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May 16, 1938.

I hereby recommend that the thesis prepared under my supervision by Albert A. Kondritzer

entitled Solubility of Serum Proteins in Phosphate Solutions.

be accepted as fulfilling this part of the requirements for the degree of Doctor of Philosophy.

Approved by:

Albert P. Mathews.

Shiro Tashiro

FORM 668—G.S. AND ED.—1M—7-33

THE PRECIPITATION

OF

HUMAN AND ANIMAL SERUM PROTEIN

BY

PHOSPHATE

A dissertation submitted to the

Graduate School
of the University of Cincinnati

in partial fulfillment of the
requirements for the degree of

DOCTOR OF PHILOSOPHY

1938

by

Albert A. Kondritzer

A.B. University of Cincinnati 1931
M.S. University of Cincinnati 1932

TABLE OF CONTENTS

The estimation of the various fractions of the serum proteins, the quantities present and their ratio, is of importance not only to the clinical but to the research laboratory as well. This is not surprising when one considers the fundamental role played by the serum proteins in the maintenance of physiological processes. The known functions of the serum proteins, emphasized in all textbooks of Biochemistry and Physiology (Mathews, 1936; Best & Taylor, 1937) are briefly outlined as follows:

(1) They serve to maintain the osmotic pressure of the blood.

(2) They give a certain viscosity to the blood which is a factor in the maintenance of the normal pressure.

(3) They aid in the regulation of the acid-base balance of the blood.

(4) The globulin and fibrinogen (of the plasma) fractions influence the tendency of the corpuscles to adhere to one another and form rouleaux or clumps.

(5) They aid the body in combating disease, immune substances being associated with the globulin fractions which is increased during the process of immunization.

It is apparent that to have available an accurate, reliable method for the quantative determination of the

individual protein fractions in serum is not only important but is a necessity. For example, albumin contributes a greater value than globulin does to the maintenance of the osmotic pressure in serum. Govaerts (1927) has shown that globulin is only one-fourth as important in oncotic pressure as albumin, since 1 gram of albumin in 100 cc of human plasma exerts an osmotic pressure of 5.5 mm of mecury, while 1 gram of globulin exerts only 1.4 mm pressure. Therefore, a knowledge of the amounts present in the circulating blood of nephrotic or other edematous patients is desirable. Under conditions, either pathological or experimental, during which the serum proteins have been depleted below their normal value it has been found that the rate of their regeneration varies with the different proteins.

Here at least two questions arise:

Do methods which have been used for the separation of albumin and globulin in normal serum retain the same degree of accuracy when applied to pathological serum; or in other words, will the same fixed concentration of an electrolyte, arbitrarily used for the separation of the normal protein complexes, serve equally well for abnormal sera in which the various complexes may be qualitatively and quantitatively altered?

In like manner can a method whose applicability has been determined for one animal species be applied indiscriminately to any or all animal species?

This last question is of particular importance due to the variety of experimental animals that have been and are being used in researches on the origin and regeneration of the serum proteins.

Finally, the ability to separate globulin from albumin finds an important practical application in the field of immunology. As has been mentioned above during the process of immunization immune substances become associated with the globulin fractions. A sharp separation of the globulins from immune serum experimentally produced is highly desirable since the purer the globulin fraction with which the immune bodies are associated the more effective the protective action bestowed.

The separation of the serum proteins in order to estimate quantitavely each kind is a problem which has engaged the attention of many investigators for almost a hundred years. When one goes back and traces the development of this phase of our knowledge of the serum proteins the difficulties and obstacles which prevent the bringing about of such a separation are readily apparent. For this purpose the following historical account of the nature of the serum proteins is presented.

A comprehensive review of the earlier work and ideas on the nature of the proteins is given by Berger and Petschacher (1930). The progress made up to the close of the nineteenth century and the opinions existing at that time is given by

Hammarsten (1902). Rimington's article (1934), "Recent Advances in the Chemistry of the Plasma Proteins and their Complexes," is an excellent presentation of the subject. These reviews, together with some older textbooks have been used freely in the preparation of this survey. Other reviews that have been of aid in this study are given in the bibliography.

HISTORICAL

Up to the middle of the last century it was generally held that the proteins of the blood serum and other similar fluids was a single homogeneous substance - albumin. Berzelius (1840) stated that blood contained four constituents of an albuminous nature, fibrin and albumin which were dissolved in the 'Blut-serumwasser,' and globulin and hematin which were contained in the red corpuscles. The name globulin, which Hoppe-Seyler proposed as a group name for proteins requiring salts for their solution, thus came to be associated with cell protein. Due to some experiments, by Liebig, Zimmerman and others, who found that on diluting blood serum and neutralizing with acetic acid a precipitate was produced, it was suspected that serum contained more than one protein. The discoverer of serum globulin, however, was Panum (1851) of Copenhagen.

Panum showed that when normal serum was diluted with water and slightly acidified with acetic acid or a stream of carbon dioxide gas passed through the diluted serum a precipitate of protein material was invariably formed. He also demonstrated that the substance occurs constantly in human blood both in health and disease, and called it 'serum casein' from its resemblance to milk casein. Shortly afterwards the carbon dioxide gas method of separation was published by Zimmerman (1854).

A few years later Alexander Schmidt (1862) in the course of his extensive observations on serum showed that coexisting with the more soluble albumin there was always the more insoluble material which he named 'globulin'. He considered

this to be a single substance. Since Schmidt thought the paraglobulin of the plasma entered into the fibrin he called the globulin in plasma fibrino-plastic substance, but usually referred to it as globulin.

Following the publication of Schmidt's views considerable activity ensued. Different methods by which protein precipitates from serum could be produced were discovered; and there was some confusion both as to whether there was more than one substance and as to nomenclature.

Kühne (1868) believed that two different substances were produced depending upon the method of preparation. He considered that the precipitate obtained on simple dilution of the serum differed from that formed subsequently by the addition of acetic acid. The first precipitate he called 'paraglobulin' and the second 'sodium albuminate'.

Heynsius (1869,1876) demonstrated, however, that, whether precipitated by simple silution, carbon dioxide gas, dilute acids, or saturation with sodium chloride, there was only one substance - paraglobulin, as it was then called. He determined the solubility curves of the two globulin fractions in sodium chloride. The two curves coincided, showing that so far as the property of solution is neutral salts is concerned, the two fractions are identical.

In the opinion of Weyl (1877) the precipitated consisted of a single substance which he named 'serum globulin'.

O. Hammarsten (1878) introduced the use of magnesium sulphate for the precipitation of this substance which he

also called 'paraglobulin'. He showed that this salt produced a much larger precipitate than the reagents previously employed. Hammarsten stated that saturation of serum with magnesium sulphate precipitated globulin completely and, globulin only. In other words, the precipitated protein was a single substance.

Some years later, however, Burckhardt (1883) challenged the validity of this view. Burkhardt stated that although Hammarsten had conclusively shown that saturation of a globulin solution with magnesium sulphate precipitated that protein completely, yet, he had not proved that the addition of this salt in similar concentration to serum precipitated globulin only and no albumin. He repeated Hammarsten's experiment by saturating serum with magnesium sulphate, dissolving the precipitate in water and dialysing the salt away. Complete precipitation did not take place; even dilution and addition of acetic acid could produce no further quantities of precipitate. It appeared to Burckhardt, therefore, that in addition to the globulin which was insoluble in water, a water-soluble substance was contained in the magnesium sulphate precipitate. Thus the probability of the existence of a third protein in serum was shown.

Hammarsten estimated the total globulin obtained by dilution and acidification as 10% of the total protein of serum, while using magnesium sulphate the quantity increased to 63%. To explain this discrepancy and the result obtained

by Burckhardt he (1884) assumed that there were present in the serum substances which influenced the solubility and precipitation of globulin. He based this assumption on experiments in which he treated serum with sodium chloride. Afterwards on dialysis the quantity of precipitate produced was increased. The sodium chloride was supposed to have altered the substance which kept the globulin in solution. However, it was evident that the magnesium sulphate precipitate did consist of two parts. But instead of reverting to globulin as that portion of serum which was insoluble in water but soluble in dilute salt solution the extended definition of precipitation by magnesium sulphate was retained.

Burckhardt's observation was, however, confirmed by E. Marcus (1899). There thus came to be recognized two globulins: (a) a globulin soluble in water (pseudoglobulin); (b) a globulin insoluble in water (euglobulin). E. Marcus stated that these two globulins had the same coagulation temperature, composition and rotatory power and differed only in their solubilities.

As for the albumin fraction evidence indicating its heterogeniety was soon presented. Halliburton (1884) differentiated three albumins in serum by variations in their temperature of coagulation. The first, A-serum albumin, coagulated at 70-73°, B-serum albumin at 76-78°, and the γalbumin at 82-85°. Although one may doubt the existence

of individual albumins when based on such evidence these results are of interest in the light of the recent work of Hewitt (1936,1937). This author, observing that horse serum albumin can be separated into fractions of widely different properties has prepared two proteins, namely crystalbumin, which is carbohydrate free, and seraglycoid, a new serum protein, to which is due the greater part of the polysaccharide content of horse serum albumin. These proteins were also obtained from, ox, rabbit, and chicken blood sera.

Meanwhile Hofmeister had conceived the idea of dividing serum and other liquids into fractions by means of taking precipitates at different degrees of concentration of one and the same solution, and thus isolating the different proteins. The first attempt was carried out by Kauder (1886) when he precipitated globulin by half-saturation of the filtrate, the globulin roughly corresponding to Hammarsten's. Kauder came to the conclusion that the precipitate produced from serum by magnesium sulphate was a single substance since fractional precipitation yielded no evidence of a double nature.

Later this system was elaborated and extended by Fuld and Spiro (1900), and Pick (1902) and others.

Fuld and Spiro, working on the effect of blood on the coagulation of milk, found that the rennetic ferment and anti-rennetic substance of serum was contained in the

in the fraction of serum brought fown by half-saturation with ammonium sulphate. This globulin fraction could be differentiated into three parts by fractional precipitation:

(1) precipitated by 21.5% saturation with ammonium sulphate - no effect on the rennet coagulation of milk.

(2) precipitated by 28-33% saturation with ammonium sulphate - increased the effect.

(3) precipitated by 28-33% saturation with ammonium sulphate - diminished the effect.

The first fraction was found to resemble the original water-insoluble substance of Panum, Schmidt, Heynsius and others, and was named euglobulin; the other two were more soluble and were called pseudoglobulins. Thus these authors first applied the terms euglobulin and pseudoglobulin which are still in use today.

Later, Porges and Spiro (1903) thought they had identified three distinct fractions in serum, differing in temperature of coagulation and in elementary per cent composition.

Reiss (1902) believed that there are no sharp limits of precipitation for the globulin components, but that they coprecipitate to some extent. At the same time he pointed out that between the upper precipitation limits of the globulins and the lower limits of the albumins, there is an interval of no precipitation from serum by ammonium

sulphate. From this time onwards, the separate individuality of two main fractions of the serum proteins - Albumins and Globulins - has been generally conceded.

Freud and Joachim (1902), on the other hand, taking the two fractions, euglobulin and pseudoglobulin, showed that each contained a water-soluble protein. As a result of their analysis they stated that the precipitate of protein of serum which is precipitated by half-saturation with ammonium sulphate consisted of four fractions, exclusive of fibrin globulin:

I. Precipitated by one-third saturation with ammonium sulphate,

(a) insoluble in water-paraeuglobulin,

(b) insoluble in water-euglobulin;

II. Precipitated by one-half saturation with ammonium sulphate;

(a) insoluble in water-parapseudoglobulin,

(b) soluble in water-pseudoglobulin.

However, Haslam (1905) showed by direct experiment that no single precipitation, both in the case of albuminoses and serum proteins, whether by acids, salts of heavy metals, salting out or alcohol, ever produced a complete separation; that in the precipitate the substances of the filtrate could always be demonstrated, often up to 20-30%, and similarly, vice versa with the filtrate.

J. Mellanby (1907), from a determination of the percentage of protein precipitated with gradually increasing concentrations of alcohol, drew the conclusion that there were three different proteins in serum.

