THE PROPERTIES AND FUNCTIONS OF THE PLASMA PROTEINS, WITH A CONSIDERATION OF THE METHODS FOR THEIR SEPARATION AND PURIFICATION[1]

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I. INTRODUCTION

The characterization of blood as a part of the "milieu intérieur" was developed by the great nineteenth-century French physiologist, Claude Bernard. The circulating fluid of the body constituted, from his point of view, the environment of the other tissues. Its functions are now associated with the transport of oxygen as well as of the substances necessary as elements of tissue structure; with the removal of carbon dioxide and waste products; with the balance of water and electrolytes; and with the hormonal control of bodily processes.

Succeeding generations of physiologists have added greatly to our knowledge regarding blood. Meanwhile, the phenomena of immunity were discovered. These phenomena have been correlated on the one hand with resistance to infectious diseases and on the other with changes in the composition of the blood, involving both its cellular and its extracellular constituents. The microscope reveals blood cells of various kinds, among them white cells, or leucocytes, associated by Metchnikoff with phagocytic activity, and red cells, or erythrocytes, which contain hemoglobin, the protein of the blood which, by virtue of its complex prosthetic, iron-containing group, carries oxygen to the tissues. Besides hemoglobin, the red blood corpuscles contain a number of other proteins present in much smaller amount and performing quite different functions, many of them

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[2] So many of my colleagues in this department have contributed to our knowledge of the plasma proteins that this report reflects the activities and thoughts of all of us. Dr. J. L. Oncley has made most of the ultracentrifugal analyses and Drs. S. H. Armstrong, Jr., J. M. Newell, and J. A. Luetscher, Jr., most of the electrophoretic analyses that are reported on the fractions purified by them or by Drs. T. L. McMeekin, J. D. Ferry, L. Pillemer, W. L. Hughes, L. E. Strong, R. M. Ferry, A. A. Green, Mrs. M. H. Blanchard, and Mr. J. H. Weare.

enzymatic. Among the latter are such enzymes as a phosphatase and a carbonic anhydrase. These and other constituents of blood cells may be considered as parts of a tissue circulating in and in equilibrium with the non-cellular constituents of the blood. Many of the substances concerned with the physiological and the immunological functions of the blood are among its extracellular constituents and are also protein in nature.

The cells of the blood are readily separated from the rest of this tissue by permitting the blood to clot. The proteins involved in this complex coagulation phenomenon include prothrombin and fibrinogen. The retreating clot yields most of the other plasma proteins in an amber-colored, transparent, aqueous serum. Provided the blood is withdrawn with care and citrate or oxalate added, clotting is prevented. The cells may then be separated from the blood by sedimentation or centrifugation, yielding a clear, transparent fluid, the plasma.

The main constituent of plasma, as of serum and most other tissues, is water. Each liter of plasma contains over 900 cc. of water.[3] By far the most copious constituents of plasma, other than water, are the proteins. These nitrogenous molecules are of such large molecular size that, under normal conditions, they do not pass through the walls of cells which are freely permeable to water, electrolytes, and smaller organic molecules. They are diverse in form and function. Some, as we have seen, are concerned with the clotting of the blood; certain others with immunity from disease. Some are primarily concerned in maintaining the osmotic pressure and thus the water balance of the body; some have pronounced amphoteric or dielectric properties. Some are hormones, others are enzymes, and the functions of many remain to be discovered. The last quarter-century has been marked by great advances in the chemistry of the proteins. It is our purpose in this discussion to consider the plasma as a system with many protein components and to explore, in the light of recent advances, both the chemical nature and the physiological functions of the protein components.

II. THE FIBRINOGEN COMPONENT OF PLASMA

The protein present in plasma varies somewhat in amount from species to species. Thus normal human plasma contains between 6 and 7 per cent protein, and fibrinogen is present to the extent of approximately 6 per cent of the total protein (table 1) (24, 41). The amount of this protein varies, like that of other proteins, not only from species to species, but also in certain diseases (72, 2, 44, 41, 40). The observation that there is wide

[3] In the drying of frozen plasma, a large part of the cost of the process depends upon the work necessary to freeze and evaporate this much water for every 60 or 70 g. of protein.

variation in a protein constituent of the plasma suggests that we should consider carefully on the one hand the chemical nature of the molecule, and, on the other hand, the function that it serves.

