionization is repressed and hydrophobic surfaces of the molecule are exposed (F15). At this pH, a solvent of appropriate dielectric constant (DC) is required for solubilization: 1 ml methanolic solution (DC: 33) containing 0.1 ml water and 0.1 g TCA will dissolve 30 mg albumin; similar ethanolic (DC: 25) acetone (DC: 21) and ether (DC: 4) solutions will dissolve 3, 1, and 0.1 mg albumin, respectively. Other solvents and acids have been employed, e.g., dichloroacetic acid–acetone (R1), dichloroacetic acid–ethylene dichloride (Y1), and hydrochloric acid–methanol (M20). With the use of phosphate buffer, pH 2.4, and ethanol (P6), albumin may be extracted quantitatively from liver ribosomes.

If membranes containing electrophoretically separated serum proteins are treated in TCA-alcohol solution before being stained with bromphenol

blue or Eriochrome Black T, only the globulins take up the dye. Although it has been suggested that TCA treatment blocks the dye-binding sites of albumin (C11), almost complete elution of albumin from the membrane is the cause of the above phenomenon.

The TCA–alcohol-treated albumin, after removal of acid and solvent by dialysis, shows no change from the untreated material in electrophoretic mobility, sedimentation, solubility, crystallizability (S24), precipitin curves (K1), or rate of degradation in the rabbit (F9). In the case of bovine albumin, a slight increase in susceptibility to proteolysis by trypsin has been reported (R3). This effect may have been due to removal of traces of calcium, which normally exerts a stabilizing action on albumin toward trypsin hydrolysis (G14).

2.3. Immunochemical Precipitation

The combining site on the antibody (γ-globulin) molecule may be conceived as a cavity formed by the helices of that molecule with its van der Waals contour complementary to and therefore selective for the antigenic determinant group. While the mechanism of the antibody precipitin reaction remains poorly understood, proper conditions for maximum precipitation (salt concentration, temperature, and pH) have to be determined experimentally. Effects peculiar to the species producing antibodies have to be considered. It has been shown that avian antisera against protein antigens precipitate best in 8% NaCl and poorly in 0.5% NaCl, while with mammalian antisera to the same proteins the reverse situation is true (G11). Avian specific precipitates have been reported to contain an α-globulin coprecipitated in amounts up to one half of the total nitrogen content (D10), but this has not been confirmed by others (B2, G10). Coprecipitation of protein with specific immunoprecipitates is, nevertheless, a real possibility. Inhibition of precipitate formation is brought about by salicylate when present at a concentration sufficient (80 mg/ml) to modify the configuration of the protein (F24). The precipitation which occurs when an α-globulin is made to move electrophoretically over its specific γ-globulin antibody does not occur if the speed of migration and potential applied are sufficiently high to enforce a dislocation of the complementary combining sites (W11). Since the sensitivity of precipitation-in-gel reactions can be increased by the addition of cadmium, nickel, and other salts to the antigen solution (C19), similar ionic factors may influence the aqueous immune reaction in the absence of agar.

Immunological techniques enable small amounts of distinct antigenic impurities to be detected in purified proteins, such as albumin (C16, O6, S54). They also enable antigenic proteins to be analyzed for multiple identi-

fying characters (F6). On the other hand, the role of immunoelectrophoresis is essentially one of high resolving power for complex mixtures (W33). Although less suitable for quantitation (F4, W28), the double diffusion two-dimensional technique of Ouchterlony offers the most sensitive means of studying antigen-antibody reactions (O5). The resolving power of the Ouchterlony plate method is due mainly to the immunospecific barrier effect of the precipitate, although this is absolute only for balanced or nearly balanced systems (F6). When two antigens are compared, using as a source of antibody a serum prepared against a third cross-reacting antigen, it is assumed that the presence of one antigenic determinant more or less than exists in a related antigen will give rise to spur formation extending beyond the point of fusion of precipitates.

Other immunochemical means of observing isolated proteins include the spraying of filter paper electropherograms with antisera against individual globulins. The nonprecipitated proteins are removed by elution and the precipitated ones are then stained (B34, G25). The "electroprecipitin" technique of Watson and Whinfrey (W11) has been developed in agar gel (R5) and as a quantitative procedure (J11); with a variety of antisera it will reveal individual glyco- and lipoproteins after electrophoresis on cellulose acetate (Watson, unpublished observations). Complete separation of some protein antigens from their specific antibody precipitates is not easily achieved, but albumin may be extracted from the antialbumin complex with cold TCA in ethanol (L15). The albumin in the extract can then be reprecipitated with ether (M20). An albumin-binding protein (or peptide) has been demonstrated in extracts of human liver (T12) and of rat liver (D6). This protein is precipitated together with albumin by the addition of antiserum, but can be separated from it by electrophoresis (D6).

As a tool for the clinical chemist, the chemical approach embracing immunological techniques will certainly prove increasingly useful. Among its first fruits has been the immunoelectrophoretic detection of several individual "globulins" present in abnormal amounts in some disease states (H3). Methods for their determination have been developed (S38). They vary in complexity and accuracy from semiquantitative estimates by serial dilutions (O6, W11) or packed precipitate volumes (H7) to highly sensitive antigen-antibody nitrogen determinations (K36, M4). In preparation for the latter, the antigen is precipitated with a slight excess of antibody at 37°C, allowed to stand several hours at 0°C, washed with ice-cold saline, and dissolved in dilute alkali (M4). Some control over possible losses during the washing process seems desirable (F16).

Precipitation of soluble antigenic proteins by antigen-specific antibody has been studied fairly intensively by diffusion techniques in stabilizing

gels. Diffusion of proteins with molecular weights up to 200,000 is not impeded by agar at concentrations under 1%, and the laws of free diffusion apply (A6). In the technique of Oudin, the protein solution is layered on the gel containing the relevant antiserum, and the distance moved by the leading edge of the precipitate is measured after several days. Using 1.7-mm diameter tubes containing 45 mm antialbumin serum in 0.3% agar gel and 20 mm albumin solution, the height of penetration of the precipitation zone after a period of 3–7 days is a linear function of log C (concentration of albumin). When the value of $h/(t)^{1/2}$ is large (h is the distance traveled during time t), $h^2/(t)^{1/2}$ approaches a linear function of log C (O6). If the precipitate is required for analysis it can be removed by a slicing technique, for which purpose diffusion may be carried out in columns up to about 10 cm in diameter (G5). Area of precipitate in a single radial diffusion method has also been used for immunochemical quantitation of proteins. Applied to albumin, the accuracy is about 5%, and the sensitivity may extend down to 0.3 μg/ml of antigen (M10).

The earliest albumin assays performed by mixing diluted serum with antialbumin serum were apparently satisfactory (C7–C9). These simple turbidimetric measurements gave results which agreed with those found by other means (M13), but the technique has not been generally adopted. Schultze and Schwick have recently expanded the technique to quantitate the following proteins: albumin, tryptophan-rich pre-albumin, ceruloplasmin, α_2-macroglobulin, α_2-lipoprotein, β-lipoprotein, transferrin, γ-globulins, and fibrinogen. Only a few microliters of serum are required, but careful standardization of each batch of antiserum is essential (S23).

2.4. Electrophoretic Separation

Separation of albumin from globulins, whether by free electrophoresis or by zone electrophoresis on paper, membranes, or agar (B33), is based essentially on net charge distribution. Preparations of albumin by "curtain" electrophoresis may therefore be contaminated with lipoprotein (O3) and with certain glycoproteins having the same net charge as albumin. Starch-gel electrophoresis, and to a slight extent agar electrophoresis, depends on molecular weight and shape, in addition to net charge. Because of this, a protein separating on paper as an α-globulin may migrate with the albumin in starch gel (P13). An α_1-globulin thought to originate from the necrosis of tumor cells also migrates with albumin in agar gel (D2). A technique commonly used to demonstrate a particular property of a plasma protein has been to prepare duplicate electrophoretic separations and then to visualize the protein on one strip and to examine the other for the property of the chosen protein. Further evidence that the protein in question is

specifically responsible for the activity being investigated would, however, appear essential. Thus, the failure to distinguish between properties of proteins and of nonprotein substances migrating with them has led to some dubious findings (A12, H23, M27), a recent example of which is the esterase activity of serum albumin (T1).

Separation of serum proteins on cellulose acetate membranes, originally introduced by Kohn (K29), has been thoroughly tested by a number of workers (B22, B24, F23, K30, O1). The negligible amount of protein absorption accounts for the cleaner separation of the various fractions and the almost complete absence of albumin "tailing." It also permits a more rapid separation, and makes possible a higher degree of precision than can be obtained with paper (B22). It is important to note the shorter migration distance traveled by albumin during microelectrophoresis on cellulose acetate. If the membrane is used after the manner of conventional paper electrophoresis, little advantage is gained (K30). The inheritance of an atypical albumin was studied recently by serum protein electrophoresis at pH 8.6 on cellulose acetate; the abnormal protein could not be observed on filter paper or in starch gel at the same pH (T3).

2.4.1. *Paper Electrophoresis*

Since 1958 when paper electrophoresis of serum proteins was reviewed in this series (P3), there have been no major technical developments in the art. The incorporation of nonionic detergents (Tween 80, Span 20, etc.) in the conventional barbiturate buffer (D14, G18, L14) and the use of "tris"-borate as buffer (A14, S27) have led to the separation of 7 to 11 globulin fractions. After zone electrophoresis in borate-containing buffer, an increased number of bands can be visualized by staining, but these are not revealed by the schlieren scanning technique after free electrophoresis in similar buffers (S27); the effects of this buffer may be due to interaction between borate and proteins having a high carbohydrate content.

In the more differential techniques, as in those dividing the serum proteins into the conventional (and arbitrary) five or six fractions, the migration lengths of the individual fractions and the degree of subfractionation often differ from day to day in the same laboratory (P3). The conditions under which the whole blood is incubated, the storage and pretreatment of serum (A11), and the presence of drugs in the serum (P2) can influence the migration speeds of some globulins. Electrophoresis of fresh serum under conventional conditions normally results in the separation of two β-globulins, but this does not occur if the blood has been drawn several hours before testing (Y5). The presence of diphtheria toxin in serum may

retard the migration of α_1-globulin and also decrease the amount of protein migrating as α_2- and β_1-globulins (P12).

As in all electrophoretic procedures, a rigidly standardized technique is essential for consistent results. However, the variable absorption of the proteins in paper, and their different protein dye-binding capacities used for quantitation (see Section 6.5), necessitate several corrections which are both tedious and impractical. Such corrections are frequently ignored on the ground of simplicity. In the opinion of the writer, filter paper electrophoresis of serum albumin and globulins is an inaccurate separative procedure which is now outmoded.

2.5. Gel Sieving

Column chromatography of serum has not proved suitable for direct fractionation of proteins. However, after preliminary fractionation by other means, a fine subdivision of the globulins can be achieved on DEAE-cellulose, CM-cellulose, or cellulose phosphate with the apparatus and gradient elution scheme described by Sober *et al.* (S36). For example, after removing the γ-globulin and most of the α_2-globulins in 1.3 *M* ammonium sulfate, Tombs *et al.* (T13) were able to elute four fractions from DEAE-cellulose—transferrin, haptoglobin, albumin, and α-globulins. No fraction was homogeneous, but the method revealed increased amounts of α-globulins in the sera of patients with advanced cancer and in a proportion of patients with rheumatoid arthritis, nephritis, and infections. Attempts to obtain albumin free of α_1-globulin by chromatographic procedures have not been successful (T9, T13).