Haslam (1913), and, independently Wiener (1911), showed that dilution of serum before precipitation resulted in less of the more soluble protein being carried down in the precipitate of the less soluble protein, and that with the sufficient dilution such inclusion caused only a small error. But Haslam also demonstrated that dilution would not lessen the relative concentration of the less soluble protein remaining in solution in the filtrate of the more soluble protein. The solubility of the less soluble protein in salt concentration at which the latter commenced to be precipitated was such at dilutions of 1 to 8 or more, that as much as 25% of the euglobulin in ox serum was always found in the pseudoglobulin filtrate, and some euglobulin was always found in the albumin filtrate. He interpreted his results as indicating quantitatively the degree of error involved in separation of this kind.

However, against the growing idea that the various distinct proteins which had been isolated were discrete substances pre-existent in the serum was the opinion expressed by Hardy (1905). He believed that the balance of probability is against the view that serum is a mixture

of certain proteins in solution "and in favor of there being in serum some complex proteid which breaks down readily into fractions whose composition and properties depend upon the degree of dilution and the reagent used." Furthermore, he contended that the globulin fraction "can be split by saturation with neutral salt or by acidification into fractions differing in properties according to the mode of preparation." It may be mentioned that such a picture of the serum proteins agreed with the so-called nucleus hypothesis of the structure of the protein molecule which was advanced by Kossel (1901) and was based upon his fundamental studies of the Protamines and Histones.

The question arises as to whether the individual protein fractions separable by salting out procedures are distinct individual proteins, and are the fractions so separated similar to the protein complexes existing in serum.

Haslam had called attention to his observation that purification of the globulin fractions beyond a certain point caused unmistakable changes in the properties of the proteins. Similar changes were also noted by Chick (1914) who, furthermore, believed that euglobulin in serum was a complex substance formed from pseudoglobulin by association with some phosphorous-containing serum lipoid. Hartley (1914) analyzed the three purified serum protein

fractions and found no difference in the amino acid composition of eu- and pseudoglobulin, but did find a difference between albumin and globulin.

Howe (1921) working with sodium sulphate, a salt whose value as a protein precipitant was first recognized by Pinkus (1901), presented evidence to substantiate the presence of three globulins in blood serum: euglobulin, pseudoglobulin I and pseudoglobulin II, whose precipitations are complete at approximately 13.5, 17.4 and 21 to 22% of sodium sulphate respectively. It is largely on this work of Howe that the presence of a pseudoglobulin II in serum is accepted; although Howe himself (1922, pg.67) while recommending that this fraction be retained as a probable entity, admitted that the evidence upon which the existence of this fraction is based is rather meagre.

This work of Howe has resulted in the rather widespread adoption of the use of a 22% concentration of sodium sulphate at 37°C for the 'complete' separation of the globulin from albumin.

Electrodialysis experiments carried out by Adolf and Pauli (1924) showed that serum proteins could be separated into an albumin fraction, soluble in water and not precipitated by half-saturation with ammonium sulphate, and a globulin fraction which was completely insoluble in water.

From their experiments on the molecular weights of the serum proteins by the ultracentrifugal method,

Svedberg and Sjörgen (1928) concluded that there are probably only two proteins in serum, i.e., globulin and albumin. According to them decomposition of the proteins occurs during salting out procedures resulting in the formation of such artificial products as euglobulin and pseudoglobulin.

Sørensen's researches from the Carlsberg Laboratory have done much to clear our views concerning the individuality of the serum proteins. He has discussed at length (1930) both parts of the question raised. He agrees with Svedberg and Sjörgen that the changes in the composition of the protein solutions brought about by salting out procedure alter the composition of the protein complexes. However, Sørensen makes a sharp distinction between reversible dissociation changes and irreversible decomposition or denaturation. His opinion is that the serum proteins are an association of mutually interacting complexes the condition of whose solubility, association or dissociation are governed by factors such as concentration of the solution, the presence or absence of electrolytes, etc. He says, "within each complex all the atom groups are interlinked by main valencies, whereas the components are reversible linked by means or residual valencies." It is such reversible changes that occur during the proper fractionation of the serum proteins to euglobulin, pseudoglobulin and albumin. Any attempt to separate the proteins from serum probably alters the protein component systems and

and it is likely that the fractions obtained will differ from the component systems existing in serum.

In support of Sørensen's theory that the serum proteins are composed of easily associable and dissociable components is the work of Block (1931). This author has shown that the various protein fractions, as isolated from fresh cattle serum by fractionation with different concentrations of neutral salts, did not have the same chemical composition based on the determination of the amino acids, arginine, histidine, and lysine. "These studies," according to Block, "lend weight to the conception that serum does not contain several independent proteins; that the fractions isolated by physiochemical methods are not pre-existent in the serum but are produced by the technic employed in their preparation."

Saturation with concentrated salt solutions has for long been the only available, convenient method for the separation and subsequent study of the serum proteins. In recent years the ultracentrifuge and greatly improved cataphoretic technic have offered more exact and accurate means for investigating these systems in their native state as well as under artificial conditions. Using the ultracentrifuge, McFarlane (1935) has conducted a series of investigations of extreme interest and importance upon serum proteins and native serum. He found that when fresh undiluted horse serum, without previous treatment

of any kind, was examined in the ultracentrifuge a separation occured which indicated the presence of about 79% of a light fraction and 21% of a heavier fraction. Half-saturation of this serum with ammonium sulphate had indicated 51% globulin and 49% albumin, values ordinarily accepted for horse serum. On simple dilution of this horse serum the ratio of the two fractions obtained by ultracentrifugal analysis approaches the albumin to globulin ratio obtained by ammonium sulphate precipitation. Only at a concentration of 0.25% of the total protein does the diluted serum behave ideally, i.e. give value comparable to half-saturation with ammonium sulphate values! Also, starting with mixtures of 'albumin' and 'globulin' and increasing the concentration, the proportion of the light to heavy fractions increased until the state of affairs present in the original serum is reached.

The same phenomenon was found to occur in human and bovine sera. Furthermore, three protein types are distinguishable in all cases, the third present in small concentrations only. For human serum, undiluted and untreated, the following fractions were obtained:

Light (albumin) - - - --65%

Medium - - - - - - - - -24%

Heavy (globulin) - - .- -10±5%

McFarlane has made it strikingly clear that the state of affairs existing in normal serum is very different from

that which would be inferred from estimation of the albumin globulin ratio as ordinarily carried out.

Electrophoresis experiments, by Arne Tiselius, on horse serum diluted four-fold, revealed five components. The fastest of these components could be identified with serum albumin. Three other fractions are found in varying amounts in all serum globulin preparations investigated and are more or less completely precipitated by half-saturation with ammonium sulphate. The slowest moving fraction, not yet isolated, appears to be formed from the others in varying amounts, depending upon the concentration i.e., increasing when passing from dilute to concentrated serum solutions. Normal sera from man, horse and rabbit gave similar pictures, although the relative amounts of the components varied considerably.

Some serum protein fractions prepared in the usual way by ammonium sulphate precipitation were studied according to this method. The results demonstrated that the precipitate obtained by 30% saturation contained appreciable amounts of the fastest component (albumin), whereas the proteins which remain in solution after precipitation of serum with 55% saturation contained 25% of the globulin fractions, all of which were present.

Ultrafiltration studies of horse serum by Elford, Grabar and Fischer (1936), resulted in the production of broken curves which led these authors to conclude that the albumin and pseudoglobulin fractions of serum do co-exist as individual molecular species in the native state.

It is quite evident that there are variations in the amount and kind of protein complexes that exist in the sera of human and other animal species. These protein complexes may be altered, by whatever mechanism, in pathological states so that not only may one or more of them increase or decrease, but new complexes may appear with changed physicochemical properties, including that of solubility. Therefore, the significance of the albumin to globulin ratio, as ordinarily determined by precipitation at some definite, fixed concentration of electrolyte, is doubtful. Since the study of the differences of the serum proteins has become of great clinical importance in the understanding of disease conditions it was felt that further experimental evidence upon the precipitation by electrolytes of the proteins of normal and pathological human sera and also that of various animal species was desirable.

It occurred to us that a study of the solubility-precipitation curves of such serum proteins, such as was carried out by Butler and Montgomery (1933), Roche, Dovier and Samuel (1936) Lin and Wu (1934) would be of value because it presents a continuous composite pattern* of the various protein fractions. We believed that an investigation of such protein patterns might reveal much interesting and pertinent information concerning the extent, and variation

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*By 'serum protein patterns' is meant the per cent or quantities of different proteins (globulins, albumins) occuring in the serums examined).

among the different animal species of coprecipitation that takes place; the factors, if any, that influence this phenomenon, the behavior of these systems under the influence of pathological conditions and, furthermore, the accuracy of any fixed concentration of electrolyte in determining the amounts of albumin and globulin present. Finally, on the basis of difference in solubility behavior, new proteins or protein complexes which make their appearance might be found.

Roche, et al, using ammonium sulphate, at pH 5.7, constructed solubility curves for human normal and pathological serum from hypertensive and cirrhotic patients. However, since the determination of protein nitrogen in the presence of an ammonium salt has obvious disadvantages and difficulties, the use of ammonium sulphate was considered inadvisable.* Liu and Wu employed increasing concentrations of methyl alcohol for the separation of the proteins. Experiments carried out with this method proved to be very unsatisfactory due to the extreme slowness of filtration and to the fact that many filtrates repeatedly came through cloudy. Butler and Montgomery (1932) studied the dependence of the solubility of the plasma proteins on the salt and plasma concentrations in concentrated solutions of potassium phosphate. As pointed out by these authors the data

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*Jameson and Roberts (1937) qualitative evidence indicating that ammonium sulphate has a denaturing effect on serum proteins.

presented by Cohn(1927) , concerning the variation of the ionic strength of potassium phosphate solutions with the maintenance of constant pH, and vice versa, makes this salt particularly suitable for salting out experiments. Also it is readily available and solutions of it are easily prepared.

EXPERIMENTAL METHOD

A 3.0 Molar potassium phosphate solution of pH 6.5 was prepared by weighing out equi-molecular amounts of the mono- and dibasic salts, dissolving in redistilled water on the steam bath, cooling and making up to volume in a volumetric flask. The solution propared in this way, usually being murky and turbid, was filtered. The phosphorus content was then checked by the method of Fiske and Subbarrow (1925), and the pH determined by the glass electrode.

A series of phosphate solutions ranging in concentration from 1.0 to 3.0 M phosphate, with a 0.1 M increment, was then prepared by appropriate dilutions. Fifteen cc of these solutions were then measured into tubes and 0.5 cc of serum added by means of a parafin-tipped one cc Mohr measuring pipette attached to a Neale-Forbes apparatus, resulting in a serum dilution of 1 to 31 and a phosphate molarity equal to $\frac{15}{15.5}$ times the molarity of the phosphate solution taken. In some earlier experiments dilutions of the 3 M phosphate were made so that addition of 0.5 cc of serum would result in 15 cc of the desired phosphate concentration with a serum dilution of 1 to 30. The serum and phosphate were mixed by gently rotating the tube and inverting slightly. Vigorous or rapid shaking was scrupulously avoided due to the surface denaturation of the protein that such treatment causes. The tubes were then allowed to stand for approximately 14 hours, usually over-

night, in a dark place at room temperature which is approximately 25° ± 2°C. In the lower concentration of phosphate the precipitate of protein settled to the bottom of the tubes, while in the higher concentrations it rose to the top of the liquid. The precipitate was kept in contact with the solutions as much as possible by gentle inversion of the tubes. This is advisable because, since the protein precipitate occludes and adsorbs phosphate, the precipitating liquid will not otherwise have the desired phosphate concentration and as a result a true equilibrium for the specific phosphate molarity will not be reached.

The next morning the contents of the tubes were again mixed. The solutions were then carefully filtered through Schleicher & Schull filter paper, #602, the first portions of the filtrate always being allowed to filter into the tubes in which the precipitation took place, until the filtrates were clear, when they were then collected in 50 cc. Erlenmeyer flasks. The funnels were covered with watch glasses to prevent evaporation during the filtration.