Double refraction of flow and the shapes of molecules

Among the characteristics of the fibrinogen molecule is its long, rod-like shape. Its molecular weight is not especially large, being of the same order as that of serum globulins. Fibrinogen reveals double refraction of flow (3). That is to say, its molecules are asymmetric and are oriented in a stream flowing with a sufficient velocity, precisely as are the logs in a fast flowing stream (54, 48). This streaming birefringence, in so far as it does not vanish when the refractive index of the medium is equal to that of the molecule, indicates that fibrinogen has a microcrystalline structure and that its molecules are many times longer than they are broad. If the fibrinogen of the blood may be compared to the oriented logs of a fast flowing stream, the clot may be thought of as a log jam.

Solubility and the "salting out" of proteins

One of the conventional methods of separating the proteins from plasma and from each other is by their fractional precipitation with neutral salts. The precipitation of proteins by neutral salts has been employed ever since the middle of the last century (59, 78, 1). "Salting out" depends upon the character of the neutral salt as well as of the protein (71, 29, 16, 19, 22, 53, 28, 30). Thus ammonium sulfate, when added in sufficient amount, will bring about precipitation of essentially all plasma proteins (4, 69). Sodium chloride will precipitate only a few, but fibrinogen is readily "salted out" by sodium chloride and is under most circumstances the first protein to be separated from plasma by salt or by most other protein precipitants.

The solubility of fibrinogen or of any other purified protein in concentrated salt solution is given by the relation

$$\log S = \beta - K_s\mu \qquad (1)$$

where S is the solubility of the protein and μ the ionic strength of the solution (5). In figure 1 the logarithm of the solubility of fibrinogen in solutions of various salts is plotted as ordinate and the ionic strength as abscissa. The slope of each curve, K_s, is a function of the protein and the neutral salt and is independent over wide ranges of pH and temperature. Among neutral salts those with monovalent cations and polyvalent anions precipitate proteins at lower concentrations. Ammonium sulfate and potassium phosphate solutions are conveniently used, the latter having the property of simultaneously controlling the acidity or pH (6, 20).

Whereas the slope, K_s, appears to be independent of pH and of tempera-

ture over wide ranges, the constant β defines the change in solubility with change in temperature or in pH (5). In general, it is found that the solubility is minimal when the protein is in an isoelectric condition or at a

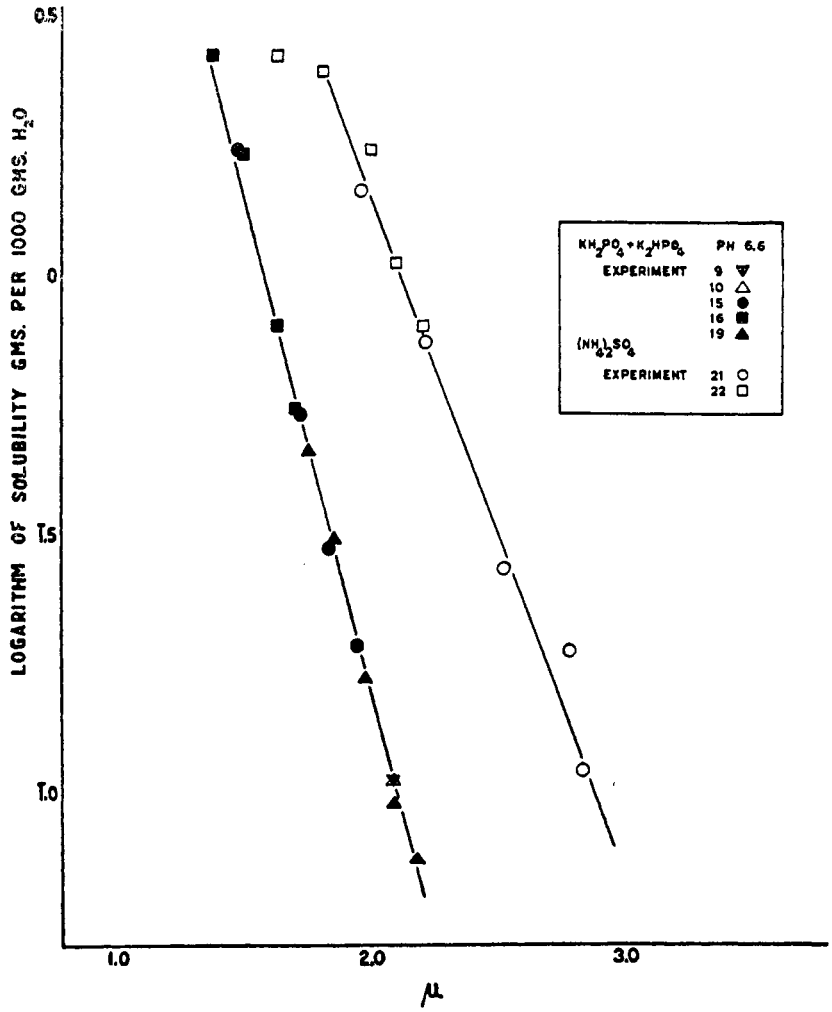

FIG. 1. Solubility of fibrinogen in concentrated phosphate and sulfate solutions at varying ionic strength. From Florkin (16).