Gel sieving or filtration makes use of sterically conditioned distribution behavior between water in two physical states: readily displaceable water in the intergranular space, and water immobilized in the macromolecular network. The nature of the gel matrix is important only insofar as it determines the swelling, and thus the penetrability of the solute. By using solutions of sufficiently high ionic strength, charge effects are suppressed and the fractionation becomes a kind of molecular sieving, the larger solute molecules moving faster and having lower gel penetrability. In contrast to ion-exchange columns which need frequent regeneration, the cross-linked dextran gel column may be used repeatedly without regeneration. Separation of serum proteins on strongly cross-linked gel (Sephadex G-25 and G-75) usually results in three fractions, whether this is performed by the column technique (F10, R28) or by the thin-layer technique (J10). The fractions eluted appear in order of decreasing molecular size, and the peaks correspond roughly to separations obtained by ultracentrifugal force.

Thin-layer chromatography of isolated serum proteins on a weakly cross-linked dextran gel (Sephadex G-200) has been reported to give R_f values linearly related to the logarithms of the molecular weights of the proteins (M28).

Serum proteins have also been fractionated in the phase systems polyethylene glycol/potassium phosphate/water (L4) and diethylene glycol/diethyl ether/glycerophosphate/water (T6).

3. Plasma Albumin and Its Variants

3.1. Normal Adult Human Albumin

Crystalline albumin was first prepared by Hewitt 30 years ago. Hewitt's preparations were contaminated with small amounts of carbohydrate, yet he pointed out that carbohydrate-free albumin might be obtained if recrystallization was repeated many times (H16). Later attempts to isolate the "albumins" with insufficiently concentrated salt solutions perpetuated the idea that albumin was a mixture of glycoproteins (K7), and also that its composition was altered in chronic nephritis (F1).

The purest available specimens of albumin are now known to be homogeneous (F15), except in one unique respect. The native protein appears to contain only 0.6–0.8 mole SH per mole albumin, i.e., in approximately 3 of 10 molecules there is no detectable reduced sulfhydryl group. Two explanations for this phenomenon have been put forward. In the first, the absent SH is thought to be the result of a sulfhydryl-disulfide exchange in the presence of cystine:

$$\mathrm{ASH} + \mathrm{CsS{-}SCs} \rightarrow \mathrm{ASSCs} + \mathrm{CsSH}$$

Complete conversion of albumin (mercaptalbumin) to non-SH albumin will occur during its incubation with cystine in neutral solution, and oxidized glutathione will to a lesser extent also remove the SH from albumin (K18). In the second explanation, loss of titratable SH is thought to be due to the burial of the occasional SH group in the interior of the molecule. This is suggested by the rise in titratable SH found after the addition to albumin of urea or sodium dodecyl sulfate. When albumin is treated with 8 M urea and subsequently diluted in neutral solution, the argentometrically determined SH titer rises from 0.7 to 1.0 SH equivalent per mole (B8). After similar treatment, an examination of the renatured protein by a low-angle X-ray scattering technique (E2) also suggests a slight alteration in the tertiary structure, without an increase in molecular size.

Purified albumin from several species has been analyzed for amino acids by a number of workers. Even allowing for recognizable error, their findings

leave no room to doubt the individuality of albumins peculiar to the species (Table 1). Phelps and Putnam (P8) have recently reviewed the evidence that albumins of all species examined have but a single peptide chain stabilized by 16 or 17 interchain disulfide bonds (P8). In the human pro-

TABLE 1
AMINO ACID GROUPINGS IN ALBUMINS[a]

Amino acid	Albumin groupings in:							
	Man			Ox		Dog	Rat	Horse
Glutamic acid	79	80	81	78	74	83	78	76
Aspartic acid	51	62	52	54	54	51	50	51
Threonine	25	23	28	34	32	23	31	21
Serine	19	23	24	26	26	25	23	26
Proline	26	27	25	30	27	31	29	25
Glycine	12	15	12	16	16	23	17	21
Alanine	62	62	62	46	46	62	59	59
Valine	42	45	41	37	33	41	33	23
Methionine	6	8	5	4	4	4	6	—
Isoleucine	8	—	8	14	14	6	13	—
Leucine	60	—	60	62	62	64	54	—
Tyrosine	18	14	16	20	18	21	22	15
Phenylalanine	30	33	30	27	27	30	23	33
Histidine	16	—	15	17	17	12	14	—
Lysine	62	—	56	62	57	58	51	—
Arginine	24	24	23	23	22	23	23	20
Tryptophan	1	—	1	2	2	1	1	—
Cystine/2	37	—	33	36	34	33	30	—
Total nitrogen	16.3	—	—	16.4	16.5	16.1	15.8	—
Reference	(S38a)	(S37)	(H6b)	(S38a)	(S44)	(A5)	(P7)	(S37)

[a] Calculated number of residues per molecular weight of 66,000.

tein, the N-terminal sequence is Asp-Ala-His (I1), while the C-terminal sequence appears to be (Val-Ser-Glu·NH_2-Asp·NH_2-Leu-Ala)-Ala-Gly-Leu (I1, K18, W21), each sequence differing from those so far determined in other mammals and birds (P8).

Albumin shows less tendency to polymerize than many other proteins. Nevertheless, as commercially produced, it contains about 5% of dimers and traces of other polymers. Aggregation can occur when an intramolecular S-S link is replaced by an intermolecular S-S link, under the catalytic influence of a very small amount of cysteine (J7).

$$A_1SH + \begin{matrix} S \\ | \\ S \end{matrix}\!\!>A_2 \rightarrow A_1SSA_2SH + \begin{matrix} S \\ | \\ S \end{matrix}\!\!>A_3 \rightarrow A_1SSA_2SSA_3$$

Dimer formation without sulfhydryl participation may also occur (H6, T8), but the mechanism of nonsulfide aggregation is not clear.

Sulfhydryl-initiated disulfide interchange occurs during the denaturation of albumin, whether effected by heat, alcohol, or urea treatment. Substances which block the SH group inhibit intermolecular disulfide bonding and tend to prevent polymerization and the formation of an opaque gel on heating (J7, S51). The effect of chloroquine resembles that of other compounds which inhibit sulfhydryl-disulfide interchange, e.g., marcaptans, iodoacetate. When buffered at pH 7.4, and in concentrations such as are employed therapeutically, chloroquine phosphate inhibits the rise in viscosity which ordinarily occurs during urea-induced denaturation of albumin (G2). A similar protection is afforded to albumin by Congo red at pH 6.4 (T7a), and by deoxyribonucleic acid (DNA). In the latter case, the effect is nullified by the presence of 0.04 *M* NaCl. Below pH 5, the addition of 1 part DNA to 15 parts by weight of albumin in aqueous solution can completely prevent the thermal coagulation of the protein. Ultracentrifugal studies indicate the formation of a complex in which 1 mole of DNA binds 1800 moles of albumin (Z2).

As with other proteins, albumin is more reactive in its denatured state. However, native albumin can bind many organic anions which other proteins fail to bind, even after denaturation. No satisfactory explanation for this can be derived at present from a knowledge of its primary structure (Table 1). After deamination of albumin, the extent of binding by azo dyes (E4) and by the sulfophthaleins (F17) is decreased. Binding is thus dependent on the presence of free NH_2 groups in the protein, but the presence of a substituent, especially in position 1 in the characteristic ring of a sulfophthalein, is also important (F17). Quinoline derivatives interact with albumin, and the binding of quinidine to human albumin depends primarily on interaction with the basic quinoline ring (C17). L-Thyroxine unites with the N-terminal aspartyl group of albumin. Binding of L-tryptophan has been shown to be highly stereospecific. In this case the ionizable N-terminal group of albumin and the COOH group of L-tryptophan are involved. Part of the indole ring is a strong point of attachment (M6).

By studying the competitive effects of structurally related colorless anions, it has been demonstrated (K5) that the binding of each isomer of the dye, phenyl-[*p*-(*p*-dimethylaminobenzeneazo)benzoylamino]acetate, involves a three-point attachment. Since the isomeric dyes utilized the same combining regions of albumin, it was concluded that these regions have a high degree of "configurational adaptability" (K5).

Studies on the binding of cations and small anions to albumin have been reviewed by Foster (F15). Contrary to earlier suggestions (K24), the

combination of Cu^{++} with albumin is not chiefly a mercaptide interaction (K31); it appears that the N-terminal aspartyl residue may bind the first molecule of copper (P5).

A highly idealized model has been postulated for the albumin molecule to account for the behavior of albumin at low pH, and for the destruction, upon isomerization, of the 10 or 12 strong binding sites for detergent ions (F15). Observations of the X-ray diffractions of bovine albumin at pH 3.6 also indicated that the molecule was not uniform or compact. It was considered that at acidic pH about one third of the polypeptide chain unraveled and assumed a loose random-coil-like structure while the rest of the molecule remained folded in a compact particle (L21). Under natural conditions, i.e., in blood plasma, albumin exists partly in the α-helix and partly in the random form (D13).

3.2. *In Vitro* Modifications and Degradations of Albumin

The splitting referred to as "heterogeneity" and demonstrated in 1939 by Leutscher (L7) by electrophoresis of impure albumin at low pH has been confirmed many times with pure preparations. In acid solutions of pH 3–4 and at low ionic strength (0.02), albumin shows two or three components after electrophoresis (A10, S1), changes in titration curve data (T2), and increase in intrinsic viscosity (N3, Y2). These changes have been attributed to an equilibrium between normal albumin (N) and a new form of albumin (F) of higher mobility. From a combination of viscosity and diffusion data for the acid-treated albumin, it has been concluded that "swelling rather than uncoiling of polypeptide chains" occurs (S13). A curve relating the percentage F formation to pH can be fitted to the equilibrium:

$$N + 3H^+ \rightleftharpoons F + 2H^+ \rightleftharpoons E$$

where E is an expanded molecular form of albumin (F15). The nature of the pH dependence of the solubility curve in the presence of concentrated salt solution indicates that the F form is much less soluble than the normal. In addition to this induced "heterogeneity," a sulfhydryl-disulfide dimerization, also reversible, occurs in the same acid pH zone, at a higher ionic strength (0.1).

On electrophoresis at pH 5.4, the albumin of the domestic fowl separates into three components—fast, slow, and mixed types (M5). It is not known whether these are true isoalbumins with slight differences in amino acid composition or artificially induced forms with realigned intramolecular hydrogen bonding. Some electrophoretic separation of fowl albumins is

seen even at pH 7.3 (M5). Fast-moving albumin has been noted in stored pooled blood-bank serum after conventional electrophoresis (K3).

Recent investigations on the human hemoglobins and various groups of isoenzymes indicate that each group comprises a limited number of proteins which, although closely similar, are nevertheless distinctly different proteins. The atypical albumins seen in "bisalbuminemia" may represent examples of variants (isoproteins) such as are seen in the hemoglobins, but the evidence that a spectrum of "subspecies" exists within the *normal* human albumin "species"—according to the concept of microheterogeneity (C16)—is not convincing.