Aliquots of the filtrate, always in duplicate, were measured by means of Normax pipettes into 100 cc Kjeldahl flasks. The volume of the aliquots varied from 2 cc to 5 cc, depending upon the quantity of protein suspected in the filtrates. Two and one half cc of digestion mixture

were then added. This digestion mixture had the following composition: 0.6 cc conc. H_2SO_4, 0.015 gm Cu SO_4, and 0.0005 gm Se in 1 cc. A clean, acid-washed glass bead was dropped into the flask and the contents digested over an electrically heated coil especially constructed. When the water was boiled off and the flasks became filled with white fumes a glass funnel was inserted in the neck of each flask. Heating was continued until the digestion liquid became pale blue-green in color. At this point the flasks were allowed to cool for about five minutes, after which time 2 drops of Merck's Reagent Superoxol were added to insure completeness of digestion. Heating was resumed and continued for 25-30 minutes in the case of those flasks containing aliquots of the lower molarity of phosphate, and for about 15 minutes for the higher molarity of phosphate aliquots. When digestion was complete the flasks were cooled, a pinch of acid-washed talc added and the funnel and neck of the flask washed down with 25-30 cc of redistilled, ammonia-free water. Six cc of 13% solution of NaOH was added through a glass tube placed inside the flask so that the alkali would reach the bottom of the flask without touching the surface of the acid digest. The flask was then connected to an all-glass still (Scott & West, 1937) agitated to mix thoroughly the contents and heated by a micro burner. The ammonia was trapped in boric acid according to the method of Brecher

(1936). After 8 minutes distilling and 2 minutes draining the boric-acid ammonia solution was titrated with 0.01 N HCl, using a mixture of Methylene Blue and Methyl Red as indicator. In the beginning of this study the distillation was carried out by the method of Pregl as modified by Goeboel, the ammonia being received in a measured amount of N/100 HCl and the excess acid determined by titration with carbonate-free N/100 NaOH. This latter method, however, was abandonned in favor of the former one.

The non-protein N was determined on a Folin-Wu filtrate by nesslerization after acid digestion. This value was subtracted from the total nitrogen to obtain the protein nitrogen, which was then multiplied by the factor 6.25 to get the protein content. The protein could be calculated either as the grams per cent dissolved at each concentration of phosphate or as the grams of protein that could be dissolved per liter of each phosphate solution. From either value the per cent of the total protein soluble at the various molarities of phosphate was calculated and was plotted as the ordinate against the molarity of the phosphate solution as the abscissa.

Since Sørensen (1925) and Cohn (1925) have established that in a mixture of proteins the logarithms of the solubilities, which are a function of the concentration of salt solutions saturated with respect to the protein, form straight lines whose intersection corresponds to the end

of the precipitation of one protein and the beginning of the precipitation of another, logarithm curves of the per cent of total protein soluble at each concentration of phosphate were also drawn.

A. COMPARISON OF SERA OF DIFFERENT MAMMALS.

The blood serums of a variety of animals were obtained and the solubility of the proteins of each serum in the different concentration of phosphate buffers was determined. The animals studies were human, dog, rabbit, rat, horse, beef, and sheep. The curves for human and for horse are those of an average of four normals, that of the rabbit an average of two. To secure sufficient blood of the rat it was necessary to bleed four rats and pool the sera. Two separate groups of rats were used in this manner at several months interval. The two rat curves coincided almost exactly. Therefore, only one of them is shown in the chart. Due to the fact that beef and sheep are not slaughtered near Duke it was possible to obtain only one sample of serum from each of these animals.

Insert Chart I

In studying these graphs we note first the similarity between the serums of man and rabbit, and also between dog and beef. Examination of the upper portions of these curves shows definite differences in the molarity of phosphate required to initiate precipitation. The extreme example is the case of rat and of sheep and horse. Whereas only a few per cent of the protein of rat serum are precipitated at 1.55 M phosphate approximately 23% is precipitated from sheep and horse serum at the same phosphate concentration.

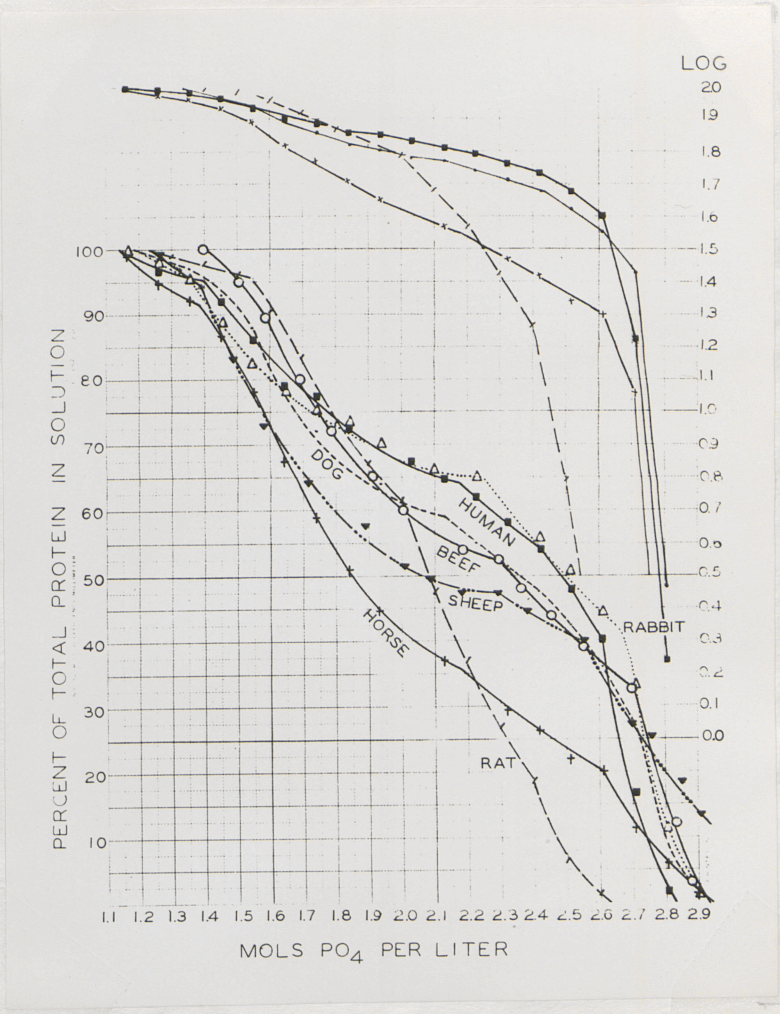

CHART I

Sera of Normal Mammals

The mammals represented by the log curves (upper curves) are identified by the use of the same insignia as used for the lower curves.

Inspection of the graphs also shows a definite break in the curves of the serum of the rabbit, human, dog, beef, and sheep within the range of phosphate concentration of 2.1 - 2.3 M. The range of the per cent of total protein precipitated from the various serums within these concentration limits are: rabbit - 4%, human - 6%, dog - 7%, beef - 4%, and sheep - 2%.

The interpretation of this break in the curve which would ordinarily be taken as distinguishing between globulins and albumins is interesting. In the case of these animals, if one were to select, as an arbitrary concentration, 2.2 M for the separation of albumins from globulins, one could estimate these fractions with a maximum error of 1% of the total protein for the dog, and only if there were no pathological abnormalities present which would alter the protein complexes.

In the case of the rat and horse it is quite different. As for the rat there is no break in the curves between 2.1 - 2.3 M phosphate, but at 2.0 M instead. In fact the amount of protein precipitated in the range 2.1 - 2.3 M is 21% of the total protein present at the start, obviously an amount sufficient to cause serious error in fractionation of the proteins. It is clear that in different animals the break between the precipitation of globulins and albumins does not come at the same concentration in all. Even Howe's method of salting out the globulins with sodium sulphate,

perhaps the method most widely used and which usually corresponds in precipitation power - as will be shown later - to approximately 2.1 M phosphate at this pH, differs by 13% from the value indicated by the graph. What is the nature of this complex in the rat which precipitates between 2.0 - 2.4 M phosphate and which comprises 48% of the total protein? Is it globulin or albumin? Concerning the nature of this fraction we can only hazard a guess.

The graph of horse serum offers another exception. Although a slight, but definite, break appears in the curve at about 2.2 M phosphate the separation is by no means sharp. A more distinct break occurs at 2.6 M phosphate. In fact Butler and Montgomery extended the range of precipitation of globulin from horse serum to 2.5 - 2.6 M. It is difficult to decide whether the fraction precipitating between 2.2 and 2.6 M should be placed with the globulin or albumin fractions. Here again the fixing of any definite concentration of electrolyte even as an arbitrary point of separation would be of doubtful validity.

For a more exact comparison of the solubility behavior of the proteins of the serum of each animal species each curve was divided into segments corresponding to the individual fractions which comprised the total protein. For this division the log curve of each graph was used. These log curves prove to be of great aid in the analysis of the precipitation

curves because they indicate the range of precipitation of the various fractions or complexes, that is, a change in direction - slope - of the log curve signifies the end of the precipitation of one component and the beginning of the of the precipitation of another. To some extent, however, such a division must be arbitrary, since we are dealing with a solution of proteins of which the various fractions not only have different solubilities in concentrated salt solution which overlap, resulting in the phenomenon of co-precipitation. However, it is possible to pick out fractions which, at least on the basis of the log curves, appear to precipitate as individual components. By extension of the log curves it is usually possible to locate the concentrations of phosphate at which their separation occurs.

Insert Table 1.

The first column on the left shows the animal used together with the gm% of serum protein. The other columns contain the various fractions precipitated over the range of phosphate concentration indicated. Both the per cent of the total protein and the gram per cent of protein precipitated are given in the same square. Occasionally subdivisions appear which are of sufficient prominence to warrant tabulation, as the last fraction in the case of the sheep.

From the data in this table it may be said that on precipitation of the normal serum of these animals with

TABLE I

Mammal Total Protein	Range at PO_4 % of Tot. Prot.-gm%				
Human 6.8gm%	1.15-1.4 M 5% 0.34gm%	1.4-1.9 M 24% 1.6gm%	1.9-2.23 M 9% 0.61gm%	2.23-2.6 M 22% 1.5gm%	2.6-3.0 M 40% 2.7gm%
Rabbit 6.4gm%	1.1-1.35 4% 0.25	1.35-1.65 18% 1.15	1.65-2.2 13% 0.83	2.2-2.62 22% 1.4	2.62-3.0 43% 2.75
Beef 5.7gm%	1.4-1.55 8% 0.46	1.55-1.83 23% 1.3	1.83-2.3 16% 0.9	2.3-2.7 20½ 1.2	2.7-3.0 32½ 1.8
Dog 6.4gm%	1.2-1.35-1.55 3% 9% 0.19gm% 0.58gm%	1.55-1.84 21% 1.3	1.84-2.13 8% 0.5gm%	2.13-2.68 29% 1.85	2.68-3.0 30 1.9
Sheep 6.1gm%	1.2-1.39 6% 0.37	1.39-1.65 25% 1.5	1.65-2.25 21.5 1.3	2.25-2.55 7.5 0.46	2.55-2.73-2.92 15% 11.5% 0.9gm% 0.7gm%
Horse 7.3gm%	1.15-1.39 9% 0.66	1.39-1.93 46% 3.36	1.93-2.18 9% 0.66	2.18-2.6 16% 1.17	2.6-3.0 20% 1.46
Rat 6.6gm%	1.2-1.55 4.5 0.3	1.55-2.0 34% 2.2	2.0-2.24 25% 1.65	2.24-2.43 18% 1.2	2.43-2.70 18.5% 1.2gm%

1st Column:

Mammal
Total Protein
gm%

Other Columns and Squares
Range of Phosphate concn. over which fractions precipitate.
Figures in lower left corner of square is % of total protein precipitated.
Figure in lower right corner of each square is gm% of protein precipitated.

phosphate buffers at least five protein fractions appear. It is noted that the range of phosphate concentration over which they precipitate varies for almost each species studied. In the dog and Beef the protein fractions precipitate at almost the same phosphate concentration. The same is true for sheep and rabbit.

From a consideration of the protein graphs as a whole it is evident that by the use of even three or four fixed concentrations of an electrolyte one can not hope to determine accurately the amounts and kinds of protein complexes as they appear to exist in the normal sera of different animal species.

B. PATHOLOGICAL SERUMS

For the selection of pathological serums for study a large choice was offered. Obviously those diseased conditions in which a study of the serum proteins is either of diagnostic value or clinical importance should be chosen. Hyperproteinemia, when it occurs, is always of great interest, first because it generally is an accompaniment of specific abnormal states such as chronic infections of the tubercular and syphilitic types, multiple myeloma, sarcoid of Boeck, lymphogranuloma inguinale, Kala azar, cirrhosis of the liver, and dehydration; secondly because the association of a kind of hyperproteinemia with the same abnormal state might throw some light on the origin of these protein fractions; and finally, due to the increased quantity of a particular fraction of fractions the solubility behavior might indicate something concerning its nature. Accordingly serums from as many patients with high proteins as we could obtain were studied. All available cases of such unusual diseases as multiple myeloma and sarcoid of Boeck were also examined.