reaction somewhat acid to the isoelectric point. This is true both in the absence and in the presence of salt. Separations between proteins can thus often be effected by neutral salt precipitation if they differ sufficiently (*a*) in K_s, (*b*) in β, (*c*) in the dependence of β upon temperature, or (*d*) in

the dependence of β upon pH. Of these, (*c*) is related to the heat of solution and (*d*) to the isoelectric point of the protein.

The alcohol precipitation of proteins at low temperature

Fibrinogen is also the first protein to be precipitated from serum or plasma by such organic solvents as acetone or the alcohols. That these conventional organic solvents should be protein precipitants depends upon the strongly polar nature of most proteins. Not only do acetone, alcohols, and many other organic solvents of low dielectric constant precipitate proteins, but when this operation is carried out at ordinary temperatures they bring about changes in the molecule of such a kind that their solubility in water all but vanishes, and the protein is said to be denatured, much as it is by high temperature.

If alcohol precipitation is carried out at sufficiently low temperatures (close to -5°C.), denaturation is often prevented, as was suggested by the earlier studies of Mellanby (49), of Hardy and Gardiner (26), and of many subsequent investigators (13, 80, 38), some concerned with the preparation of antibodies. Thus even egg albumin, a readily denatured protein, has been maintained in ethanol–water mixtures at -5°C. for protracted periods of time, the ethanol removed before the temperature was raised, and the unmodified protein recrystallized (15). Provided denaturation is prevented, alcohol precipitation methods can be substituted for salt precipitation methods. The same dependence of solubility upon pH obtains.[4] Alcohol precipitation methods, although more likely to denature labile proteins, have the decided advantage that precipitates from ethanol–water mixtures at low temperatures can be readily dried under a vacuum. This yields solid, salt-free, water-soluble, purified proteins which can be prepared in any desired amount by the large-scale methods of industry.

III. THE γ-GLOBULIN COMPONENTS OF PLASMA

If the concentration of salt or of ethanol is further increased after the removal of the precipitated fibrinogen, there separates a fraction of the plasma proteins which has been called in the recent literature the γ-globulin. This characterization is based upon the method of electrophoresis perfected by Tiselius (75).

Electrophoretic mobility

The observation that proteins move in an electric field was first made toward the end of the last century (64). The late Sir William Hardy (25) recognized the significance of the phenomenon as due to the amphoteric

[4] The dependence upon temperature is, however, quite different and is discussed in the last section of this paper.

properties of proteins, and he and subsequent workers,—notable among whom were Pauli (61, 62, 37) and Michaelis (52, 51),—studied a variety of proteins. Whereas electrophoresis in the hands of workers before Tiselius revealed the isoelectric point and the magnitude of the electric charge borne by the protein at varying acid and alkaline reactions, the further development of powerful optical tools, for which we are so largely indebted to Svedberg and his colleagues in Upsala (73), permit the observation of a series of moving boundaries, each revealing protein moving through the solvent with a characteristic electrophoretic mobility. In its most convenient form, the so-called Töpler schlieren (shadow) phenomenon (63, 74, 39) is employed to project on the photographic screen a pattern which can be resolved into a series of skewed probability curves, the area under each of which measures the concentration of the protein moving with each mobility. The mobility, u, calculated from the change with time of the center of mass of each area, is generally expressed in centimeters per second, when the protein is in an electric field with a gradient of 1 volt per centimeter. Such photographic diagrams for human, horse, bovine, and guinea pig serum are reproduced in figure 2, which thus illustrates differences between and similarities in the sera of different species.

Although there are differences in these schlieren diagrams, there are also very definite similarities, and the analysis of the proteins into electrophoretically different fractions is both convenient and valuable. At neutral reactions the fastest moving component of either plasma or serum is the albumin. Tiselius named the three other major components of serum the α-, β-, and γ-globulins. The last move most slowly in the electric field. Tiselius studied horse serum, but it was soon pointed out (72) that the behavior of human serum was comparable, and this is true in varying degrees of the other species that have been studied.