Some earlier experiments revealing apparent heterogeneity of albumin in neutral solution (S36) were made with impure albumin preparations, as evinced by the finding of differences in the carbohydrate contents of the separated components. Other separations achieved by chromatography on calcium phosphate were either not reproducible (H18), or resulted in subfractions containing polymers (T11). These fractions may be formed during the preparation and purification of albumin. When samples of albumin prepared according to Cohn's procedure were run by conventional paper electrophoresis and then re-electrophoresed in starch gel, several components could be distinguished (S3). However, the same procedures, applied to normal and abnormal sera, revealed only one albumin component (S3). When the rear and the forward fractions of this component in starch gel were further examined, they were found to contain no globulins and to be immunologically identical with the major fraction (R10). "Incomplete" precipitation against antialbumin serum has been reported for late fractions obtained by elution of desalted albumin chromatographed on DEAE-cellulose (R8), but not for fractions separated by agar electrophoresis at pH 8.2 (G17).

Degradation of human albumin by proteolytic enzymes has been studied by Lapresle and colleagues (L1–L3, W13). Trypsin digestion at pH 8 produced two groups of compounds, one of which resembled the nondegraded protein, while the other was of much lower molecular weight and carried only part of the antigenic structure of albumin (L2). Chymotrypsin digestion at pH 4.5 split the molecule into three different immunoprecipitating components. Pepsin gave a similar result, but in this case the three precipitating components differed from those produced by chymotryptic digestion. The action of a spleen proteinase at pH 3.5 was to liberate a smaller fragment (molecular weight about 16,000). Unlike the trypsin-produced unit, it did not precipitate with antialbumin serum, although a positive passive hemagglutination reaction was obtained with the antiserum. An interesting property of this fragment was its ability to inhibit

the specific precipitation of rabbit antiserum to albumin by human albumin (L3). Antiserum to albumin "inhibitor" fragment was prepared in the rabbit, and this agglutinated both "inhibitor"-sensitized red cells and albumin-sensitized red cells, but did not give precipitates with either "inhibitor" or albumin. It was assumed that, for specific precipitation, tervalent bonding was necessary for the formation of a three-dimensional lattice, and that the antibodies to albumin "inhibitor" fragment reacted with only two sites on the whole albumin or on the albumin fragment (W13).

Modification of human albumin by blocking the free NH_2 groups does not affect its reaction with antialbumin serum, but causes the protein to have a higher electrophoretic mobility at pH 8.6 (S46). Blocking of the free COOH groups results in decrease in electrophoretic mobility, increase in dye-binding capacity, and suppression of immunological activity in proportion to the degree of esterification (S46).

3.3. Occurrence of Atypical Albumins in Man

Some earlier experimental data have been interpreted as evidence that a structurally abnormal or altered protein may exist in the blood plasma in certain disease states. Examples of this are the decrease in the arginine/lysine ratio for total serum protein which occurs in famine edema (F11), the decrease in the protective power of the albumin obtained from patients with parenchymal liver disease toward the colloid flocculating activity of γ-globulin (M2, M27), and the increase in the calcium-binding capacity of albumin in the plasma of patients with renal damage and parathyroid hyperplasia (M14). More recent observations also suggest the possibility of qualitative alterations in the plasma proteins. During ill health, the following changes have been reported: a reduction in the number of polarographically active groups which can be released by alkaline denaturation from the plasma proteins (H23, K35), a change in optical rotation, and a difference in the variation of optical rotation with pH of the plasma albumin (J8). In neonates with fetal erythroblastosis, a decrease in bilirubin-binding of albumin has been noted (W5). However, since the preparations of the presumably atypical albumins were not demonstrated to be free from contaminants, these and other observations (B18, J9) may equally well be explained in terms of abnormal binding of lipids, peptides, aminophenols, porphyrins, etc., to the protein. Ott has recently shown that in alkaline solution the molar extinction coefficient of albumin at 285 nm depends on the free fatty acid (FFA)/albumin ratio and has a minimum value for a FFA/albumin molar ratio of 6 (O4).

Acid-alcohol treatment is said to induce in bovine albumin a slight change in the protein's susceptibility to trypsin (R3). Evidence for the existence

of fetal albumin in cord blood rests likewise on its resistance to proteolysis and to alkaline denaturation, which are found to be slightly greater than for normal adult human albumin (M26). As mentioned above, these changes can be explained in terms of absorbed lipids or other impurities, and it must be concluded that the existence of a fetal variant of human albumin has not been proved at the present time. (See also S54.) Albumin produced in health and in hepatitis is immunologically indistinguishable (B32).

Whether the amount of free fatty acid bound to albumin *in vivo* has physiological repercussions remains to be settled. It has been shown by electrophoresis that FFA will displace bilirubin from the albumin of icteric neonatal plasma. But the concentration of FFA (5 mEq/liter) necessary for partial displacement of the pigment was almost 10 times that normally present in blood plasma (N6).

3.3.1. *Isoalbumins and Quasi-Albumins*

Albumin fractions with electrophoretic mobilities differing slightly from normal have been found in the blood of several families of European descent. No clinical abnormalities are associated with this congenital manifestation. The plasma of these individuals contains two demonstrable albumin fractions, neither of which can be distinguished immunologically from normal human albumin (A8, B10, E1, F19, K26, R14, W23, W24). On electrophoresis of serum at pH 8.6, one albumin (albumin A) migrates at the normal rate while another moves either more slowly, as in the cases described by Knedel and others (E1, K26, K27, R14, W23), or more quickly as in the cases described by Nennsteil and Becht and others (N2, T3, W24, W32). The anomalous protein in the earliest descriptions was designated albumin A_2 (K26) or albumin B (E1).

In all these individuals with double albuminemia (bisalbuminemia), the level of total albumin in the plasma is normal, and the two albumin fractions are present in approximately equal concentration, with a slight bias in favor of the normal component, such as might be due to less efficient protein synthesis by an abnormal gene. Trypsin and chymotrypsin digestion of the albumins suggests that, in the molecule of one atypical albumin, a basic amino acid residue, probably lysine, replaces an acid amino acid residue (G4). It seems clear, from the studies of Earle *et al.*, Sarcione, and Tárnoky (E1, S9, T3), that either albumin may, on a supporting medium *in vitro*, be normal in some respects and abnormal in others.

Double albuminemia has recently been described in the blood and ascitic fluid of an 11-month-old infant suffering from ascites of unknown etiology. The plasma concentrations of the two albumins were unequal, that of the presumably normal albumin being 15 g/liter and that of the faster albumin

only 6.1 g/liter; the two albumins were indistinguishable immunologically (V3). After recovery, the electrophoresis pattern was found to be normal. The protein abnormality could not be found in the patient's parents, grandparents, or relatives, so that in this respect also the condition differed from the more commonly described one. It is not possible to decide on the present evidence whether this means that the trait for an atypical albumin is latent in the patient and his forebears.

Of special interest are discoveries of transient atypical albumins which may be observed only in disease. Scheurlen (S14) and Wuhrmann (W32) have described a patient with diabetes mellitus, who during several years showed the double anomaly only when her diabetes was out of control. On the first occasion when it was found, she had acute cystopyelitis. The additional albumin band disappeared after treatment, but appeared again at renewed decompensation. On later occasions the anomaly appeared during precomatose episodes, after which it disappeared again. More lately, however, the double albumin band has become constantly present in the plasma of this patient (W32).

What was thought to be another variant of normal albumin has been reported as a hereditary anomaly. This has been found in 13 relatives over three generations, but in each individual the amount in the plasma was less than 4 g/liter (F20). It had the same electrophoretic mobility as albumin at pH 8.6 on paper, but moved more slowly than albumin in starch gel. The protein has not been examined immunologically (F20), and it is probably present in trace amounts in normal persons (P13). This minor, presumably albumin component disappeared in the presence of mercaptoethanol, so that it may possibly be an albumin dimer.

An abnormal protein which migrates in starch gel with the β-globulins is present in the plasma of some patients with myelomatosis; it can be precipitated by an antialbumin serum. This protein "β-albumin" has been isolated and shown to be a macromolecule composed of albumin, a β-globulin, and a γ-globulin (B1).

The protein moiety of α_1-lipoprotein—"lipalbumin" (U1)—has been shown to be antigenically distinct from albumin (S10).

It must be pointed out that the so-called quasi-albumins described by Poulik and Smithies (P13) are immunologically unrelated to albumin and would be more appropriately named quasi-α_1-globulins. These proteins migrate just behind albumin in the vertical starch-gel electrophoretic technique of Smithies (S34) and include the group-specific (Gc) components. A fetal plasma protein of similar electrophoretic mobility is known (B12, B13), whose carbohydrate content is 23 mg/g, which clearly distinguishes it from the glycoprotein fetuin. Occasionally the protein persists and can be observed in some neonatal blood plasmas.

4. Hypoalbuminemia in Health and Disease

In the absence of dehydration, a raised plasma albumin level is very rare. However, factors other than the protein intake per se can bring about an increase in the serum protein levels above normal averages, even in the presence of mild to moderate protein deficiency (S25). In one tropical community, raised plasma albumin levels in the face of protein deficiency have been reported. This elevation was not the result of dehydration, or of racial differences, but was associated with a high proportion of vegetable protein in the diet (S25). Thus, plasma protein levels may not be a reliable indicator for the detection of mild protein deficiency.

4.1. Hypoalbuminemia and Plasma Volume Changes

Hypoalbuminemia in the adult may be defined as an albumin level of below 35 g/liter plasma. As has previously been pointed out, the practice of expressing albumin and globulin concentrations as percentages of the total plasma protein can be misleading (O7). In serial investigations on individual patients, or in comparing groups of patients (D15), a substantial alteration in the plasma albumin concentration may occur, even though the percentage albumin of total protein is normal. A parallel situation has been quoted by Agneray *et al.* (A2). Their patient had a grossly elevated plasma γ-globulin level. The albumin level as a percentage of total protein was much below that laboratory's normal range, yet the absolute concentration of albumin was in fact a high normal one.

Sick persons commonly have some degree of hypoalbuminemia. The hypoalbuminemia frequently associated with elevated γ-globulin levels in chronic inflammatory states probably results from an increase in the plasma volume (J3). The well-known depression in both plasma albumin and γ-globulin occurring toward the end of the second trimester of pregnancy is usually regarded chiefly as the result of an expansion in plasma volume, but may be due to a primary alteration in metabolism. A suggestion that increased renal protein catabolism contributes to pregnancy hypoalbuminemia (T15) remains unsupported by evidence. Depression in plasma albumin concentration during normal pregnancy fails to occur in some tropical communities (S25). In severe toxemia of pregnancy the plasma albumin level may drop below 30 g/liter and continue to fall. The condition responds to intravenous albumin infusion with significant diuresis, and this treatment is particularly valuable in toxemic subjects resistant to common diuretics (B26a).

In many acute conditions, priority on the part of the blood volume regulation as compared to regulation of albumin metabolism must account

for the low plasma albumin concentration in these states. In severely burned patients, seepage of plasma from the denuded areas is associated with a decrease in both the plasma volume and the albumin concentration of the plasma. Occasionally there is an initial dehydration of the blood, probably due in part to the passage of water from blood to tissues, as may occur when there is no marked external loss of fluid. The transitory rise in albumin is followed by a fall, precipitous at first and then more gradual, until a gradual rise occurs with recovery (P14). In following recovery from the effects of plasma protein loss through hemorrhage, burns, exudates, or intestinal disease, the albumin concentration and the hematocrit, poor though the latter may be as a plasma volume indicator, can yet provide useful clinical criteria.