In view of the great interest in the liver as a possible place of formation of some of the serum proteins it was thought advisable to collect solubility data on the serum proteins from a number of patients with diseases of this organ. Also, hepatic damage or failure may be accompanied by alterations in the serum proteins. The determination of the protein fractions in liver disease is of

diagnostic value and importance as shown by the work of Myers and Keefer (1932), Snell (1935) and Foley, Keeton, Kendrick and Darling (1935, 1937).

Blood serum was also secured from patients with nephrosis, a condition in which the determination of the proteins, particularly albumin, is of value to the clinician. The albumin content of the serum of such patients is low. Here the accuracy of the separation of the protein fractions by precipitation with a fixed concentration of a salt solution is doubtful owing to the uncertainty of the extent of coprecipitation. It was hoped that by constructing protein solubility curves of the serum of such patients the significance, if any, of such an analysis could be estimated.

The serum proteins precipitation results presented here have been arranged on four charts; on two of them according to the kind of disease or the involvement of a common factor, indicating the curves obtained in the same abnormal conditions; and on the other two in order to contrast the variety of curves it is possible to obtain in different pathological states.

1. DISEASES OF THE LIVER

Ward patients with evidence of definite liver damage or liver dys-function were selected at random for study. Hepatic abnormalities were shown clinically, by such signs as palpability and tenderness of the organ, and physiologically by laboratory determination which include cholesterol, cholesterol-esters, Van den Bergh reaction, and bromsulphalein (BSP) tests. A brief summary of their clinical histories is attached.

The blood was drawn, usually late in the afternoon, with as little stasis as possible. The serum was used in all cases. When fibrinogen was determined about 3 cc of the blood was collected in an oxalate bottle. The fibrinogen was determined by recalcifying 1 cc of plasma diluted 1 to 35 with 0.8% NaCl and determining the nitrogen in the centrifuged and washed clot by the method already described.

Insert Chart II

Four cases from among those studied were chosen for these graphs. Selection was based on the presence of as little complication by other pathological factors as possible. An average (4) normal curve is shown for contrast and comparison. Log curves calculated from the per cent of total protein in solution at the given phosphate concentration are also presented.

A qualitative analysis of this chart reveals several facts. The curves parallel each other and their pattern,

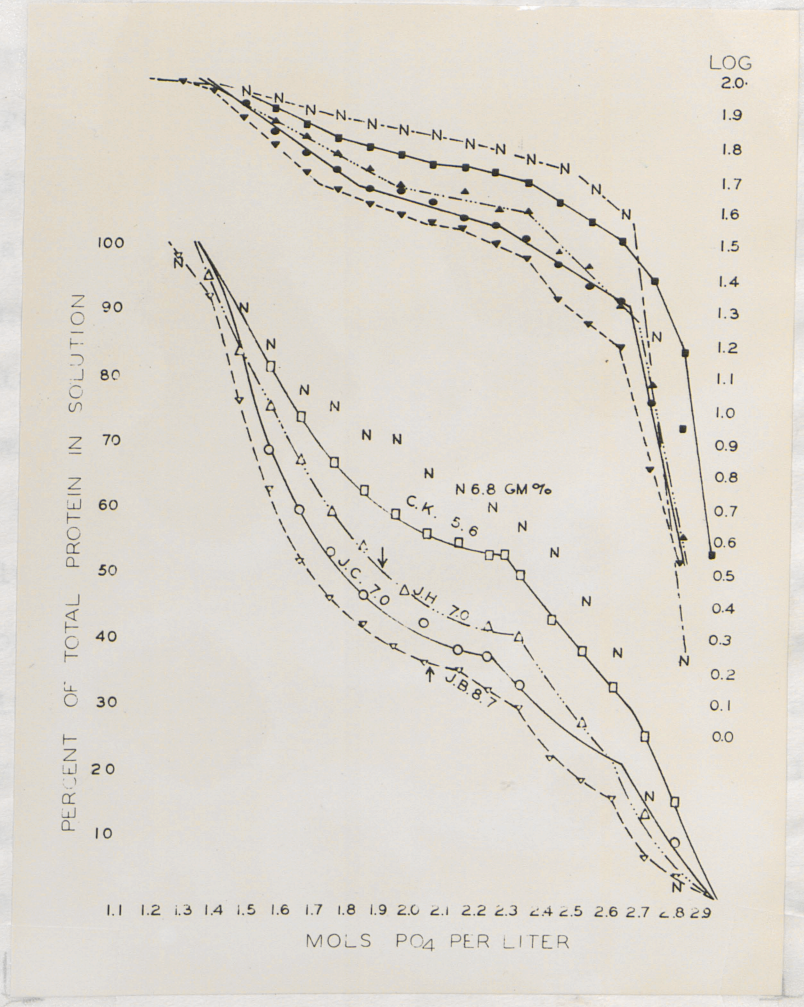

CHART II

LIVER DISEASES

The upper curves with the black-faced insignia are the log curves of the lower curves with the open faced insignis.

with a slight exception in the case of J.B., is similar to that of the normal. It must be kept in mind that since these curves are calculated on a per cent of solubility basis, the position of the curve on the chart is determined by the ratio the various fractions bear to one another. Two curves coinciding in position and paralleling each other indicate similar percentages of the same kind of protein, regardless of what the total protein may be. It follows from this that curves crossing each other indicate differences in solubility behavior for that particular range of phosphate concentration and, therefore, show the presence of different kinds of protein, with the assumption, of course, that solubility behavior characterizes proteins. In this graph we note the crossing over of the middle curves at two places. The differences, however, in protein concentration in the areas enclosed by these curves above the upper and beneath the lower points of intersection are too small to be significant.

The globulin fractions of these sera precipitate more sharply than those of normal blood, due no doubt to their greater relative and absolute concentration, just as the albumin of normal serum precipitates more rapidly than does the albumin, greatly decreased in percentages and quantity, of these sera. There is also a clearer separation of the globulin complexes from each other than is found in normal serum. The log curves emphasize this fact. Whereas in the

normal log curve there is a slight break at 1.74 M phosphate in curves of the pathological sera this division is much more evident although not at the same phosphate concentration. This observation will be dealt with in greater detail later.

The range of phosphate concentration over which a division of the principle complexes occurs is 2.1 M to 2.3 M. The average of this range, 2.2 M, would be as favorable a concentration as possible for the precipitation of the globulins for the clinical determination of the protein of these sera.

In two of these cases, J.B. and J.H., precipitation was carried out with the sodium sulphate method of Howe by adding one-half cc of serum to 15 cc of 22.5% sodium sulphate at 37°. All apparatus used was kept in the incubator and all measurements and manipulations were carried out there.

The arrows indicate the position on the curves where the separation by Howe's method occurred (21.8% Na_2SO_4). In the case of J.B. this point agrees very well with the break in the solubility curve and differs from the 2.2 M average by about 3%. The agreement, however, with respect to J.H. is not so good. This point corresponds to 1.9 M phosphate, whereas the break in the curve comes at 2.3 M with a difference of 10% in the total protein.

Regarding the albumin portion of the curves, little can be said. Not only the percentage, but the actual amount of this fraction present is low. Thus it is possible that the

influence of the globulins on the precipitation behavior of the albumins is greater than in the normal serum. Also small errors in analysis of the fractions present in lesser amounts are greatly magnified, particularly in the log curve. From a qualitative viewpoint it may be said that the albumin complexes in these sera are similar, with the slight exception of J.B., to that in normal. However, one does notice a slight increase in the solubility of the albumin fraction in the region of highest phosphate concentrations. This may be more significant and real than is apparent when one considers the fact that this increase in solubility is maintained in the presence of comparatively high globulin concentration which would have the affect of decreasing the solubility of the albumin, due to the factor of coprecipitation. It is quite possible that in these sera there are albumin complexes of smaller molecular size than those present in normal serum.

In order to arrive at a more quantitative interpretation of these solubility patterns an attempt was made to break down each curve into its component fractions. One of the difficulties met with in an attempt of this kind is the apparent presence of a large number of protein complexes, many of them formed no doubt by coprecipitation of the globulin and albumin fractions. However, from the shape of the solublility curve and the change in the slope of the log curve one can pick out the principal complexes which make

up any one complete pattern.

Insert Table II

The results of this analysis are summarized in Table II. The data includes the ranges of phosphate concentration over which the complexes precipitated, the per cent of total protein and the grams per cent protein precipitated over these ranges.

In normal human serum it is possible to identify at least five protein complexes or coprecipitation systems. Concerning the exact nature or individuality of these little can be said except that three precipitates over the range descriptive of the globulin and tow over that of albumins. For convenience the various fractions have been labeled I, II, III, IV, V.

The data on the pathological liver cases shows several interesting facts. In two cases, J.C. and J. H. the small initial fraction either has disappeared or is so reduced in quantity that it has escaped detection. In the other two cases it has increased in amount, both proportionately and actually over that in the normal. In all cases the percentage of fraction II has increased significantly and, with the exception of C.K., whose total protein was low (5.6gm%), the actual amount also has increased. Fraction III, on the other hand, has decreased, both in per cent, in all cases, and in amount, with the exception of J.B. whose total protein

TABLE II

	I	II	III	IV	V
Normal 6.8gm%	1.2-1.4 M 5% 0.3gm%	1.4-1.75 M 20% 1.4gm%	1.75-2.27 M 15% 1.0gm%	2.27-2.65 M 30% 2.0gm%	2.65-3.0 M 30% 2.0gm%
C.K. 5.6gm%	1.3-1.45 0.6gm% 10%	1.45-1.75 24% 1.34gm%	1.75-2.1 11% 0.62gm%	2.1-2.6 22% 1.23gm%	2.6-3.0 33% 1.85gm%
J.C. 7.0gm%	0%	1.32-1.81 51% 3.6gm%	1.81-2.24 12% 0.84	2.24-2.65 17% 1.2gm%	2.65-3.0 20% 1.4gm%
J.H. 7.0gm%	0%	1.3-1.91 49% 3.4gm%	1.91-2.3 11% 0.75	2.3-2.61 19% 1.3gm%	2.61-3.0 21% 1.5gm%
J.B. 8.7gm%	1.25-1.35 8% 0.7gm%	1.35-1.7 44% 3.8gm%	1.7-2.1 13% 1.1gm%	2.1-2.32-2.61 6% 14% 0.52gm% 1.2gm%	2.61-3.0 15% 1.3gm%

1st column:
Patients initials,
Total Protein
gm%

Other Columns and Squares
Range of Phosphate concn. over which fractions precipitate.
Figure in lower left corner of square is % of total protein precipitated.
Figure in lower right corner of each square is gm% pf protein precipitated.

is the highest of all, 8.7 gm% and whose quantity of fraction III is 1.1gm% as compared to 1.0gm% for the normal. An interesting observation with respect to fraction III is that regardless of the total protein the per cent present is constant in these cases. The significance of this is not clear at present.

Fractions IV and V include the complexes that precipitate over the albumin range. Here these fractions are decreased in all cases both in per cent and actual amount with the single exception of fraction V of C.K. in whom the per cent is increased above the normal while the gm% present is below the normal. The constancy of the quantity of proteins present in both of these fractions should be noted.

CASE HISTORIES OF PATIENTS

WITH

LIVER DISEASE

C.K. #96087 49 year old - white - male

Admitted 1/25/38

Physical Examination: Chronic alcoholism since 1929, liver enlarged and palpable, nodule felt in epigastric region, spleen palpable, tenderness over liver and spleen.

Blood: Hb. 7.4gm%; RBC 3,640,000; WBC 5,200.

Blood Chemistry	1/26/38	1/27/38	2/1/38
NPN	16mgm%		100mgm%
Van den Bergh	Direct reaction		
Bilirubin	3.5mgm%		5.8 "
BSP			
5 min		70% retained	
30 min		60% retained	
Cholesterol			114mgm%
" esters			55mgm%
Esterfied			48%

Final Clinical Impression: Laennec's cirrhosis of the liver with ascites. Secondary anemia.

J.H. #95590 53 year old - negro - male

Admitted 1/7/38

Physical Examination: Well developed, well nourished.
Liver margin barely palpable, not tender.

1/9/38 Hb. 12.6gm%; RBC 5,200,000; WBC 5,160.