One of the great advantages of the electrophoretic method of analysis inheres in the convenience, reproducibility, and rapidity of the method. This may be illustrated by comparing the analysis of normal human serum into these four components that have been reported from three different laboratories, namely by Svensson (74) from Upsala, by Longsworth and his colleagues (44, 41, 40) from the Rockefeller Institute for Medical Research, and by Luetscher (42) from our laboratory. The ratio of the concentration of each globulin to the concentration of albumin in plasma is tabulated, thus avoiding both the factor of dilution and the factor of differences between plasma and serum. The good agreement between these results is quite gratifying and is none the less significant because each of the fractions revealed represents not a single protein but a population of proteins varying in size, shape, solubility, physiological and immunological function, and in many other respects.

The difference between the plasma and serum of the same species, depending on the absence in the latter of the characteristic fibrinogen peak, is represented in figure 3. Although fibrinogen is precipitated by lower concentrations of salt or of ethanol than the other serum proteins, it has

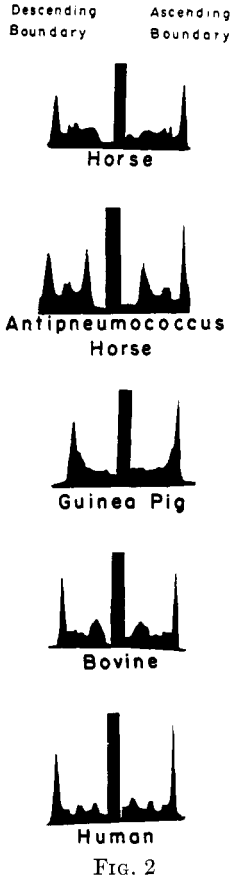

FIG. 2

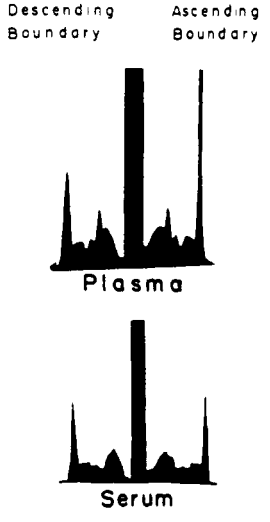

FIG. 3

FIG. 2. Electrophoretic schlieren patterns of human, bovine, guinea pig, and horse sera

FIG. 3. Electrophoretic schlieren patterns of bovine plasma and serum

a higher electrophoretic mobility at neutral reactions than γ-globulin, presumably owing to its more acid isoelectric point.

A comparative electrophoretic study, such as that upon serum and plasma, may reveal the diminution in concentration of a protein, in this case fibrinogen, but cannot be used to prove its absence. A more satisfactory analysis of a separation than that depending upon differences in

solutions containing many protein components can be carried out with the separated precipitates. Thus the diagram of fibrinogen separated from bovine plasma by precipitation with ethanol at low temperature is reproduced in figure 4. Here we have but a single peak with a mobility close to that of fibrinogen in the original plasma. The absence of proteins moving with grossly different mobilities is demonstrated by such an electrophoretic analysis, but here, too, electrophoretic analysis alone cannot prove that but one protein is present, since even appreciable amounts of proteins with closely the same mobility as the main component could not readily be detected.

The electrophoretic diagram of γ-globulin prepared from bovine plasma is also reproduced in figure 4. Although completely different from fibrinogen, both in form and function, and conveniently characterized by its electrophoretic mobility as representing approximately 12 per cent of human and 18 per cent of bovine plasma, the γ-globulin fraction contains

TABLE 1

Ratio of α-, β-, and γ-globulins to the albumin of human serum as revealed by electrophoretic analysis (42)

	$\frac{\alpha\text{-GLOBULIN}}{\text{ALBUMIN}}$	$\frac{\beta\text{-GLOBULIN}}{\text{ALBUMIN}}$	$\frac{\gamma\text{-GLOBULIN}}{\text{ALBUMIN}}$
Svensson	0.13	0.26	0.17
Longsworth	0.12	0.23	0.20
Luetscher	0.11	0.21	0.19

a fair number of proteins differing in isoelectric point, in molecular weight, and in solubility.