Relatively more albumin and less β- and γ-globulins exist in the connective tissues than in the plasma (B19). While the amount of albumin in the human skin is not known, the amount present in the skin of the rat, rabbit, and ox is in each case about 30% of that found in the circulating plasma (H27). The corresponding figure for muscle extracellular space albumin is about 25% (G3). The source of the bone matrix protein also may be plasma albumin (A4). A store of albumin therefore exists outside the blood compartment, but few studies have been made on the movement of this albumin to and from the vascular compartment. In normally active working men and women, while there is no variation in the cell plasma distribution in the blood during the day, Whitehead *et al.* have reported that an appreciable increase in plasma albumin (2–8 g/liter; mean 4 g/liter) occurs between the time of rising in the morning and the mid-afternoon (W22). These workers found no concurrent alteration in the total globulin concentration (W22), although observations to the contrary have been reported (A16). The cause of this diurnal variation is not clear, but is at least partly related to the postural change (A16). A seasonal variation may also exist. The mean albumin plus α-globulin (Howe fraction) of 400 adults was found to be lower in the summer months than in the winter (T16).

Persons domiciled at high altitudes tend to have low plasma albumin levels (J2, S25). This may be a result of the lower atmospheric pressure, but it is of interest that a barometric depression equivalent to that found at an altitude of 8 kilometers was required to cause significant hypoalbuminemia in rabbits (P1).

A fall in the plasma albumin level occurs in patients with lesions of the hypothalamic region. With recoverable lesions, the albumin level returns to normal. The mechanism by which these changes take place is unknown (L16).

4.2. Decreased Synthesis of Albumin

The sole plasma protein present in the human fetus of 6-weeks development is albumin. By 12 weeks, traces of γ-globulin, several α-globulins, and a β-globulin have appeared in the fetal blood plasma (T7). On the other hand, the first embryonic proteins to appear in the blood plasma of the rat are γ- and β-globulins; albumin is not detected immunologically until shortly before birth (G25). In the frog, a rapid production of albumin has been shown to coincide with the progress of spontaneous matamorphosis. At this time, the level rises from about 0.07 to 7 g/liter (H14). Because of the much reduced albumin, conclusions drawn from experiments on fetoplacental transport mechanisms in animals may not be directly applicable to the human fetus.

Contrary to earlier indications (D3, K25), plasma albumin levels in normal newborn infants are equal to or higher than the corresponding maternal levels (J6, J12, S40, S48). After birth, the plasma albumin level in the infant falls slightly, and it has been suggested that albumin synthesis may be defective until about the third month of life (J12). No metabolic studies have been made at this stage.

Since albumin is synthesized in the liver (M23), the reduction in albumin found in most forms of liver disease has been attributed to decreased synthesis. The isolated microsome fraction of liver is capable of incorporating amino acids into albumin, but the ribonucleoprotein particles do not seem to be the site of protein synthesis (D6). A reduction in albumin synthesis may be one result of a shortage of ATP (adenosine triphosphate) (K34).

4.2.1. *Analbuminemia*

Patients of all age groups with idiopathic hypoalbuminemia have been investigated by Gordon and colleagues (G13). These investigators attempted to classify their cases on the basis of clinical and laboratory findings. A hypercatabolic group consisted of patients with edema, normal erythrocyte sedimentation rate (ESR) and normal cholesterol levels, low plasma globulin levels, and a rapid rate of albumin degradation or loss. Characteristic of another type of idiopathic hypoalbuminemia was a failure of albumin synthesis, hypercholesterolemia, raised ESR, and a minimum or absent edema. It seems certain that at least two of their patients with the latter type of idiopathic hypoalbuminemia were subjects with recessive hereditary analbuminemia, a congenital abnormality described by Bennhold *et al.* (B11). In investigating these cases, the small amount of "albumin" found soluble in 1.5 *M* sodium sulfate would in fact be largely globulin.

Hereditary analbuminemia was first reported in a female farm worker who regularly suffered, prior to menstruation, from slight edema of the ankles. Her brother had the same protein abnormality. No other member of the family was affected but the parents were second cousins, which suggested that a rare recessive gene was involved (B11). As compensation for the effects of a slightly decreased plasma oncotic pressure, the renal plasma flow was found to be reduced below normal and the tubular reabsorption of water and sodium slightly decreased (B9). In the absence of the normal carrier, disappearance of intravenously administered Congo red was more rapid than normal. However the rate of disappearance of intravenously administered I^{131}-labeled albumin (O3) was no greater than normal, which indicates that increased albumin catabolism was not involved (F22). Two other individuals with analbuminemia have been noted in the literature (B5, S29). Occurrence of this condition in a patient with rheumatoid arthritis appears to be a chance association (S29).

Although traces of the relevant components are found in congenital protein deficiencies, it seems appropriate to retain the term analbuminemia for this unexpected abnormality of synthesis. Various figures (from nil to 0.8 mg/ml) for the trace levels of albumin determined immunochemically have been given (L15, O3, S30). Whether the albumin in these individuals is of the normal human variety is unknown, but sufficient of the antigenic part of the usual structure exists intact *in vivo* to permit infused albumin to be "recognized" as a normal body constituent. Owing to a lack of clinical consequences the analbuminic state may be commoner than supposed.

It is not known how much albumin enters the fetal from the maternal circulation (A1), or to what extent albumin-transported materials are important to fetal growth and homeostasis. One biologically important albumin-bound peptide is the "synalbumin" antagonist to insulin. Increased insulin antagonist is present in the plasma of diabetic and prediabetic patients (V1) and its passage into the fetus might explain the pancreatic islet hypertrophy of the stillborn infant of a prediabetic mother.

4.3. Increased Catabolism and Loss of Albumin

Severe deficiency of dietary protein produces a marked lowering of plasma albumin, and this is usually considered to occur as a result of depressed synthesis due to the deprivation of essential amino acids. A marked hypoalbuminemia in the rat and dog (F7) and in the cat (G24) has resulted from experimentally induced chronic choline deficiency. The effect was not reversed by a dietary supplement of methionine, but was reversed by choline supplementation. This situation bore no direct relation to the fatty

infiltration of the liver, which took 2 months to clear up after choline replacement (G24).

The conclusion drawn from I^{131}-albumin studies, that there was no increase of albumin catabolism in ill-nourished subjects, is now considered to be in error (P9). Calculation of the rate of albumin breakdown from the slope of the plasma decay curve after an intravenous injection of I^{131}-labeled albumin is now thought to be inadequate, because no allowance was previously made for the progressive dilution of the labeled albumin by albumin newly formed during the early stages of treatment of malnutrition (P9). Some selective breakdown of denatured labeled albumin has also been demonstrated to occur immediately following injection of I^{131}-albumin (M1). Picou and Waterlow (P9) demonstrated that the rapid return to normal of the plasma albumin level, which occurs in ill-nourished subjects in response to nutrition, is mainly the result of decreased catabolism rather than increased synthesis. They are of the opinion that increased catabolism contributes substantially to the lowering of the plasma albumin level in severe malnutrition.

However, Hoffenberg *et al.* (H21) have demonstrated that decreased, not increased, albumin catabolism occurs after short periods of protein deprivation in man. Volunteers given a low protein diet for several weeks (H21) or kept on a semistarvation diet for 6 weeks (K9), suffered a negligible change in plasma albumin levels and total circulating albumin. Of more interest was the observation that these individuals undergoing protein restriction exhibited an increase in the half-life of plasma albumin and a decrease in the size of the extravascular pool of albumin, and that these responses to protein deprivation commenced after the loss of only 50–100 g body nitrogen (H21). A homeostatic mechanism, therefore, controls the rate of albumin breakdown. The size of the albumin pool appears to be strictly limited, as is indicated by both animal experiments and human surveys which show that a high protein diet provides no safety margin for situations of stress or deprivation (H22). In the face of a high protein diet, both synthesis and catabolism of albumin are increased (S45).

MacFarlane has made the suggestion that albumin and probably other proteins may be diffusely catabolized in the endothelium all over the body (M1). The bulk of the "catabolism" of albumin probably occurs via the gastrointestinal tract, being lost as protein to the body. This has been demonstrated for rabbits given trypsin inhibitor orally (T4). The amount of albumin lost, in normal man, by way of the intestine is still in dispute (F21), although it seems likely that a figure of 4–6 g per day—more than half the amount of albumin synthesized daily—will prove correct (J4, T4, W19, W20). Using an intravenous I^{131}-labeled albumin technique in experi-

mental animals, Wetterfors found the normal protein leakage from the various parts of the intestine to be correlated with the extent of the blood flow to that part (W19).

The low plasma albumin levels found in ulcerative colitis and regional enteritis may be ascribed to loss of albumin from the wall of the intestine (S47). In cancer of the stomach, there are both an abnormal leak and a pathological distribution of albumin (B16). Total body irradiation of rabbits by a medical cobalt-60 γ-irradiation source resulted in excessive loss of albumin escaping into the intestinal lumen. Excessive albumin leakage has been considered the chief mechanism underlying the shock which characterizes the acute radiation injury complex (B16). In the condition of protein-losing gastroenteropathy, large amounts of protein (20–30 g) are excreted into the gut daily. A persistent loss of albumin into the sputum of patients with chronic bronchitis has recently been reported (B19a).

The extreme hypoalbuminemia of the nephrotic syndrome can be attributed to the sustained urinary loss of albumin (S39), but it is doubtful whether albumin deficiency plays a causal role in the hyperlipemia and hypercholesterolemia found in this condition. Low-density lipoprotein (LDL) (90% lipids) is converted by lipoprotein lipase *in vivo* to high-density lipoprotein (HDL) (70% lipids), and the liberated fatty acid anions are bound and transported by plasma albumin. In normal nonlipemic serum the mean nonesterified fatty acid/albumin molar ratio is 0.95 ± 0.04, while in "nephrotic" sera—lipemic because of accumulation of LDL-triglycerides—the corresponding ratio is about 3 (C4). The plasma hyperlipemia which is observed in the nephrotic syndrome, and can be induced in rats by injections of antikidney serum, has been considered to result from albumin deficiency (R26). However this suggestion is not borne out by more recent studies (R25) and is contradicted by the failure of LDL to accumulate in the blood of analbuminic subjects (O3). Plasma lipemia in the nephrotic syndrome apparently is due to loss or inhibition of lipoprotein lipase activity.

A new physical sign, the appearance of paired narrow white bands running parallel to the lunule in the finger nails, has been described in patients with persistently low plasma albumin levels. The bands appear in a variety of disease states, and following albumin infusions become less marked and disappear (M30). They are not found in healthy subjects or in patients with albumin levels above 22 g/liter.