1/21/38 Biopsy of liver during an exploratory laparotomy showed portal cirrhosis, with heavy replacement by scar tissue.

J.B. #97153 17 year old - negro - male.

Physical Examination: Weakness, lungs clear, abdomen rounded, slightly protuberant. Liver palpable, edge tender. Spleen palpable, not tender.

Blood: Hb. 8.4gm%; RBC 2,610,000; WBC 6,600.

Blood Chemistry	2/14/38	2/15/38	2/23/38
Van den Bergh	Direct		Direct
Bilirubin	5.8mgm%		6.6mgm%
NPN	25 "		
Glucose	100 "		
Plasma albumin	1.9gm%		2.3gm%
Plasma globulin	6.4"		7.0gm%
Plasma cholesterol	148mgm%		
Plasma " esters	66mgm%		
Esterfied	42%		
BSP	5 min	30% retained	
	30 min	20% retained	

Final Clinical Impression: Splenic Anemia; question of portal cirrhosis.

J.C. #97110 29 year old - white - male.

Physical Examination: No generalized glandular enlargement, abdomen rounded and protuberant. Liver palpable and tender, spleen palpable.

Blood: Hb. 11.8gm%; RBC 3,990,000; WBC 9,560.

Blood Chemistry:	2/12/38	2/17/38	3/4/38
NPN	30mgm%	32mgm%	
Van den Bergh	Indirect		
Plasma albumin	2.0gm%		
" globulin	4.4gm%		
Cholesterol	92mgm%	101mgm%	
" esters	41mgm%	47mgm%	
Esterfied	45%	46%	
BSP	5 min		30%
	30 min		Negative

Final Clinical Impression: Laennecs cirrhosis, chronic alcoholism, Beriberi heart.

2. CASES OF HYPERGLOBULINEMIA INCLUDING SARCOID OF BOECK

Among the diseases in which hyperproteinemia occurs and in which the fractionation of the serum proteins may be of clinical value and a diagnostic aid is sarcoid of Boeck. It has been noted by Jeghers and Selesnick (1937) in their review of Hyperproteinemia that "subglobulin fraction determinations are not available in cases of Boeck's sarcoid." Three cases have come to our attention and have been included in our study. While the total protein was not as high in these patients as in those studied by Salvesen (1935), being 8.2, 8.35 and 8.7gm% in ours compared to the values of 9.0 to 9.7gm% given by this author, the globulin fraction was definitely increased. In the chart presented here are two of these cases of Boeck's sarcoid, two cases of hyperglobulinemia, J.B.M., on whom a diagnosis was made of generalized syphilis with cirrhosis of the liver, and L.L., whose afflictions were many - diffuse goitre with hyperthyroidism, secondary anemia, hypertension. Data on a patient with sarcoid of Boeck, J.C., whose protein solubility curve is omitted because it coincides with that of another, F.M., (whose curve is shown), is given in Table lll.

Insert Chart III

It is noticed with regard to these curves, that although the general pattern is almost the same for all the sera there are differences in the range of separation of globulin from albumin. A definite break is seen in the curve of F.M. at 2.05 M phosphate. However, in the curve of J.B.M.

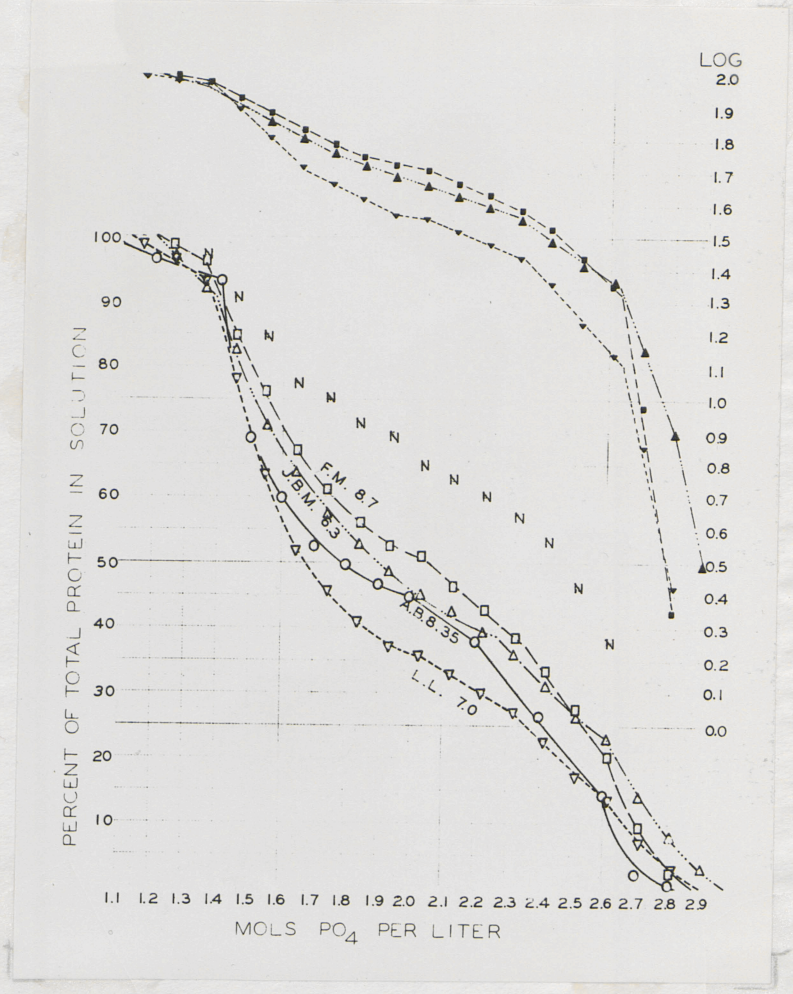

CHART III

Hyperglobulinemia, including

Sarcoid of Boeck.

The upper log curves with the black-faced insignia correspond to the lower curves with open-faced insignia.

F.M.
: Sarcoid of Boeck
A.B.

J.B.M.
: See text for description
: of disease.
L.L

the change in direction comes at 2.27 M. Fractionation of this serum by Howe's method gives an albumin value of 45.6% of the total protein which corresponds to approximately 2.0 M by the method used here. The curve of A.B. presents the difficulty of interpretation due to the appearance of a probable coprecipitation complex between 2.0 and 2.2 M, although a break does occur at 2.0 M phosphate. Also in the case of L.L., the slight break at 2.0 M and the presence of a fraction precipitating between 2.0 and 2.3 M phosphate makes questionable the arbitrary selection of a fixed point for the separation of the serum protein entities.

A quantitative analysis has been attempted and the data for the various fractions is listed in Table III.

Insert Table III (p.53)

Some difficulty was met with in the allocation of the protein components into various fractions because of the presence of extra, intermediary coprecipitation complexes. It must be kept in mind that where the number of fractions exceed the arbitrary five the placing of excess fractions in any particular group is optional. Usually the question is whether to place a component with fraction III, which would suggest that it is an albumin. Fractions I, II, and V usually are rather definite.

It is noticed that in all cases fraction I increased in quantity, althought only slightly in the serum of F.M.

Fraction II is considerably greater than normal both relatively and actually. In the case of J.B.M. with a total protein (6.3gm%) below the normal average given this fraction is 2.1gm% while in the normal it is 1.4gm%. For the others the value ranges from 2.5gm% to 3.3gm%.

Regarding the other fractions: for V it can be seen that it has decreased in amount and in percentage. This is rather surprising in the case of F.M. and J.C. when one considers that from the solubility curve it is apparent that the albumin to globulin ratio, if determined at 2.0 M phosphate where a break occurs, is 1. This means an albumin concentration of 4.2gm% (average of both) which is definitely a normal quantity. This finding would make it appear that the fraction precipitating from these sera between 2.0 M and 2.25 or 2.3 M phosphate is really globulin or contains very much more globulin than albumin. The calculated total albumin would then be lower and would harmonize with the decreased value of fraction V in these two cases.

On the other hand if these complexes precipitating between 2.0 and 2.25 - 2.3 M phosphate are composed in large part of albumin and that therefore the total albumin is normal or higher, then we must conclude that the presence of the increased amount of globulin - fraction II has exerted to such an influence on fraction V as to cause the latter to have a decreased solubility.

Thus it is apparent that the situation in pathological sera such as presented here, with regards to the proteins is such that it can not be revealed by single precipitation at a fixed concentration of an electrolyte.

TABLE III

	I	II	III	IV	V
Normal 6.8gm%	1.2-1.4 M 5% 0.3gm%	1.4-1.75 M 20% 1.4gm%	1.75-2.27 M 15% 1.0gm%	2.27-2.65 M 30% 2.0gm%	2.65-3.0 M 30% 2.0gm%
F.M. (B. Sar.) 8.7gm%	1.2-1.35M 4% 0.35gm%	1.35-1.8 M 38% 3.3gm%	1.8-2.05 M 8% 0.7gm%	2.05-2.36-2.61 13% 17% 1.5gm% 1.1gm%	2.61-3.0 M 20% 1.7gm%
J.B,M. 6.3gm%	1.2-1.4 M 9% 0.5gm%	1.4-1.75 M 33½% 2.1gm%	1.75-2.27 M 19% 1.2gm%	2.27-2.6 M 15½% 1.gm%	2.6-3.0 M 23% 1.4gm%
A.B. (B. Sar.) 8.35gm%	7% 0.58gm%	1.4-1.64 36% 3gm%	1.64-2.05 M 13% 1.1gm%	2.05-2.6 M 30% 2.5gm%	2.6-3.0 M 15% 1.2gm%
L.L. 7.0gm%	1.1-1.35 M 7% 0.5gm%	1.35-1.66 43% 3gm%	1.66-2.03-2.34 14% 9.5% 1gm% 0.67gm%	2.34-2.64 M 13.5% 0.95gm%	2.64-3.0 M 13% 0.9gm%
J.C. (B.Sar.) 8.2gm%	1.3-1.4 M 8% 0.65gm%	1.4-1.7 M 31% 2.5gm%	1.7-2.0 9% 1.74gm%	2.0-2.25-2.6 M 7% 27½% 0.5gm% 2.3gm%	2.6[illegible]3.0 M 17.5% 1.4gm%

1st Column:
Patients initial
Total Protein
Gm%

Other Columns and Squares
Range of Phosphate conc. over which fractions precipitate.
Figure in lower left corner of square is % of total protein precipitated.
Figure in lower right corner of each square is gm% of protein precipitated.

3. CASES OF VARIOUS PATHOLOGICAL CONDITIONS

In this group are included the patterns of the serum protein from a number of patients of varied pathology as follows:

B.H. Generalized hypertensive cardiovascular heart disease.

E.C. Lymphogranuloma inguinale.

M.P. Atrophic arthritis, anemic secondary to chronic infection.

D.R. Generalized syphilis, pellagra, secondary anemia.

N.G. Chronic nephritis, nephrotic syndrome.

~~A brief summary of their clinical histories is attached.~~

Here again we note the uncertainty and difficulty of attempting to determine the relative concentration of the protein fractions by a single precipitation. Omitting the curve of N.G. for the moment it is evident that the concentration of phosphate at which the globulin fractions are separated from the albumin fractions is not constant.