Isoelectric precipitation

Just a century ago a French physiologist, Denis, reported to the group of protein chemists who were collaborating with Liebig in Giessen that certain of the proteins of the blood, which we now call globulins, or euglobulins, were soluble in salt solutions, but not in water (67). The precipitation of fractions of plasma proteins by dilution and acidification has often been employed since then. Globulins are, however, readily denatured in dilute solution, and so our practice in the separation and purification of globulins has generally been to avoid dilution, i.e., to avoid lower concentrations of salt and of protein than are absolutely necessary in order to effect the separation. It is accordingly an advantage that the very convenient methods of dialysis now available permit the precipitation of proteins, when sufficiently free of salt, near their respective isoelectric points.

The γ-globulin fraction of plasma is rich in euglobulin. Here again, however, there are differences between species. Thus, roughly one-third of the γ-globulin fraction of the horse, but over two-thirds of that of the cow, is euglobulin. The bovine γ-globulin, the electrophoretic analysis of which is reproduced in figure 4, despite its apparent uniformity, yielded proteins isoelectric near both pH 6 and pH 7. That there was euglobulin

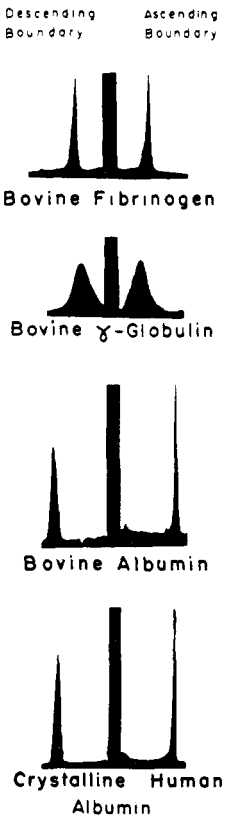

FIG. 4. Electrophoretic schlieren patterns of human and of bovine plasma proteins purified by ethanol–water fractionation.

isoelectric near pH 7 was first demonstrated in connection with the separation and purification of the pneumococcus antibody (13, 23). Euglobulins of isoelectric points near pH 5 and 6 have also previously been demonstrated (65, 21, 28). When the γ-globulin fraction is further fractionated by isoelectric precipitation into various euglobulins and pseudoglobulins, their electrophoretic mobilities prove not to be identical, although all are

generally within the area covered by the probability curve of the mean mobility of the γ-globulin.

Sedimentation constants and molecular weights

Proteins with the same net charge should have the same electrophoretic mobility, provided they have the same size and shape. The size and shape of proteins which appear to be electrophoretically homogeneous are, however, often very different. The ultracentrifuge, for which we are indebted to Svedberg (73), is by far the most powerful tool that we have to permit discrimination between molecules of different sizes and shapes, but the ultracentrifuge alone does not permit evaluation of molecular weights. These can be determined from the rate of sedimentation in the ultracentrifuge, only provided the shape of the molecule or its diffusion constant is known. The sedimentation constants of the proteins are, however, revealed directly by ultracentrifugal analysis, and those recorded here are for solutions containing 1 per cent of protein in potassium chloride of ionic strength 0.2, corrected to the density and viscosity of water at 20°C. but not to zero protein concentration.

The largest part of the γ-globulin of horse serum sediments with a constant of 6.2×10^{-13} cm. per second in unit centrifugal field (1 cm. per sec.^{2}), and bovine γ-globulin sediments with a constant estimated to be very nearly the same,—namely, 6.4×10^{-13}. Sedimentation constants of the order of 9, 12, 18, 32, and even 62×10^{-13} have been observed for euglobulins isoelectric at neutral reactions. This euglobulin fraction is rich in antipneumococcus antibody, and an increase in the amount of this and other antibodies is associated with a large increase in globulin, which often involves not only a relative increase but also an absolute increase in the total serum proteins. Certain of these antibodies have been considered γ-globulins, but some are thought to have slightly different mobilities (76, 77, 43, 60). Studies of Heidelberger and others (27, 34, 33) yielded a sedimentation constant for horse, pig, and cow antipneumococcus euglobulin of 18×10^{-13}, which, with measurements of free diffusion, suggested molecular weights of approximately 900,000, or close to six times the molecular weight of most other γ-globulins (27, 34, 33). The molecular weight of the γ-globulin of a sedimentation constant of 12×10^{-13} could, in fact, be smaller and that of 36×10^{-13} greater than 900,000.