5. "Total Globulin" Fraction of Blood

The average composition of the nonalbumin fraction of blood serum is shown in Table 2. It will be seen that, if we abide by the original definition

of globulin (L10) on grounds of insolubility in 50% saturated ammonium sulfate solution, the normal blood serum proteins are made up of approximately 40 g albumin, 30 g globulin, and 10 g of other proteins per liter. It is clear, too, from the multiple nature of the nonalbumin fraction, that the

TABLE 2
MAIN COMPONENTS OF NORMAL "TOTAL GLOBULIN" OF HUMAN BLOOD SERUM

Glycoprotein	Approximate concentration (mg/100 ml)	Protein content (%)	Precipitation in 2.05 M $(NH_4)_2SO_4$	Reference
γ-Globulins 7 S	1100	97	+	(S38)
γ-Globulins 19 S	20	90	+	(W3)
$\gamma_{1A}(\beta_{2A})$-Globulin 7 S	150	91	+	(B33, H11)
$\gamma_{1M}(\beta_{2M})$-Macroglobulin 19 S		—	+	(B33)
Properdin	50	—	+	(W15)
Transferrin	400	94	+	(S23)
Prothrombin	15	89	+	(S20)
Ceruloplasmin	30	93	+	(S20)
Plasminogen	20	99	?	(S31)
Haptoglobin I	100	77	−	(R15)
Haptoglobin II			+	(H13)
Hemopexin	100	—	+	(N7, H6a)
α_2-Macroglobulin 19 S	240	92	+	(B29, S19)
α_2-Glycoproteins 2.4 S	100	84	−	(S17)
α_1-Trypsin inhibitor	290	69	−	(B30, S50)
Orosomucoid	75	66	−	(S16, S32)
α_1-Glycoprotein 3.5 S	30	86	—	(S21)
"Pre-albumin"	25	99	—	(A7, S22)

Lipoprotein	S_f in NaCl (1.063 g/ml)	Approximate concentration (mg/100 ml)	Protein content (%)	Precipitation in 2.05 M $(NH_4)_2SO_4$	Reference
LDL (β)	20–100	260	10	+	(D1, L12)
LDL (β)	4–20	360	20	+	(S23)
HDL_1 (β)	0–4	20	30	+	(S23)
HDL_2 (α_2)	—	60	35	+	(D1, L12)
HDL_3 (α_1)	—	180	60	—	(S10)

concept of serum globulin as a clinical entity can no longer justifiably be held. Likewise, the use of the A/G ratio as a clinical index (E5) is theoretically unnecessary and in practice of low precision and accuracy (W6).

Since 1905 when Hardy (H4) first observed separation of serum proteins by electrophoresis, there have been repeated suggestions that the plasma proteins are not independent components *in vivo;* that there exist molecules

of an albumin-globulin complex or "combined protein" which are in equilibrium with molecules of free albumin and free globulin (H5), or that the individual proteins are part of a giant molecule (orosin) which can be fragmented in a manner dependent in part on the separative process (B17). This concept received some support from the finding of a consistent relationship between several pairs of amino acids in each of several salt-fractionated plasma proteins (B17). However, some methods now available for the specific determination of albumin in the blood of healthy adult subjects yield the same result, irrespective of the nature of the separative techniques (W10). Plasma albumin seems therefore not to be an integral part of the "macromolecule orosin." A better case could be made out for the "orosin" nature of some of the non-γ-type globulins of plasma, since there is a fair positive correlation between the electrophoretically separated α_1-, α_2-, and β-globulins in normal individuals (B21, B24).

Estimation of globulins subdivided by paper electrophoresis into the various α, β, and γ types is of very limited value in clinical medicine (O7). Furthermore, information obtained by it can usually be obtained with greater ease and accuracy by simple chemical and clinical tests (O7). Sera from patients that might be expected to have abnormal proteins give no characteristic patterns that can be distinguished from normal controls, even when starch-gel electrophoresis is used (B6).

It has been suggested that a statistical measure of the electrophoretic lack of homogeneity ("entropy") of the serum globulins might allow pathological processes to be further differentiated (H10). This mathematical device seems unlikely to be of real value, because paper electrophoresis is an arbitary process dependent chiefly on one physical parameter, namely, molecular charge.

Subfractionation of globulins by immunochemical methods (H3) can be confidently expected to provide more useful and significant information about the course of a disease than previously has been available. Newer chemical methods for individual groups of plasma proteins are also being usefully applied to diagnostic and prognostic problems (K13). Intraglobulin fractional analysis, according to Greenspan (G22, G23), compares favorably with other established chemical aids in the differential diagnosis of jaundice. The analyses carried out were for seromucoid, acid-precipitable globulins, zinc sulfate turbidity, and total protein-bound hexose.

One effect of heating serum at 56°C for 30 minutes—as is done to inactivate complement—is to cause a component which normally moves electrophoretically as a fast α-globulin to migrate as a slow α-globulin. Such heating has no effect on the determination of albumin or total globulins (A11, H19) and the erroneous use of 1.5 M Na_2SO_4 for albumin frac-

tionation probably accounts for earlier reports of appreciable changes in the A/G ratio after heating serum at 56°C (C2, K28). Another effect of this treatment is to decrease the amount of globulin which can be precipitated at a given pH by certain acid anions, such as phosphotungstate and vanadate (C15). In the case of preheated serum from patients with rheumatoid arthritis, the amount of vanadate-induced precipitate is abnormally decreased to an extent which can be correlated with the patient's degree of resilience, and is said to give a measure of "tissue reactivity." Rheumatoid "disease activity" may be assessed from the pH at which an orthovanadic acid solution produces a given amount of insoluble globulin. Alteration in this pH provides an index claimed to be "more stable and less sensitive than the ESR and therefore of greater clinical significance" (C15).

6. Methods for Determining Albumin and "Total Globulin"

Described in this section are different ways of determining albumin previously isolated (Sections 2.1 to 2.5) from not more than 200 μl whole plasma. Newer direct procedures for albumin and globulins which do not require prior separation of the proteins are also given.

While it cannot be denied that the soundest methods for the estimation of pure albumin in aqueous solution are likely to be those based on elementary composition (i.e., carbon or nitrogen) or measurement of peptide bonds (e.g., far-ultraviolet spectrophotometry) the oft-repeated statement that the Kjeldahl nitrogen method for determining plasma proteins is theoretically more sound than others is untrue. This method suffers no less than others from the disadvantage that the individual plasma proteins possess different nitrogen contents (Table 3).

While erythrocytes have been shown not to absorb albumin from plasma (B28), washed blood platelets absorb and firmly bind radioactive albumin (S5). Although human blood platelets absorb albumin and fibrinogen, specifically from among the plasma proteins, the amount of albumin precipitated on centrifuging platelet-rich plasma is extremely small (B15).

Standardization of albumin analysis can be carried out with either human or bovine albumin. Each is commercially available in a degree of purity approaching that of several other organic substances used as reference standards by clinical chemists. Unlike the globulins, albumin does not absorb appreciable amounts of water vapor on normal exposure to air (A13). Current batches of crystalline human albumin (Light) contain no carbohydrate, less globulin than 15 mg/g, ash 3 mg/g, moisture 15–35 mg/g, and fatty acids 4–8 mg/g. Allowance may be made for moisture content. Absorbed fatty acid ions can be removed by extraction with acetic acid in iso-octane (G9), but this is usually not necessary and will decrease the life

TABLE 3
NITROGEN CONTENT OF HUMAN PLASMA ALBUMIN AND "GLOBULINS"

Protein	Nitrogen (g/100 g protein)	Reference
Albumin preparation		
Fraction soluble in 3 M $(NH_4)_2SO_4$	15.5	(G8)
Fraction soluble in 22% Na_2SO_4	15.4	(C18)
Cohn V fraction	16.0	(A13)
Crystallized from Cohn V fraction	15.95	(B25)
Recrystallized several times	15.7	(K8)
By paper curtain electrophoresis	15.3	(S57)
By agar electrophoresis	14.91	(S52)
Isolated plasma proteins		
α_1-Lipoprotein	8.0	(A13)
β-Lipoprotein	4.2	(A13)
γ-Globulin	15.2, 15.4, 16.0, 16.1	(A13, B25, S53, S57)
Mixed preparations of "globulins"		
Crude Howe precipitate	14.4	(C18)
α_1- + α_2-Globulins	14.3, 15.0, 15.1	(A13, S53, S57)
Mainly γ-globulins	14.7, 14.8, 15.2, 16.3	(A13, B25, S53, S57)

of the preparation in solution, since fatty acid ions tend to stabilize the protein against denaturation.

Since the most reliable protein nitrogen analysis (Table 3) gives a value of 157 mg N/g for albumin, conversion of N to albumin requires the factor 6.4. Convenient corresponding factors for N to "globulin" and total protein are 6.5 and 6.45, respectively.

6.1. NITROGEN DETERMINATION

The digestion method devised by Kjeldahl in 1883 at the Carlsberg Laboratory, Copenhagen—and modified 2 years later by the introduction of a metal catalyst—remains to-day the sole means of converting protein N to NH_4^+. Numerous modifications have been proposed to shorten the period of boiling and to ensure complete degradation of organic N. Hydrogen peroxide can be used as an oxidizing agent without causing loss of ammonia.

One "micro-Kjeldahl" method (10–20 mg protein) requires a minimum heating time of 4 hours with HgO as catalyst (S57), while another (J1)—carried out in a sealed tube at 460–480°C for 30 minutes without a catalyst —has been proposed. An ultramicro method (0.02–0.1 μg N) can be completed in 1 hour using Se as catalyst (E6).

After conversion of organic N to NH_4^+, steam distillation of NH_3 fol-

lowed by titration with 0.01 *M* HCl remains the method of choice for quantities down to 20 μg N. The ammonia is conveniently trapped in 1% solution of boric acid (S35) containing the indicator devised by Sher (S28). Correction must be made for any N present in the sulfuric acid, oxidant, and catalyst.

Successful attempts have been made to replace titration by direct colorimetric determination of ammonia. Berthelot's reaction has been carried out on the neutralized Kjeldahl digest (E6, L19), but interference by H_2O_2 and by Ca^{++} can occur (R13). The Berthelot reaction has been speeded up by the introduction of a catalyst, sodium nitroprusside (L19); it must be carried out at pH 10–12 (C5) under which conditions it is specific for NH_4^+ (W12). The reaction between NH_4^+ and indanetrione hydrate may also be used; the digest is neutralized, buffered at pH 5.0, heated with the reagent at 110°C for 30 minutes, diluted with 50% ethanol, and the color measured at 570 nm (J1).

Urea (W16) and tris(hydroxymethyl)aminomethane (R18) have been recommended as convenient stable primary N standards for protein N analysis.

6.2. Ultraviolet Absorption Spectrophotometry

Spectrophotometry in the region 270–290 nm has frequently been recommended as a method of choice for determining the concentration of an individual protein in solution; it has been less extensively employed for estimation of total protein in mixtures such as serum and cerebrospinal fluid (CSF). For the former purpose this is a reliable practice, provided allowance is made for contamination by extraneous substances which may also have appreciable ultraviolet absorption (O4). At 280 nm, the specific absorptions of the various normal plasma globulin and fibrinogen fractions vary only a little among themselves, yet each is almost 3 times the absorption of the albumin fraction (L5). This is due to the low content in albumin of those aromatic amino acids responsible for the absorption. The method is therefore unreliable for the determination of serum protein of unknown albumin concentration. The albumin content of suitably diluted serum can be calculated from the absorption at 280 nm after the total serum protein concentration has been determined by another method (L6, S33). An average allowance has to be made for normally occurring nonprotein material absorbing at 280 nm, such as uric acid and bilirubin. Unfortunately, the molar extinction coefficient of albumin decreases as the FFA/protein ratio increases (O4). In addition, too many sera containing interfering substances are encountered in clinical practice for the success of this approach.