Insert Chart IV

From a study of these solubility patterns certain facts are evident. In three curves, those of B.H., E.C., and M.P., there is a rather sharp separation of complexes occuring at phosphate concentrations of 2.3 M, 2.1 M and 2.3 M respectively. It is obvious that while 2.3 M phosphate can be used for the separation of albumin and globulin in two of these cases its use in case of E. C. would

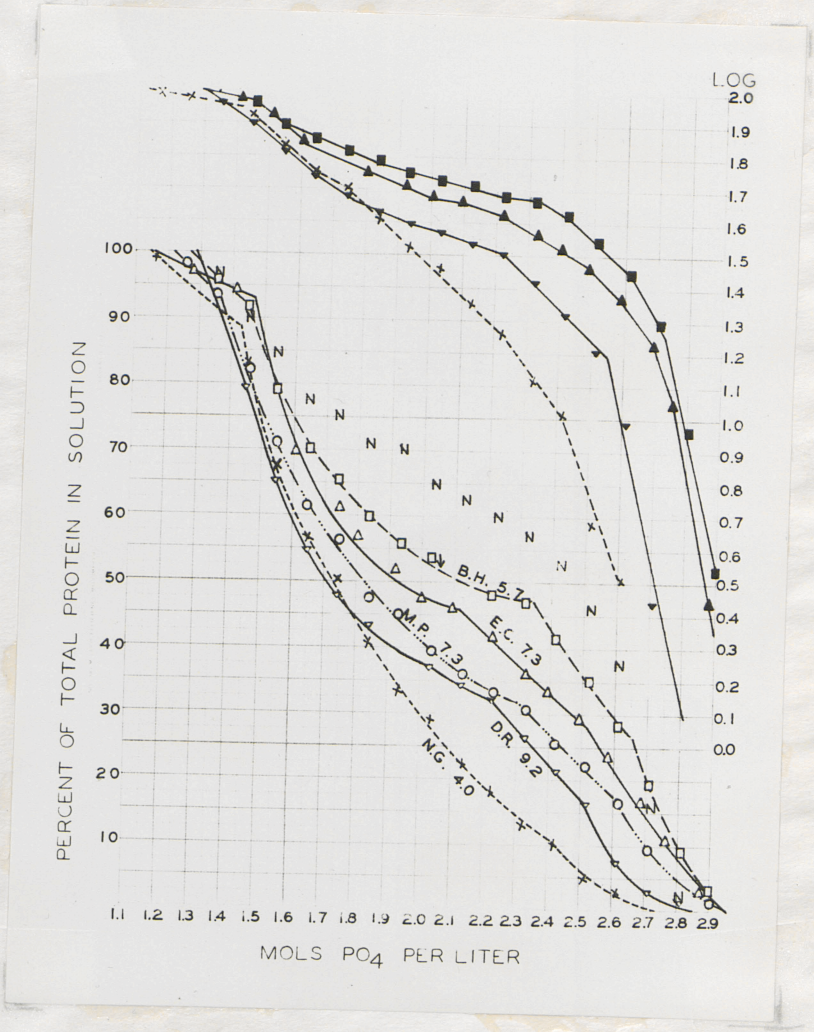

CHART IV

Various Pathological Conditions

The upper log curves with the black-faced insignia correspond to the lower curves with the open-faced insignia and same type of line.

See text for description of disease.

introduce an error of at least 10% of the total protein, which, vice versa, the use of 2.1 M would mean a maximum error of only 5%. The average of this range, 2.2 M phosphate, causes a maximum error of about 4% and that only in the case of E.C.

Howe's method of fractionation was carried out with the serum of B.H. The albumin filtrate contained 52% of the total protein, thus corresponding to 2.06 M phosphate concentration. As shown by the solubility curve the separation occurs at 2.3 M phosphate or 47% of total protein.

The serum of D.R. offers another peculiarity that is often met with and which increases the difficulty of interpretating protein analysis. In the curve of this serum there is a slight, but definite break at 2.03 M. However there is a sharper, more definite break at 2.2 M. The small fraction precipitating during this range is perhaps globulin on account of the increased amount of total ~~globulin~~ protein in this serum. It can not be denied that, in regards to the curves on this graph so far, a fair approximation of the relative amounts of albumin and globulin can be ascertained by using a single, fixed concentration of phosphate solution - here 2.2 M. However, when the serum protein pattern of such patients as N.G. is considered it is seen that the situation here is entirely different. The greater portion of the curve, between 1.4 and 2.4 M is unbroken. The break at 2.4 M is definite but slight. There is not doubt that the

bulk of the protein here is globulin, but some albumin is present as shown by the extension of the precipitation to 2.7 M phosphate. It is practically impossible to select any point on this curve for even an approximate separation of the protein fractions. Examination of the log curve shows a definite separation of the complexes between 1.65 M and 1.75 M phosphate and a much less definite separation at 2.2 M and also at 2.4 M. The factor of coprecipitation, though, still remains. Since the exact nature or composition of these coprecipitation complexes cannot be determined the difficulty of selecting the region of separation of albumin from globulin still remains.

By means of the solubility curves, together with their log curves, the proteins of these sera were divided into definite fractions or coprecipitation systems. As before they fell into five main groups, designated again as fractions I,II, III, IV, V.

Insert Table IV

A study of data presented in this table reveals some interesting facts.

Fraction I is increased in per cent and amount in all cases, being greatest in the nephrotic N.G. in the serum of whom this fraction is 11% of the total protein or 0.44gm%. It cannot be said whether this is an actual or apparent increase. It is possible that the quantity of albumin normally present in serum is able to exert such an influence on this fraction that its solubility is increased,

TABLE IV

Normal 6.8gm%	1.2-1.4M 5% 0.3gm%	1.4-1.75M 20% 1.4gm%	1.75-2.27M 15% 1.0gm%	2.27-2.65M 20% 2.0gm%	2.65-3.0M 30% 2.0gm%
B. H. 5.7gm%	1.3-1.45M 8% 0.46gm%	1.45-1.73M 26% 1.5gm%	1.73-2.35M 19% 1.1gm%	2.35-2.62M 20% 1.1gm%	2.62-3.0M 27%-1.5gm%
Es. Cr. 9.3gm%	1.2-1.47M 7% 0.5gm%	1.47-1.65M 28% 2gm%	1.65-2.12M 19% 1.4gm%	2.12-2.53M 18% 1.3gm%	2.53-3.0M 28% 2.0gm%
M. P. 7.3gm%	1.2-1.37 7% 0.5gm%	1.37-1.75M 39% 2.8gm%	1.75-2.31M 23% 1.7gm%	2.31-2.6M 14% 1gm%	2.6-3.0M 17% 1.2gm%
D. R. 9.2gm%		1.3-1.75M 53% 4.9gm%	1.75-2.03-2.22M 10% 5% 0.9 gm 0.46gm%	2.22-2.54M 17% 1.6 gm%	2.54-3.0M 15% 1.4gm%
N. G. 4.0gm	1.15-1.43 11% 0.44gm%	1.43-1.7M 37% 1.5gm%	1.7-2.2M 32% 1.3gm%	2.2-2.4M 7% 0.28gm%	2.4-2.7M 11% 0.44gm%

1st Column:
Patients initial
Total protein
gm%

Other Columns and Squares
Range of Phosphate over which fractions precipitate.
Figure in lower left corner of square is % of total protein precipitated
Figure in lower right corner of each square is gm% of protein precipitated.

while in nephrosis when the albumin is greatly diminished this influence is gone, and, provided no alterations occur in the other globulin components, the solubility of fraction I will come closer to its true behavior.

Fraction II is definitelyincreased in percentage over the normal in every case. In 3 cases E.C., M.P., and D.R. this fraction is also definitely greater in actual quantity while in the sera of B.H. and N.G., both with low total proteins the value is 1.5gm% compared to 1.4gm% in the normal.

Fraction III, in direct contrast to the similar fraction in the sera of the liver cases, is increased relatively and actually in all of these cases, provided in the case of D.R. we consider the protein precipitating from 1.75 to 2.22 M phosphate to be one complex, or two complexes so very similar that they may be regarded as one. It is surprising that even in those sera where the total protein is low this increase, slight though it may be, is found. There again it is possible that the decrease in albumin has resulted in the loss of an influence which normally holds this complex in solution. The fact that such spheres of solubility-influence exist, that the presence or absence of one fraction can so markedly affect the solubility of another makes all the more futile any attempt to determine the quantities of the various protein complexes by the use of three or four fixed concentrations of an electrolyte.

The albumin fractions IV and V have decreased in all the sera, with the single exception of V of E.C. in whom it

remained constant. It is to be remembered that the figures for the albumin of N.G. are purely speculative and subject to large error.

It is apparent that this attempt to individualize the various protein components merely emphasizes the complex state in which they exist and the variations in complexity that is possible in abnormal conditions.

4. CASES INVOLVING PATHOLOGY OF BONE INCLUDING MULTIPLE MYELOMA

One of the abnormal conditions in which it is known that proteins may show striking and significant variations is that involving the pathology of the bones. Multiple myeloma is perhaps the one type of bone disease in which the most dramatic changes in the serum proteins may occur. The findings of Bence-Jones protein in the urine of myelomatus patients, the presence of remarkably high globulin content in the serum, the association of these two factors, either singly or together, with the pathological processes that take place in the bones makes this disease of peculiar interest and perhaps of great value in the study of the nature and even of the origin of the serum proteins. This disease is not a common one, in fact since this study was begun only four cases have been found in Duke Hospital.

Since it is possible for such significant alterations to be associated with bone changes as occurs in multiple myeloma a search was made of other abnormal conditions involving bone for possible alterations of the serum proteins by checking the blood specimens that came to the Blood Chemistry Laboratory. Three cases were found in which there was a definite hyperproteinemia. The solubility-precipitation curves of the serum proteins of all these cases are shown in Chart V.

Insert Chart V

Of the four multiple myeloma patients M.F.J., Wm.W., S.M. and L.M. only one, M.F.J., had a Bence-Jones proteinuria.

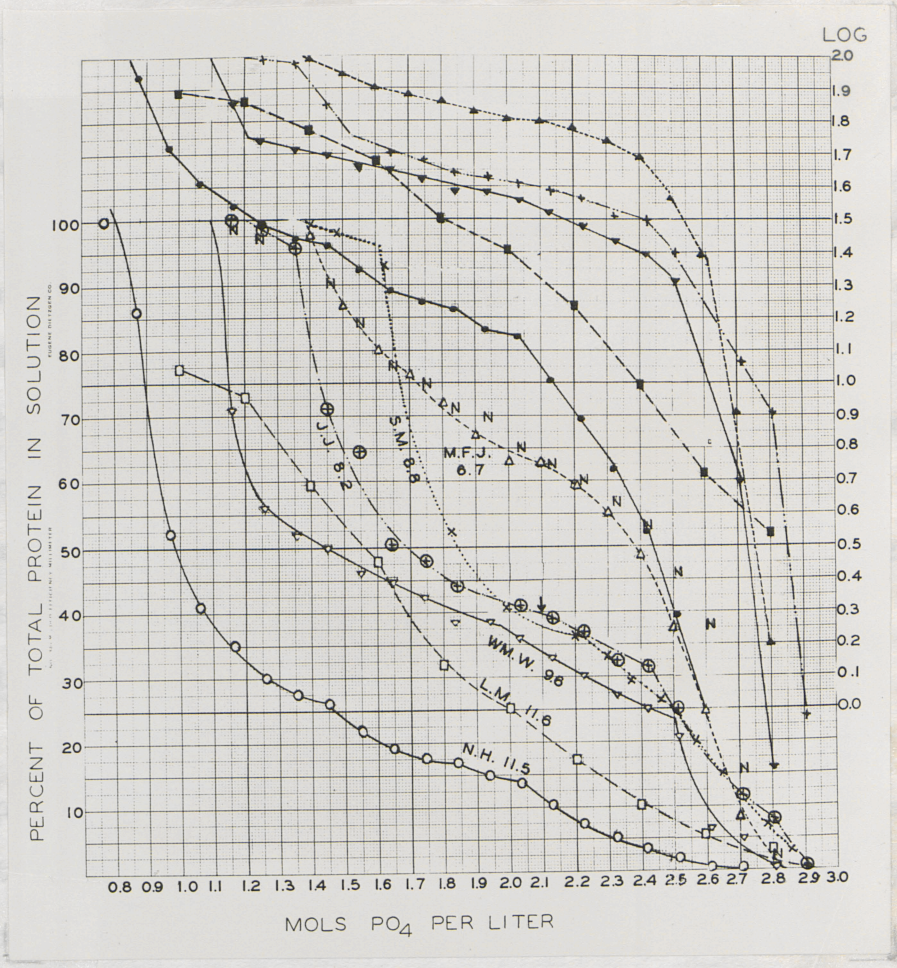

CHART V

Diseases of Bone, including Multiple Myeloma

The upper log curves with the black-faced insignia correspond to the lower curves with the open-faced insignia and same type of line.

M.F.J., S.M., Wm.W., L.M. : Multiple Myeloma

J.J.: Senile Osteoporosis

N.H.: Hypertrophic Arthritis

Repeated examination of the urine of the other three failed to reveal the presence of any Bence Jones protein. It is interesting to note that the finding of Bence Jones protein in the urine occured in the patient with apparently normal proteins, while in the urine of the three patients with the hyperglobulinemia none of this protein could be detected.

The protein patterns of these patients with bone disturbances are extremely interesting, not only do they show the similarities but the striking differences that may occur in a serum constituent - the proteins - in patients afflicted with the same disease.

A solubility curve of a multiple myeloma patient with apparently normal proteins and with Bence Jones proteinuria is that of M.F.J. Here the pattern coincides closely, with the exception of a slight increase in the albumin fraction, to the normal.

The curves of Wm.W., S.M. and L.M. (plasma) illustrates the patterns obtained in those myelomatous patients with hyperglobulinemia. Because of the lack of sufficient plasma it was not possible later to extend the curve to concentrations of phosphate lower than 1.0 M.

It is seen that not only do these patterns differ markedly and significantly from the normal but they also show definite variations between themselves. In the serum of Wm.W. there is a fraction, amounting to about 42% of the total protein, which is precipitated between 1.1 and 1.25 M

phosphate, the latter a concentration at wnich precipitation in the normal serum has scarcely begun. From here the curve extends ~~about~~ almost in a straight line with a slight, perhaps questionable break at 1.97 M to 2.5 M phosphate where a rather sharp separation of an albumin fraction takes place.

Regarding the protein pattern of L•M. it is seen that 22% of the total protein is precipitated by 1.0 M phosphate. Even though this curve is that of plasma rather than serum it seems unlikely that this fraction consists entirely of fibrinogen. If it did it would mean a fibrinogen content of about 2.5gm% which would be a most extraordinary, high value. It appears that the non-fibrinogen fraction of this precipitate is not the same as the initial fraction in the serum of Wm.W., at least as far as solubility is concerned. At 1.6 M phosphate there is a definite separation of complexes as indicated by a break in the curve, which then drops down to zero solubility with out interruption. The break at 2.5 M in the curve of Wm.W. is definitely absent here while the break at 1.6 in the case of L.M. does not occur in that of Wm.W.

The precipitation curves of the serum proteins of the third myelomatous patient (S.M.) with increased globulin presents a very interesting result. Here precipitation of the major globulin fraction begins at 1.6 to 1.65 M phosphate, in sharp contrast not only to the normal but to practically all the other pathological sera. It should be mentioned that the serum had a lipemic appearance--more so

than observed in any of the other abnormal sera. The question of whether or not the lipoids affect the solubility of the serum proteins in potassium phosphate solutions as yet has not been answered. Butler, Blatt, and Southgate, (1935) claim that the character of the precipitation curve of lipid-free horse serum is almost identical to that of the normal. This is in direct opposition to the work of Wu (1933), using sodium sulphate and magnesium sulphate, and to that of Liu and Wu (1937), using potassium phosphate, who state that the solubility of the proteins of natural serum at any given concentration of these salts is much greater than the proteins of the lipid-free serum. Results obtained by the author (1937) on normal and antipneumococcus horse and rabbit serums support the contention of Butler et al. However, it may be that the lipoids as they occur normally, do not affect the solubility of the serum proteins in concentrated salt solutions. When the lipids are increased pathologically or post-absortively either the increased total lipids or the increase of a particular lipid, such as neutral fat, may alter the solubility behavior of the serum proteins in salt solutions. This is a phase of the problem that requires further investigation. Whatever the cause of the increased solubility in potassium phosphate of this protein fraction in the serum of S.M., it is evident that, if one were to determine the globulin fractions of this serum by the accepted procedure of salting out at two

or three fixed concentrations of an electrolyte, an erroneous picture of their distribution would be obtained.

The solubility curve of the patient J.J. with senile osteoporosis indicates an increase in the globulin portion of the proteins in relation to the albumin. A separation of these two complexes occurs at 2.1 M phosphate. This division, though, is not particularly sharp due to the presence of some complex which precipitates from 2.1 - 2.42 M phosphate.

The most interesting pattern of all is that of N.H. the patient with hypertrophic arthritis and secondary anemia. Here we have the presence of a fraction that has not been met with before in these studies. This protein component comprises about 50% of the total protein of the serum and is completely precipitated at 1.0 M phosphate. Also 70% of the total proteins are precipitated when 1.25 M is reached, the concentration at which the protein of normal serum just begin to precipitate. There is a definite break in the curve at 1.4 M, and a final one between 1.85 - 2.03 M phosphate.

A qualitative inspection of this graph shows definitely that the difference that exists among the protein complexes of these sera could not be determined by the use of even several-three or four - fixed concentrations of an electrolyte.

As in the previous studies a chart of the various fractions has been made. Due to the nature and solubility behavior of the sera of Wm.W., L.M. and N.H. the fractions

did not fall into groups covering approximately similar ranges of phosphate concentration as definitely as did those of the other sera studied.

Insert Table V

Fraction I of these three sera increased greatly over that in the normal. Whether or not this fraction is the same in these cases and is identical with euglobulin of normal sera is problematical. The only property of this fraction that we have studied here is the one of solubility and, in that property, differences certainly have been shown by the solubility patterns of the serum of these patitent. This point, however, will be discussed later.

Fraction II has increased in every case except that of M.F.J. Only in the case of J.J. perhaps can it be said that this fraction represents an increased amount of a similar protein fraction existing in normal serum. As mentioned before the nature of these patterns makes comparison with the normal difficult and unreliable.

It is interesting to note that in three sera, those of J.J., M.F.J. and Wm.W., the albumin fraction V is normal as to the quantity present, while in the sera of N.H. it is definitely decreased.

TABLE V

	I	II	III	IV	V
Normal 6.8gm	5% 0.3gm%	1.4-1.75 20% 1.4gm%	1.75-2.27M 15% 1.0gm%	2.27-2.65M 30% 2.0gm	2.65-3.0M 30% 2.0gm%
M F J. 6.7gm%	0	1.4-1.6M 19% 1.3gm%	1.6-2.1 18% 1.2gm%	2.1-2.5 26% 1.7	2.5-3.0M 37% 2.5
J. J. 8.2 gm%	1.2-1.35 4% 0.33 gm%	1.35-1.53 35% 2.9% 1.35-1.55 37% 3gm%	1.53-1.84-2.13 17% 5% 1.4 gm% 0.41 gm% 1.55-2.13 20% 1.6gm%	2.13-2.43 7% 0.57gm%	2.43-2.8-3.0
Wm W. 11gm%	1.1-1.25 43% 4.7gm%	1.25-2.0 20% 2.2gm%		2.02.5 14% 1.5gm%	2.5-3.0M 23% 2.5gm%
N. H. 11.5gm%	0.8-0.99 51% 5.9gm%	0.99-1.4 25% 2.9gm%	1.4-1.84 9% 1.04gm%	1.84-2.04-2.4 3% 11% 0.34gm 1.26gm%	2.4-2.0M 3% 0.34gm%
L. M. 11.6gm%	? - 1.2M 27% 3.1gm%	1.2-1.6 25% 2.9gm%	1.6-2.0M 23% 2.7gm%		2.0-3.0M 25% 2.9gm%
S. M. 8.8gm%	1.35-1.62 100-94 6% 0.53gm%	1.62-1.87 94-48 46% 4gm%	1.87-2.2 48-36 12% 1.05gm%	2.2-2.6 38-18 18% 1.68gm%	2.6-3.0 18 18% 1.6 gm%

1st Column:

Patients initials
Total Protein
gm%

Other Columns and Squares
Range of Phosphate concn. over which fractions precipitate.
Figure in lower left corner of square is % of total protein precipitated.
Figure in lower right corner of each square is gm% of protein precipitated.

CASE HISTORIES OF PATIENTS WITH BONE DISEASE

Wm.W. 70 year old - white - male.

Admitted 6/17/37

Blood: Hb. 10.8gm%; RBC 3,990,00; WBC 6,400.

X-Ray Examination: Disclosed a large tumor in the sternum of osteolytic type, multiple areas of bone rarefaction in both iliac bones. Lungs clear. Biopsy of the sternal mass was taken, 6/26/37, and from frozen sections a diagnosis of multiple myeloma was suggested. However, there are no changes in the skull which should be apparent to x-ray.

Urine: Negative for Bence-Jones Protein. X-Ray examination, 8/29/37. Lumbar spine and pelvis show an extensive degree of bone destruction throughout the entire pelvis which is very suspicious of multiple myeloma.

Final Clinical Impression: Ewing's tumor of sternum?
Multiple Myeloma?

M.F.J. #83810 64 year old - negro - female.

Admitted 4/23/37

Blood: Hb 116gm%; RBC 3,560,000; WBC 4,240.

Blood Chemistry

NPN	38mgm%
Glucose	84" "
Plasma cholesterol	164 " "
Serum calcium	11.2 "
Inorg. phosphorus	3.1 "
Phosphatase	3.6 Bodansky units

X-Ray of bone, including skull, showed generalized decalcification, many localized areas of rarefaction, and collapse of posterior cervical vertebrae.

Study of bone marrow of both sternum and rib showed nothing remarkable.

Urine: Repeated examination showed presence of Bence-Jones protein.

Final Clinical Impression: Multiple Myeloma.

L.M. #3195 49 year old - negro - female.

Admitted 1/18/37

Physical Examination: No ulcers, thyroid not enlarged, liver and spleen not felt.

X-Ray Examination: Extreme destruction throughout entire skull, with no evidence of any new bone formation. Also large area of destruction in ribs. The findings are consistent with extreme malignant metastases or multiple myeloma. Lungs are clear. No evidence of calculi in kidneys. Kidney pelves apparently normal.

Blood: Hb. 11.1gm%; RBC 3,660,000; WBC 7,900.

Urine: Repeatedly negative for Bence-Jones protein. Albumin, one plus.

Blood Chemistry:

1/20/37		1/14/37
Serum Calcium	13.0mgm%	12.4mgm%
Inorg. phosphorus	5.0 "	5 "
Phosphatase	6.1 Bodansky units	
Plasma NPN		38 "
" albumin		2.8gm%
" globulin		8.1 "

Final Clinical Impression: Multiple Myeloma, chronic active glomerulo-nephritis.

N.H. #87295 58 year old - male

Admitted 7/6/37

Physical Examination: Slight pitting edema, liver quite firm, tender.

Blood: Hb. 4.4gm%; RBC 1.800.000; WBC 7,400

X-Ray Examination 7/10/37

Considerable calcification of arteries. There is a moderate amount of hypertrophic arthritis around both the elbow and wrist joints on the right. No evidence of metastatic malignancy.

Blood Chemistry

NPN	25mgm%
Plasma albumin	1.2gm%
" globulin	8.3mgm%

Urine: albumin --

Final Clinical Impression: Arteries sclerotic heart disease, secondary anemia, hypertrophic arthritis.

J.J. #97781 68 year old - white - male

X-Ray: Films of both femora and of the lumbo sacral spine show extreme demineralization of all the bones; bones and joints of both elbows appear to be normal. Nothing definite to suggest metastatic malignancy.

Serum Calcium 10.3 mgm%

P 3.8 "

Phosphatase 3.8 Bodansky units

Blood: Hb. 72%; RBC 3,700,000; WBC 6,000

S. M. #99478 51 year old - white - male.

Admitted 3/29/38

Blood: Hb 7.3gm;RBC2,250,000; WBC 11,800

X-Ray: Film of skull shows extensive bone destruction extending throughout all the bones. The appearance is that of malignant metastases and might well be multiple myeloma. A number of ribs show areas of rarefaction. Lumbar spine and pelvis show no destructive changes.

Blood Chemistry:

NPN	64
Serum calcium	14.9mgm%
Phosphorus	4.4 "
Albumin	2gm%
Globulin	7.8mgm%

Urine: Negative for Bence-Jones protein on 3 examinations. albumin 2+

Final Clinical Impression : Multiple myeloma, Metastatic carcinoma or hyperparathyroidism.

DISCUSSION

The view that there are definite zones of concentration of an electrolyte at which various protein fractions--particularly the globulins--can be precipitated quantitatively has been widely held since Howe (1922) published his work on the estimation of the plasma proteins with sodium sulphate, and extended it (1923) to include other neutral salts. Perhaps the most commonly used method for the separation of the total globulins of serum or plasma is precipitation by a 22% sodium sulphate solution at 37°C. No matter what the species of animal or the pathological state the method is consistently the same. Doubt as to the validity of this practice was raised by Butler and Montgomery, who called attention to the differences in solubility behavior of the globulin fractions from sera of man and horse, and on this evidence questioned the application of a method based on calf or other sera analyses to the determination of protein fractions in human sera.

In one part of this work we have studied a variety of animal species and we have shown that in the blood serum of these animals there is no common critical zone in which a fixed concentration of an electrolyte can be used indiscriminately for the separation of the globulin from the albumin fraction. It is true that there is a close agreement between some species, such as between rabbit and human, but it is the striking appearance of the pattern of the rat that

emphasizes the fallacy and errors that are inherent in the application of a method, satisfactory for one species, to all species. It is likely that the serum proteins of animal species not included here will show this same absence of uniform solubility behavior.