Although it has generally been supposed that the maximum absorption

of protein solutions will occur in the region of 280 nm (E4, R29), several workers using improved spectrophotometric instruments have shown that this is not the case. Simple peptides have absorption maxima about 190 nm, and Goldfarb *et al.* (G7), studying a series of proteins in the far-ultraviolet region, concluded that the peptide bonds contribute about 70% of the total absorptivity of the protein at 205 nm. At this wavelength, too, the specific absorption of most proteins is about 30 times their value at 280 nm. Spectrophotometry in the far-ultraviolet range therefore provides a sensitive means of determining plasma or serum proteins. Down to 1 μg/ml of protein can be measured. Because of instrumental limitations and other reasons, wavelengths higher than those needed for peak absorption have been recommended in various techniques for serum protein determination. Waddell (W1) chose to use the slope of the absorption curve between 215 and 225 nm, rather than a single wavelength, in order to minimize any error arising from nonprotein absorption. However, the optical densities of protein-free filtrates of sera prepared by the zinc hydroxide precipitation method are very low, approximately 0.5% of that of the original serum (T14). An unconfirmed report (B7) suggests that this method may give too high values with myelomatosis serum.

Over the range of serum protein levels usually encountered, the absorption of a 1:1000 dilution obeys Beer's law both at 225 nm (W1), and at 210 nm (T14). Neither NaCl nor ammonium sulfate interferes. Direct measurement at a single low wavelength (T14) seems to be a satisfactory procedure for determining either serum protein or separated albumin (T14, W8). The method is applicable to electrophoretically separated albumin and globulins (M31), and to albumin in acid-alcohol mixtures, when suitable blanks are included (W8). With appropriate blanks, concentrations of acetate, citrate, succinate, phthalate, and barbiturate up to 0.005 M can be tolerated. Absorption in the far-ultraviolet is unaffected by pH in the range pH 4–8. Outside this range, an altered state of ionization may result in a new molecular form of protein with different spectroscopic characteristics (see Rosenheck and Doty, R24).

6.3. Reactions of Characteristic Molecular Groupings

The condensation reaction between the terminal asparagine of albumin and reduced indanetrione hydrate provides a very sensitive means of determining this protein. Possibly the ϵ-amino groups of lysine react too, and these give about 10% more color than a number of other amino acids (Y3). Yemm and Cocking have described an improved method for determining primary amino groups (Y3); this can be applied to soluble protein in the absence of ammonia and free amino acids, and to the determination

of NH_2 in antigen-antibody precipitates (K36, M4). The method will detect 1–10 μg NH_2 N in a 0.1-ml sample.

The nature of the particular molecular groupings which combine in a specific manner with certain azo dyes or combine with specific antibody is unknown. Methods based on these reactions are discussed in Sections 6.4 and 2.3, respectively.

6.3.1. *Reaction with Alkaline Copper Solution*

The reaction which occurs when copper sulfate solution is added to protein dissolved in alkali has been used for quantitative analysis for more than 50 years (R12). According to Mehl *et al.* (M17), the blue-violet product is a copper complex in which each Cu atom is bound to four peptide N atoms; it has an E_{max} at 552 nm.

The "one-piece" alkaline copper "biuret" reagent was introduced by Kingsley (K21) and enables the reaction to be carried out without the precipitation of cupric hydroxide. At least 12 "improved" reagents of this type but of different composition have been proposed.

The early biuret reagents, which have limited stability, yield a final alkali concentration of 1.5–3.0 *N*, and a final $CuSO_4 \cdot 5H_2O$ concentration of about 1.7 g/liter or 0.007 *M* (K17, K21, M16). Their use with normal sera frequently gives rise to turbidity, which usually cannot be extracted with ether (K21, W16). The same objection applies to reagents containing ethylene glycol (M16) or citrate (Z1). Keyser and Vaughn (K15) circumvented the difficulty by measuring the residual absorbance after the biuret color had been destroyed with potassium cyanide. However, the incorporation of tartrate into the reagent, as suggested by Weichselbaum (W16), greatly reduces the incidence of turbidity, possibly owing to delayed formation of an insoluble phospholipoproteinate. Tartrate also produces a reagent that is stable indefinitely if stored in an alkali-resistant container.

Alkaline copper reagents containing potassium sodium tartrate can be considered in two groups. Those of the first type yield in the reaction mixture an alkali concentration of 0.1–0.2 *N* and a copper sulfate concentration of 2.0–2.5 g/liter or 0.008–0.01 *M* (R7, W16, W26). Those of the second type provide a higher final alkali concentration, 0.5–1.0 *N*, and a $CuSO_4 \cdot 5H_2O$ concentration of 1.0–1.2 g/liter or 0.004–0.005 *M* (G15, L20, R4). With either class of reagent, identical amounts of biuret compound are produced from albumin, but the reagent devised by Gornall *et al.* (G15) is recommended. In favor of this low-copper reagent is the lower blank absorbance which allows higher accuracy in low-protein assays. Color production obeys Beer's law for protein concentration up to 1 mg/ml of the reaction mixture.

The earlier alkaline copper tartrate reagents required the addition of potassium iodide to prevent their slow autoreduction. However, if the constituents are of analytical quality, tartrate-biuret reagents are stable indefinitely, and potassium iodide can safely be omitted.

The use of Benedict's qualitative reagent and NaOH solution has been suggested for protein estimation (G6, H9, H28). This practice is not recommended as opalescence may occur during an estimation, although it has been claimed that this opalescence can be removed by ether extraction (H9). Another biuret reagent contains a high concentration of ammonium hydroxide (L9), but is inconvenient to work with and has lower sensitivity to protein.

The presence of ammonium *ions* in the alkaline copper increases the blank color, but decreases the blue-violet color produced from protein. This effect occurs at concentrations down to 0.05 *M* (S41), so that albumin in ammonium sulfate solutions must first be isolated before the quantitative biuret reaction can be applied. When present in higher concentrations, sodium sulfate and most other salts used for fractionating protein solutions intensify the biuret color (R27). However, the absorbance of the blank alkaline copper solutions containing no protein is also affected to the same extent, making it unnecessary to isolate the soluble protein before applying the biuret reaction.

The presence in blood of sodium oxalate or sodium fluoride at 200 mg/ml, or of sodium EDTA (ethylenediaminetetraacetic acid) at 50 mg/ml, has no effect on protein determination with the Gornall type of reagent, but higher concentrations of EDTA or its salts will interfere. Turbidity or foreign color may arise from excessive hemolysis, severe icterus, or drugs such as Stelazine (trifluoperazine), which makes it necessary to watch for turbidity when a manual biuret method is applied to abnormal sera. A serum control with a blank reagent containing no copper sulfate has been recommended (W9). Nonprotein biuret-reacting compounds probably do not occur in blood plasma at sufficiently high levels to be detected by the technique under discussion. Biuret as a secondary standard is not satisfactory (R4).

Another approach to the determination of albumin is that based on its copper-binding capacity. Protein-complexed copper may be determined colorimetrically with oxalyldihydrazine and acetaldehyde (K6) after removing excess copper as cupric hydroxide, or with diethyldithiocarbamate after removing excess copper as insoluble phosphate (N1) or on an ion-exchange resin (W18). The latter is the better method, although special care must be observed in the preparation of the anion exchanger Dowex 1 suspension and in the mixing of it with the copper-protein solution, so as to ensure adequate contact between resin beads and solution. The procedure

of Westley and Lambeth (W18) has a sensitivity approaching that of the copper-modified Wu procedure (L18). It may be increased further by using a more sensitive reagent for copper, such as 1,5-diphenylcarbohydrazide (M21).

In the above methods, the separated albumin (or whole serum if total protein is to be determined) is reacted with excess copper in approximately 1 *N* alkali. A macromethod is also available which utilizes the capacity of albumin to react with copper(II) in a mole ratio of 1:1 (K32). This is an amperometric titration of blood serum (1 ml) in 0.1 *M* ammoniacal ammonium nitrate pH 9.2 with $CuSO_4$ (4.8×10^{-2} *M*). Under these conditions human γ-globulin did not react, so that the fair agreement by this method with results obtained by a biuret method after sulfite fractionation (K32) is probably the result of a balance of errors. In view of the interest in the small fraction of albumin-bound copper in hepatolenticular degeneration, and the paucity of information on the unique binding reaction presumably with N-terminal aspartyl residues, further investigation would be valuable.

Continuous microdetermination of protein with a Sephadex-Cu^{64} detection column has been described (G19). Radioactive copper is added to the plasma and the free Cu^{64} and the protein-bound isotope are separated at an alkaline pH by filtration through Sephadex. The free copper complexes with the hydrophilic gel and remains in the column. As little as 0.13 μg albumin in a volume of 1 ml may be determined. The main drawback of the method is the short life of the Cu^{64} isotope.

6.3.2. *Phosphotungsto-Phosphomolybdate Reduction*

The blue color reaction which occurs when urates or phenols are added to mixtures of phosphomolybdic acid, phosphoric acid, and tungstate was first described by Folin and Denis in 1912 (F13, F14). The active component of the phenol reagent is a mixed complex of the series $(P_2O_5)_x(WO_3)_y$ where $x + y = 18$ (W29). Wu improved the reagent for detecting phenols by the addition of HCl and bromine, thereby giving it higher sensitivity and lower blank color (W29). The blue color reaction product has the same spectrum (λ_{max}: 745 nm; λ_{min}: 405 nm) for all types of phenols, amino acids, etc. Addition of lithium sulfate to the reagent is said to permit the use of a higher proportion of reagent in an alkaline medium (F12).

Wu (W30) discovered that the "phenol reagent" could be used to determine serum protein by adding it directly to serum diluted with alkali and comparing the color produced with that obtained from a standard tyrosine solution similarly treated. He determined the ratio by weight of protein to tyrosine and found it to be higher for albumin than for "globulin" (W30). In spite of claims to the contrary (A9, G21, M25), this difference

in reactivity with phosphotungsto-molybdate between albumin and globulins has been confirmed (C1, T17). For this reason, Wu's method of plasma protein assay fell into disuse (S7).

Herriot observed that pretreatment of protein with alkaline copper solution subsequently results in a more sensitive reaction with the phosphotungsto-molybdate reagent (H15). Lowry and his colleagues (D4, L18) have systematized the Cu-modified Wu procedure, and established for protein estimation a satisfactory ultramicro technique which has not been improved upon or modified (R11). With this procedure, bovine albumin on a molar basis produces 105 times as much color as tyrosine (C6, L18). Color yield is probably the resultant of two simultaneous processes: a copper-catalyzed removal of electrons from chromogenic groupings in the protein to the phosphotungsto-molybdate, and a rapid destruction of the electron acceptor in the alkaline solution (C6). Maximum reduction of the reagent occurs between pH 9 and 10 (F14, L18). Since phosphate-molybdate dissociation at this pH gives the reagent a half life of only 8 seconds (L18), it is imperative to add the reagent rapidly and to immediately mix the solution. Although tripeptides and tetrapeptides are highly chromogenic, large color increments do not seem to be associated with any special or restricted set of amino acid sequences (C6). In the case of albumin, a 5-fold increase in color (C6, L18) is given by the reaction as a result of its pretreatment with

TABLE 4

CHROMOGENICITY OF SOME PLASMA PROTEINS TOWARD THE ALKALINE COPPER REAGENT OF GORNALL AND THE ALKALINE COPPER PHOSPHOTUNGSTO-PHOSPHOMOLYBDATE PROCEDURE OF LOWRY

Protein	Relative color per gram dried protein[a]	
	Biuret	Cu-phosphotungstomolybdate
Human albumin	100	100
Bovine albumin	100	100
Human γ-globulin	96; 99 (G15)	123
Bovine γ-globulin	105	130
Equine γ-globulin	98	116
Human Cohn IV-5	—	80
Human Cohn IV-6	—	91
Human Cohn IV-7	—	83
Equine α-globulins	79	85
Human α_1-glycoprotein	52	55
Normal human pooled serum	98 (L18); 100 (G15)	119; 103 (L18)

[a] Calculated from unpublished data of Watson and Farrance (1964) and from absorbancies quoted by other workers (G15, L18).

alkaline copper. The chromogenicity of some plasma proteins in the Cu-modified Wu procedure is shown in Table 4, from which it will be seen that on a weight basis γ-globulin produces 23% more color than albumin.

6.4. Albumin Determination with Dyes and Organic Indicators

The first useful application of the "protein error of indicators" arose from the early studies of Klotz (K22, K23). In the presence of low concentrations of bovine albumin, but not of γ-globulin or gelatin, the absorption spectra of a number of different dye solutions have been found to undergo pronounced shifts. Albumin-binding data and absorption maxima of many anionic azo dyes have been listed (M3). Three of these dyes have been utilized for protein estimation.

When added to methyl orange solution buffered at pH 3.5, albumin binds and effectively removes the pink anion, so that a decrease in absorbance at 550 nm provides a measure of protein content (B20). Modifications of the original method of Bracken and Klotz have improved its accuracy by the inclusion of a serum blank (K10), and have increased its sensitivity by doubling the dye ionic strength (K12). Studies on the specificity of the method (B20, W10) and most comparative studies (G12, K14) suggest there is little if any methyl orange binding to γ-globulin. However, β-lipoprotein-dye interaction is a source of error, since it has been shown that methyl orange is bound equally strongly to β-lipoprotein and to albumin (R22). The method is remarkably free from interference by dye-binding competitors. Hemoglobin (100 mg/100 ml) is without effect; a concentration of bilirubin of 20 mg/100 ml causes an error of less than 5% (F2). No interference is encountered from heparin at the normal anticoagulant levels (0.1 mg/ml blood) (K11), but 0.2 mg/ml blood plasma causes underestimation by about 7% (W10). A salicylate concentration of 60 mg/100 ml plasma is without effect on methyl orange binding of albumin (W10).

A polarographic method of measuring the methyl red-binding capacity of serum proteins has been described, which reveals abnormalities in patients with impaired renal function (B27). The free methyl red anion has a much larger diffusion coefficient than the methyl red-albumin complex. The two can therefore be separated polarographically, the concentration of the free dye anion measured, and the amount of protein-bound dye calculated. The decrease in the methyl red-binding capacity found in the serum of patients with various kidney diseases was thought to be due to the presence of increased amounts of phenolic acids, which compete at pH 7.3 with methyl red (10^{-4} M) for protein binding sites (B27, R2). However, a graphic plot of the values obtained by Breyer and Radcliffe (B27) reveals a fair correlation between the methyl red-binding capacity and the "albu-

min" concentration of their sera. Exact data on the extent of binding of methyl red by serum globulins are not available.

2-(4'-Hydroxybenzeneazo)benzoic acid (HABA) buffered at pH 6.2 shows an increase in absorbancy at 520 nm, which is a linear function of the albumin concentration within a limited range. However the methods devised with this indicator are unsatisfactory for several reasons (W10). It has a low sensitivity (R32) not appreciably improved by using a lower pH or making the optical measurements at 480 nm (W27). Decreased dye binding of albumin occurs in the case of sera from patients who have taken salicylate (N4). Low concentrations of bile pigments similarly interfere (G12). The method is inconvenient to use since the HABA-albumin interaction is thermolabile (G12). Furthermore, it is not applicable to plasma containing heparin in the usual anticoagulant amounts (N5); heparin gives rise to a turbidity which has been ascribed to a reaction between it and β-lipoprotein (M12).

The closely related dyes Evans blue (T 1824), trypan blue (T 1936), and Congo red show bathychromic shifts in their spectra in the presence of albumin (B26). Of the three dyes, only the former has no affinity for globulins at neutral pH.

The binding of several phthalein dyes to the plasma proteins has been studied by various techniques. The binding of bromphenol blue (BPB; tetrabromophenolsulfophthalein) in acid solution to protein results in the well-known spectral shift (W2) from yellow (600 nm) to a blue-green color (440 nm), depending on the dye/protein ratio and the particular protein. The binding strength of one of two binding sites on the albumin molecule greatly exceeds that of any γ-globulin binding site (S15). Useful differentiation of proteins in urines with BPB has been demonstrated (W7), but no BPB procedure for the clinical determination of albumin or individual globulins has been reported.

Bromcresol green (BCG) is also firmly bound to albumin. The difference spectrum of buffered BCG with and without added albumin shows a narrow peak at 615 nm (B3). Under suitable conditions BCG offers the most sensitive means of determining albumin by a dye-binding method. In addition, measurements at the red end of the visible spectrum suffer minimum interference by bilirubin and the blood pigments (B3, R19).

Phenolsulfophthalein (PSP) binding to plasma albumin has been studied by equilibrium dialysis. Huggins *et al.* (H26) equilibrated against water a mixture of serum and PSP contained in cellulose casing, and determined the PSP bound in μg/ml serum. With this method, it was shown that 45–55 μg PSP was bound per 10 mg "albumin" in the plasma of normal adults (H26, M22), but that the plasma of women of more than 24 weeks

of gestation was deficient in ability to bind PSP (M22). This decreased binding of PSP to albumin was thought to indicate a structural defect in the albumin molecule. Equilibrium dialysis with PSP at pH 7.4 and the Howe salting-out procedure were employed by Blondheim (B18), who found a mean critical plasma "albumin" level of 15 g/liter, below which there was no appreciable binding of PSP, and above which the extent of the same dye binding was proportional to the concentration of excess of "albumin." This was explained by supposing that the avidity of some naturally occurring substances for some sites exceeded and therefore blocked the binding of PSP. There can be no doubt that several organic sulfonates can compete successfully with PSP for albumin binding. However, in 0.05 M phosphate buffer at pH 7.4, the concentration of naphthalenesulfonate required to inhibit the binding of 3×10^{-5} M PSP to albumin (5×10^{-5} M) by 5% would be approximately 1 M (R17). If there are endogenous substances with binding constants of the same order as naphthalenesulfonate, it seems inconceivable that their concentration in the blood would approach 1 M. PSP binding of albumin in serum requires reinvestigation using improved methods. Waters and Porter (W4) recently used the same method of Huggins *et al.* (H26) and observed that normal neonatal blood plasma was capable of binding 57–80 μg PSP per 10 mg "albumin," but that the plasma "albumin" from neonates with erythroblastosis had a lower PSP-binding capacity. This reduction in binding was inversely related to the plasma bilirubin concentration, suggesting that bilirubin can compete successfully with PSP for binding to albumins (W4).

The serum albumin of cancer patients also appears to bind less PSP than normal. This is thought (B18) to be due to the occupation of PSP-binding sites by unknown substances occurring in the blood in cancerous states, since after isolation and treatment with alcohol, but not after exhaustive dialysis, the "cancer" albumin recovers its PSP-binding capacity. However, the possibility that some denaturation occurred during the alcohol treatment cannot be excluded. PSP binding to an abnormal globulin of a myelomatosis serum (H26) is probably a consequence of dehydration and precipitation of the myeloma protein.

The ability of albumin to bind eosin has been examined (G26) by observing the spectral shift from 516 to 536 nm which occurs when albumin solution is added to eosin (final concentration 6×10^{-3} M) in phosphate buffer (0.05 M) at pH 7.1. A technique was devised for the estimation of albumin in 0.02–0.20 ml oxalated plasma. The results found were lower than those obtained by carrying out *la technique classique*, and the explanation given is that the plasma fatty acids present cause a reduction in eosin binding to plasma albumin. The extent to the difference in values obtained

by the two methods can be adequately accounted for by allowing for the fact that globulin precipitation is incomplete in a 50% saturated ammonium sulfate solution, and therefore it results in incorrectly high values for albumin. An attempt to determine and make allowance for albumin-bound nonesterified fatty acids was not successful (G27).

Two methods based on the binding of related biological pigments have been proposed. In the first, the production of methemalbumin is measured at 403 nm (the wavelength of its maximum absorbance) after the addition of blood serum to a solution of alkaline hematin at pH 7.5 (R23). A relatively large increment in albumin is required to give a measurable change in optical density. The high affinity for hematin of one or more β-globulins present in the plasma of adults (N7, H6a) and in the newborn (D12) has recently been noted, but their quantitation by, or effect on, albumin determination by this method has not been considered. In the second method, a xylene solution of bilirubin is equilibrated with plasma diluted in borate buffer at pH 8.5. Loss of bilirubin in the organic layer is measured optically at 454 nm, and under the experimental conditions is proportional to the plasma concentration of albumin up to 40 g/liter (W10). This method shows a freedom from interfering substances similar to that of the methyl orange method, but has a higher sensitivity than the latter (W10).

Albumin concentrations obtained by methyl orange binding and bilirubin partition methods are too high. Part of this discrepancy is accounted for in the binding of these substances to lipoproteins. The possibility of change in configuration and of loss of dye-binding capacity during the preparation of purified human albumin must also be considered. Horse albumin loses 33% of its methyl orange-binding capacity after storage at 60°C for 1 hour (K37, K38).

6.5. Dye Binding of Albumin and Globulins on Supporting Media

It has previously been shown that the proportions of components in a protein mixture, separated by paper electrophoresis and determined by dye-binding techniques, fail to agree with the predetermined ratio, and that there is no consistent relationship enabling a simple correction factor to be applied (F18). This finding has been confirmed (R21), and it has furthermore been clearly demonstrated that the individual protein fractions of normal serum vary widely in dye-binding capacity (B23, J5, R21, S52). An earlier report that a staining technique with bromphenol blue resulted in equivalent binding capacities to albumin and β-globulin (H2) has not been substantiated (J5). Other different dyes and combinations of dyes have been proposed for the determination of serum protein after electrophoretic separation, but no dye has advantages sufficient to win universal

approval. Osborn (O2) concluded after careful study that more reliable results are obtained with light green than with bromcresol green or lissamine green. Other workers consider the latter dye superior because its binding capacities toward denatured albumin and globulin are more nearly equal (B22, G16, S8). Yet others have claimed a similar advantage for Ponceau S (M19) and for amido black (G20).

The uptake of light green by known weights of denatured albumin and β-globulin from various concentrations of the dye has been measured. When the reciprocal of the amount of dye bound to proteins was plotted against the final concentration of dye in the bath, a straight line was obtained (O2) in accordance with the equation, derived by Klotz for the binding of small anions by proteins, assuming no interference between the binding sites:

$$\frac{1}{r} = \frac{1}{Kn} \cdot \frac{1}{A} + \frac{1}{n}$$

where r = number of moles of bound dye per mole protein, A = concentration of free dye at equilibrium, n = maximum number of binding sites available on the protein molecule, and K = the intrinsic constant giving a measure of the affinity of the protein for the dye. The difference in the type or rate of the denaturing process which must be used prior to or during the dye staining will result in the proteins having different degrees of reactivity in terms of dye binding. The different albumin figures obtained by alcoholic and aqueous bromphenol blue staining can be explained in this way (S11).

Not only is the amount of dye bound to protein influenced by the nature and timing of the denaturation process (J5), but small changes in the ambient temperature may be important. An alcoholic dye bath produced 25% less bromphenol blue uptake by denatured albumin when the temperature was lowered from 20° to 0°C, but remained unaffected by a rise in temperature to 37°C (M15).