Granted for the moment that it is possible to subdivide the serum proteins into a number of component fractions a study of the solubility patterns presented here shows definitely that, when one intends to carry out an investigation of the blood proteins of one or more animal species, it is first necessary to construct curves of each species by some such method as we have given. Only by such procedure can truly comparable data be obtained. It should be kept in mind though that zones of separation established for the normal may very well be shifted or lost in the pathological state.

The fact that in diseased humans not only the concentrations of electrolyte at which the various protein complexes precipitate may change, but also that the nature of the complex precipitating at some fixed concentration may vary has been definitely established by this study. As mentioned previously, Howe extended the number of electrolytes that can be used for the fractionation of the globulins of blood serum (presumable calf). He determined the critical precipitation zones for a potassium phosphate mixture which had a ratio of dibasic to monobasic salt of 1 to 2, pH 7.0. The molarities of this phosphate buffer at which the different

globulins completely precipitated are: 1.125 for fibrinogen, 1.425 for euglobulin, 1.725 for pseudoglobulin 1, and 2.025 M for pseudoglobulin 11. It is conceivable that if such zones exist for this phosphate mixture there should be similar ones, though not at the same molarity perhaps, for the phosphate mixture used in our experiments. We have compared the precipitating action of this 2.025 M phosphate with that of the same concentration of our phsophate, ratio 1 to 1, pH 6.5 and found it to differ by only a few per cent.

Examination of the tables on which have been listed the range of precipitation of the protein fractions shows a definite lack of agreement among the various sera. The formation of coprecipitation complexes of varying composition not only among the globulins and among the albumins, but, also, between the globulin and albumin fractions as a whole, prevents the fractionation of serum into comparable components by such fixed critical zones. Particularly in those cases where a complex normally present in small amounts becomes greatly increased or what appears to be a new fraction appears, such as in the serum of N.H. (hypertrophic arthritis), Wm.W. and L.M. (multiple myeloma) is fractionation rendered meaningless. One cannot compare the nature of the protein precipitates form these sera at any range of phosphate concentration with that occuring in normal sera at the same molarity.

McFarlane found in an examination of pathological sera in the ultracentrifuge that globulin fractions obtained by

half-saturation with ammonium sulphate varied in the amount of albumin they contained--from 20% in one case to almost none in another. Our experiments may offer a partial explanation for this since they show clearly that the concentration of salt solution at which globulin separates from albumin varies from one pathological serum to another, hence any attempt to determine these fractions by precipitation at a fixed concentration will result in just such discrepancies and erratic results as these.

However, clinical laboratories must partition the serum proteins and the only practical, convenient method available to day is by the use of concentrated salt solutions. A study of the solubility patterns given here indicate that one the average a 2.2 M solution of equimolecular phosphate, pH 6.5, would be as efficient as any that might be found. This phosphate concentration would perhaps give better and more consistent separations for pathological sera than for normal as the sharper break in the curves of some of the patients studied suggest.

It is in cases of nephrosis though, where the globulin concentration remains normal but the albumin is greatly lowered by loss through the kidneys, that salting out procedures are of questionable and doubtful value. Evidence to confirm this impression can be gathered from the large literature on the colloid osmotic pressure of normal and pathological blood sera. Attempts, such as were made by

Peters and Wies (1937), to establish some relationship between the total osmotic pressure and either the total protein or the amount of albumin present have usually failed to give consistent results when applied to pathological sera, particularly those from nephrotic patients. Solubility curves give a reason for this. In the first place it is practically impossible to determine even fairly accurately the albumin content of a hypo-albuminemic patient in order to check the albumin value calculated from the osmotic pressure, secondly, additional fractions, formed from either the albumins or globulins and contributing different values to the total osmotic pressure, may be present. It appears to us that the determination of albumin in these patients can have little quantitative significance. All one can say is that the albumin in these individuals is low. Likewise colormetric methods for the determination of albumin and globulin, when applied to such sera, do not give either consistent results or values in agreement with those obtained by the Kjeldahl method. As a typical example, Tuchman and Sobotka (1932) found that the use of the colorimetric method of Wu (1922), for the determination of albumin and globulin of the serum of edematous patients in whom the serum proteins were below normal, involved serious error. The separation of the globulin was effected by half-saturation with Halliburton's magnesium sulfate-sodium sulfate mixture. The tyrosine factor for albumin and that for globulin

upon which the accuracy of the method depends, while remaining constant for normal serum, varied so widely in the serum of patients with low proteins that the results obtained for the two fractions were in error.

Tuchman and Sobotka offer two possible explanations for their observation: (1) the actual tyrosine content, or, more correctly, the actual content of amino acids reacting with the Wu reagent, is changed in these patients to varying degree; (2) the conditions governing color development with phosphotungstic acid, and not the actual amounts of tyrosine, change in edema.

We believe a third possible explanation for the failure of the colorimetric method is the same as that given for the failure of osmotic pressure measurements to indicate correctly the quantities of albumin and globulin present in the serum of nephrotic patients, As clearly shown by the solubility curve of the patient with nephrosis precipitation of the serum of such patients at a fixed concentration of salt solution does not yield fractions whose properties and behavior can be compared with those obtained from normal serum.

We had hoped when undertaking this investigation that new proteins, if they were formed by or during diseased processes, would be detected. There is evidence that in some pathological conditions the serum proteins differ from those normally present. This difference may be not only in the kind and number of amino acids that make up the protein but in their distribution throughout the molecule. Abnormal

environmental influences may affect the degree of association or dissociation of the protein complexes so that larger or smaller aggregates, having modified physico-chemical properties, are formed. Finally, entirely new proteins may be produced.

In a number of diversified pathological cases Metzger (1932) noted important differences in the tyrosine, tryptophane and cysine content in both albumin and globulin. Alving and Mirsky (1936) found the cystine content of the protein fraction remaining after precipitation of the globulin (half-saturation with ammonium sulfate), in the plasma of patients with nephrotic syndrome, to be lower than the cystine content of normal serum albumin. The presence in the serum of patients in the degenerative stages of Bright's disease of a protein fraction not occurring in normal serum has been reported by Jameson (1937). In nephrosis Ehrstrom (1936,1937) found the plasma proteins to be modified in such a way that their ability to adsorb Congo red (in vivo or in vitro) is diminished. Since this diminished adsorption could not be associated with a reduction in protein content or with a lowered albumin content, albumin to globulin ratio, cholesterol content, non-protein nitrogen or edema he attributed the phenomenon to a synthesis of serum proteins which differ in their physico-chemical properties from the original proteins.

To the extent that solubility behavior in concentrated phosphate solution characterizes and identifies a protein it appears from our graphs that as far as globulin is concerned in the majority of cases of hyperglobulinemia such as those due to chronic infection, cirrhosis of the liver, sarcoid of Boeck, the high value merely represents an increase over the normal. This agrees with McFarlane's findings and also the general opinion of most observers (Peters and Van Slyke, 1932). The conclusion of Jeghers and Selesnick, 1937) 'that euglobulin and more rarely pseudoglobulin 1 constitute the bulk of the protein increases responsible for the majority of the instances of hyperproteinemia' is not supported by our evidence.

We were impressed by the small though definite increase in the solubility of the albumin complexes in the liver cases, as already mentioned and also in several others. This indicates either that albumin-like bodies of smaller molecular dimensions have appeared, or that the albumin had been partially broken down into complexes of smaller size. Fractions sedimenting slower than the albumin of normal serum were observed by McFarlane in a number of pathological sera when ultracentrifuged.

The appearance of a very slightly soluble fraction in the serum of a patient with hypertrophic arthritis and secondary anemia was of great interest to us. The presence of increased globulin in multiple myeloma was shown by

Perlzweig, Delrue and Geschickter (1928), who demonstrated the appearance in the serum of a myelomatous patient of 9 and 11gm% of globulin, of which more than half was euglobulin. Since that time it has been generally recognized that hyperglobulinemia may be associated with multiple myeloma. This association of an increased globulin, particularly euglobulin, with a disease of the bone, and the finding of Cuthbertson and Tompsett (1935) that serum proteins - particularly the globulin - increased not only in some patients with fractures of the bone but in some who had undergone operations involving manipulations of the bone, suggested that the serum proteins might also be altered in other pathological processes of the bone. That this is possible is shown by the hyperproteinemia with hyperglobulinemia in the cases of N.H. (hypertrophic arthritis) and J.J. (senile osteoporosis). The increase in the globulin fraction in the serum of J.J. is no doubt due to pseudoglobulin. The exact nature of the fraction precipitating between 0.8 and 1.0 M phosphate in the serum of N.H. is unknown to us at present. That it is not euglobulin is indicated by the difference in solubility behavior between it and the least soluble fraction in the serum of Wm.W. (multiple myeloma). In the case of the latter approximately 40% of a total protein of 9.6gm% precipitates between 1.1 and 1.2 M phosphate. This means that 3.8gm% of this fraction--presumable euglobulin--was in solution at 1.1 M phosphate.

If the slightly soluble fraction in the serum of N.H. were euglobulin it would be expected that at least 3.8gm% would be in solution at 1.0 M phosphate. That this is not so is calculated from the solubility curve which shows that only 1.4gm% of a protein has precipitated even up to 1.45 M phosphate, the range ordinarily ascribed to euglobulin. Apparently it is of greater molecular size than any protein normally present in human serum.

It is quite likely that this new fraction or fractions very similar to it have appeared in hyperglobulinemic sera but have escaped detection because of the methods of fractionation used. In the determination of the globulin subdivisions most investigators usually have carried out precipitation at fixed concentration of some salt solution. Therefore the concentration at which a protein fraction begins to precipitate is not determined. This is an important property of proteins and lack of appreciation of this fact has no doubt resulted not only in the failure to recognize new protein fractions that may have appeared but in the confusing of them with euglobulin.

With regards to the significance of an albumin to globulin ratio in serum of this kind it is apparent that it can have little meaning. The nature of the coprecipitation complexes formed appears to be so variable that an attempt to separate globulin from albumin, for a quantitative determination, by a single fractionation is futile.

The obvious conclusion that one can draw from these experiments is that there still remains to be discovered the ideal conditions for quantitatively fractionating the serum proteins by means of concentrated salt solutions. Results obtained by such methods as electrophoresis and ultracentrifugation have done much to clarify our views about the protein complexes of serum. Even these methods, though, have their limitations and furthermore are beyond the reach of most investigators. We believe that the solubility-precipitation patterns as presented in this study have offered valuable and, to some extent, comparable information concerning the nature of the serum proteins in health and disease.

SUMMARY

1. Solubility-precipitation patterns of the proteins of the sera of a number of animal species, and of the sera of human pathological cases have been presented.

2. It has been shown that the same fixed concentration of electrolyte cannot be used for the separation of albumin and globulin in the sera of normal animal species.

3. The solubility-precipitation patterns of human pathological sera may vary greatly due to the formation of different complexes or associations of the proteins normally present, or to an increase or decrease of any one of the normally present, or to an increase or decrease of any one of the normal fractions, or to the appearance of new proteins not present normally, thus making doubtful the significance of the albumin to globulin ratio as ordinarily obtained by salting out procedures.

4. It is recommended that for clinical purposes an equi-molecular solution of mono- and dibasic potassium phosphate, pH 6.5, in a concentration of 2.2 M phosphate be used for the estimation of albumin and globulin in pathological sera.

5. The appearance in a pathological serum of a protein whose solubility in phosphate buffer solution is much less than any protein present in normal serum is reported.

6. Three cases of sarcoid of Boeck with hyperproteinemia are reported.

7. Four cases of multiple myeloma, one with apparently normal serum proteins and Bence-Jones proteinuria, three with hyperproteinemia and no Bence-Jones proteinuria, are reported.

ACKNOWLEDGMENT

The author wishes to express his appreciation to Dr. William A. Perlzweig of the Department of Biochemistry, Duke University, for his guidance throughout this study, to Dr. Shiro Tashiro and Dr. A. P. Mathews for their helpful criticism of and suggestions for the text of the manuscript, to the members of the Junior House Staff of Duke Hospital for their co-operation in securing the blood samples, to the Charles Arnold Iglauer Fellowship formerly held, and to the Lederle Company for the financial aid which made this investigation possible.

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