In order to overcome the relative error in albumin and globulin estimations due to the higher binding capacity of albumin, correction factors of 1.6 to 2.4 have been suggested (P3). However, for some dyes (e.g., nigrosin) the difference in binding capacity to serum albumin and globulin is not constant at different protein concentrations (S11).

The importance of restricting the protein density on the supporting medium has been stressed (O2). Yet even at low concentrations, the relationship between several dyes and protein is consistently nonlinear whether

carried out on paper, on cellulose acetate (O2), or on agar gel (W25). The uptake of lissamine green is reported to be more nearly linear when cellulose acetate is the medium (A3), but in general, apart from the time required for diffusion, the medium used for protein separation (paper cellulose acetate, starch, agar, or polyacrylamide gels) has little effect per se on the dye uptake of the fractions (R31, S53).

Electrophoretically separated bands of abnormal plasma proteins possess abnormal and variable lissamine green-binding capacities (B23). Part of the explanation for this lies in the heterogeneity of these fractions. Electrophoretically separated albumin is no exception and may be contaminated with lipid and lipoprotein on paper (O3) and with lipid and an α-globulin on starch gel (P13).

6.6. Albumin Determination with Fluorescogenic Substances

The introduction of a substituent anilino group into certain dyes of the acridine and naphthalene series leads to compounds which are nonfluorescent in aqueous solution, but brightly fluorescent when absorbed in solution or on the solid phase. Examples of such fluorescogenic substances are 5-(4′-carboxyanilino)-2-chloromethoxyacridine and 1-anilinonaphthalene-8-sulfonic acid. These dyes are not bound to γ-globulin, Bence Jones protein, or other native globulins. In contrast to the dye binding of acid-denatured proteins which is a general property, the dye binding of the aforementioned class of compounds in neutral solution, as measured fluorimetrically, is a specific property of native albumin (W14). Maximum binding capacity is about 3 moles/mole albumin.

Two fluorimetric techniques have been described, the only essential difference between them being their sensitivity. One method employs vasoflavine—a sulfonated methylated benzothiazole derivative (B14) which increases linearly in fluorescence in the presence of small amounts of albumin.

The more sensitive method of Rees *et al.* (R6) requires about 7 μl plasma per ml dye solution (6 mg 1-anilinonaphthalene-8-sulfonic acid per liter phosphate buffer, pH 7.6). The fluorescence intensity of the plasma-dye mixture is measured (activation peak: 370 nm, fluorescent peak: 485 nm) and the albumin concentration obtained from a standard curve. The dye is stable and has low blank fluorescence; a filter fluorimeter is satisfactory for the determination. Bile pigments in excess of about 5 mg/100 ml plasma interfere with the method; low results are presumably caused by competition between bilirubin and dye for binding. Bovine albumin has been stated to produce more intense fluorescence with anilinonaphthalene-sulfonate than human albumin (R6). This requires confirmation, since it

may have been due to lipids or other contaminants present in the less pure human albumin available at that time.

6.7. Determination of Serum Proteins and "Total Globulin"

"Total globulin" may be determined directly by dye binding, by a tryptophan reaction, and by turbidimetry.

In the dye procedure, the globulin fraction is isolated by ammonium sulfate precipitation, and allowed to react with naphthalene black in a citric acid buffer. The stained globulins are then separated by centrifugation, and the amount of dye in the supernatant fluid is measured in a colorimeter (P11). The resulting loss of soluble dye is related to the globulin concentration, and the procedure is standardized with pure γ-globulin.

The second procedure relies on the large tryptophan content of most of the globulins compared to that of albumin (S2). The total serum proteins are precipitated, and their tryptophan content is determined by the perchloric acid reaction of Tauber (T5). Allowance is made for the small contribution of albumin to the reaction (Table 5) by standardizing the

TABLE 5
Tryptophan and Tyrosine Contents of Some Purified Human Plasma Proteins

Protein	Tryptophan[a]	Tyrosine[a]	Reference
Albumin	1.9	46.4	(F8, P8)
α_1-Lipoprotein	—	33.4	(S30)
α_1-Glycoprotein	12.5	19.9	(W17)
α_2-Glycoproteins	12.0	27.2	(S18)
Pre-albumin	26.0	1.9	(S22)
γ-Globulins	27.4, 34.2	67.5	(F8, P8)
Plasminogen	37.8	59.1	(S31)
Prothrombin	33.3	45.8	(S26)

[a] All values in mg/g.

method with a serum, the globulin of which has previously been determined by another method (S2). As an alternative, the tryptophan content of the globulins alone may be estimated after first separating them in a TCA-isopropanol solution (W8). The secondary standard can then be replaced by a human γ-globulin preparation.

Incubation of diluted and buffered serum (pH 6.6) with methanol at 70% (v/v) or with potassium phosphate solution results in a suspension, the optical density of which has been used as a measure of globulin (A15, K4, S6). Standardization of the particle size of the dispersed globulins may be attempted by the use of a protective colloid and by exact control of the

reaction conditions (S6), but the latter is not always achieved, and in view of their low sensitivity turbidity procedures are not recommended.

Serum "total globulin" is more usually determined as the difference between the albumin and total protein levels and, in conclusion, a brief assessment of methods available for total protein determination will be given.

6.7.1. *Total Protein Estimation*

A densitometric method (L17) utilizes the rate of fall of a drop of serum through a bromobenzene/petroleum ether mixture of known density. This method, which also works with redissolved serum globulin (M29), is inconvenient because vaporization of solvent and gradual solution of serum lipids necessitate constant renewal of working solutions. On the other hand, the copper sulfate densitometric method of Van Slyke *et al.* (V2), designed for field use, seems more reliable (R20) than has been previously supposed (L11).

The difference between the nitrogen contents of equal weights of lipoprotein and albumin is relatively greater than the difference between the densities (or refractivity) of these two proteins. It can therefore be argued that the copper proteinate density method (and refractometry) will yield more "accurate" total protein values than a nitrogen (e.g., Kjeldahl) method when applied to serum containing an abnormally high concentration of lipoprotein. Protein values derived from serum densities might be expected to be higher than biuret values for protein, whenever the serum contains a substantial increase in a lipoprotein or a glycoprotein of low peptide content.

Determination of serum proteins by refractometry or by densitometry is each dependent upon the nonprotein solid content of the blood being constant and contributing only a small fraction of the refractivity or relative density. A measure of total solids can be obtained with a temperature-compensated hand refractometer, after which the total protein is best derived (R9) from an experimentally determined relationship such as

$$\text{Total protein (g/dl)} = 0.89\ (\text{total solids}) - 0.23$$

Automatic recording refractometry for serum protein estimation has also been described (M11).

If the densitometric method of Van Slyke (V2) is used, a more accurate measure of protein (H20, R20) can be obtained from the relationship

$$\text{Total protein (g/dl)} = 383\ (\text{specific gravity} - 1.007)$$

than from the earlier equations stipulated by van Slyke and others. This method cannot be used for patients receiving infusions of amino acids, peptides, or THAM [tris(hydroxymethyl) aminomethane]. A level of free amino N above 400 mg/liter serum prevents the formation of the envelopes of copper proteinate (Q1). If storage of serum is necessary, this should be below −10°C to prevent a rise in amine content. On the other hand, if specimens are to be stored prior to refractometry, this should be at 0°–2°C to prevent lipoprotein destruction which might otherwise occur on thawing.

Neither of the two physical methods is significantly affected by relative changes in albumin and γ-globulin contents (R20, R30), but the extent of lipoid interference has not been clearly defined (D16). Comparative figures for serum proteins in children, obtained by the copper sulfate method, the Hitachi microrefractometer, and a biuret method (J12), are shown in Table 6, from which it is apparent that neither of the physical methods is appreciably affected by neutral-fat turbidity or intense icterus. Sunderman (S55) also considered the relationship between serum protein concentration and refractivity not to be influenced by the cholesterol content of the serum. Nevertheless refractometry frequently overestimates protein in serum from patients with primary nephrotic syndrome (Table 6).

Procedures for the direct weighing of precipitated or heat-coagulated protein of serum (D8) are uneconomic. Turbidity methods have been discussed in relation to total globulin, and near- and far-ultraviolet spectrophotometry in relation to albumin. Of these, spectrophotometry at about 215 nm is the method of choice. A simple and direct fluorimetric method (K33) should also prove useful. BPB staining of serum on paper, after protein concentration by the Weiss ring-oven technique, has been tried. This is a rapid technique (F3), but the color, confined as it is to a line, is difficult to quantitate, so that the method is insensitive and unsatisfactory in its present form.

When nitrogen determination for protein is performed an allowance for nonprotein nitrogen (NPN) should be made, even though this may be inaccurate owing to the difficulty of separating protein from nonprotein. It should be noted that the concentration of total NPN in serum is greater than in plasma. Steyn-Parvé *et al.* explained this unexpected observation (S49) by supposing the fibrin clot to absorb and retain an excess of water. The liberation of peptides and amino acids by limited proteolysis of fibrinogen prior to polymerization (clotting) of fibrin, and by fibrinolysis after clotting, also contributes to the serum N content.

A method for protein carbon has been devised. After oxidizing the separated serum proteins by boiling in dichromate in sulfuric acid for 20

TABLE 6

SERUM PROTEIN VALUES COMPARED BY DENSITOMETRY (R20), REFRACTOMETRY,[a] AND BIURET REACTION (J12)

Source of serum	Description of serum	No.	Mean value[b] (and S.D.) Densitometry (D)	Refractometry (R)	Biuret (B)	100 × D / B	100 × R / B
Children (1–13 years) with acute and chronic diseases	Normo-proteinemic	100	69.95 (6.0)	69.25 (6.1)	68.82 (5.8)	102	101
	Hypo-proteinemic	50	50.1 (6)	52.1 (7)	49.4 (6)	102	106
	Hyper-proteinemic	25	83.9 (7)	82.2 (8)	83.2 (9)	101	99
	Intensely icteric	35	67.5 (19)	69.6 (17)	69.9 (17)	97	100
	Grossly lipemic	14	69.4 (5)	74.1 (8)	73.7 (7)	94	101
Children with the "nephrotic sydrome"		16	56.9 (14)	64.2 (12)	56.5 (14)	101	114

[a] With a hand protein-refractometer, Hitachi Instrument Co.

[b] Obtained with sera from unselected material routinely submitted for analysis. Unpublished work (1964) by courtesy of B. J. Stevens. All values in mg/ml.

minutes, the excess dichromate is determined colorimetrically using a cadmium iodide–starch reagent (H1).

All other available methods for determining serum total proteins have been discussed above in relation to albumin, and their limitations indicated. All methods suffer from differences in the individual protein's contribution of the parameter being measured. However, the tyrosine (Folin-Ciocalteu) and arginine (Sakaguchi) equivalence methods (S7) and the near-ultraviolet spectrophotometric methods are more severely affected in this respect than other methods, and are therefore unreliable for total serum protein analysis.

Since no means has yet been devised for sampling blood plasma *in situ* (i.e., cell-free from the blood vessel), the clean separation of plasma or serum from blood is frequently the most expensive part of the total protein analysis.

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AUTHOR INDEX

Numbers in parentheses are reference numbers and indicate that an author's work is referred to although his name is not cited in the text. Numbers in italics show the page on which the complete reference is listed.

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