Recently, Kabat (34) has observed that the molecules of this fraction, like fibrinogen, are very asymmetric in shape, revealing double refraction of flow, and it has been suggested that they seem to be polymers of smaller proteins conjugated end to end (79). These interesting results suggest how the process of immunization, resulting in molecules of large molecular weight and very small net charge at physiological reactions, can take place

TABLE 2

Estimated compositions, electrophoretic mobilities, and sedimentation constants of serum and plasma proteins

SOURCE OF PROTEINS	FIBRINOGEN	GLOBULINS			ALBUMIN
		γ	β	α	
	Proportion of total protein estimated by electrophoretic analysis, in per cent				
Human plasma (42)	6	12	13	7	62
Bovine plasma	18	18	8	16	40
Human serum		13	14	7	66
Bovine serum		22	10	20	48
Horse serum		24	21	13	42
	Electrophoretic mobilities of 1 per cent protein in phosphate buffer solutions of 0.2 ionic strength and pH 7.7 at 0°C.: $u_{20}^{1\%} \times 10^5$				
Human plasma (42)	1.9	0.9	2.7	3.8	5.1
Bovine plasma fractions (8)	2.1	1.6			5.2
Horse serum fractions (9)[7]*		1.9	2.9	3.9	4.5 5.3
	Sedimentation constants† of 1 per cent protein in potassium chloride solutions of 0.2 ionic strength: $s_{20,w}^{1\%} \times 10^{13}$				
Human plasma fractions					3.9
Bovine plasma fractions (8)	7.6	6.4 17			4.0
Horse serum fractions (27, 34, 33, 9)		6.2 17	6.3	6.8 17	3.9

* The values in serum are somewhat different. Studies thus far completed by J. M. Newell and S. H. Armstrong, Jr., suggest 1.7 for γ, 3.4 for β, 4.1 for α, and 5.6×10^{-5} for the total albumin. The crystalline carbohydrate-free albumin has a mobility of 5.3 and the 5.5 per cent carbohydrate-containing crystalline albumin of 4.5×10^{-5} cm.^{2} per volt-second.

† Svedberg and Pedersen (73) give a value of $s_{20,w}$ reduced to zero protein concentration of 7.1×10^{-13} for both total horse serum globulin obtained by half-saturation with ammonium sulfate and for electrophoretically prepared γ-globulin from normal human serum. They give the value 4.46×10^{-13} for the sedimentation constant of horse serum albumin, obtained by averaging the value for the A and B fractions prepared by Kekwick (35). McFarlane's (45) studies on diluted normal serum give 6.5–6.7 for horse and cow and 5.7 for human total globulin, and 4.2–4.4 for horse and cow and 3.9×10^{-13} for human albumin. The last value is in exact agreement with our study of purified human albumin.

without serious disturbances of water balance. These large globulins, existing close to their isoelectric points, would presumably make the smallest contributions to osmotic pressure of any of the plasma proteins.

Thus far certain antibodies have been located in the γ-globulin fraction. Another concern of the immunologist is complement, essential in certain antigen-antibody reactions such as the hemolysis of red blood cells by specific antisera. Long since recognized as associated with the physical-chemical state of the serum proteins, it would appear to depend upon the presence of but small amounts of a few components, one of which has recently been obtained as a euglobulin. Separated from serum by concentrations of ammonium sulfate generally employed in precipitating the γ-globulin fraction, and having a sedimentation constant of 6.4×10^{-13}, characteristic of most globulins, it has an electrophoretic mobility in 0.2 *M* phosphate buffer at pH 7.7 of 2.9×10^{-5}, possibly more characteristic of a β- than a γ-globulin. Its isoelectric point is close to pH 5.2 and it has been separated from other components of complement because it is quite insoluble in phosphate buffers of pH 5.2 and ionic strength 0.02, but soluble at this reaction at slightly higher ionic strengths.[5]

Prothrombin is generally precipitated from plasma with the γ-globulins, and its isoelectric point is also in the neighborhood of pH 5, at which point it is frequently separated by dilution of plasma and acidification. Its further purification from fibrinogen and other euglobulins has been carried out by the addition of calcium bicarbonate (50), or by isoelectric precipitation (10, 11, 68, 21). It is not our purpose in this survey to discuss the best methods for the preparation of each plasma protein, but rather to locate as on a contour map the various proteins which have thus far been identified, because of either their physical characteristics or their physiological functions.

As methods of separating the very labile protein molecules of physiological significance improve, it is probable that the well-characterized components of plasma will increase. Knowledge of the physical and chemical characteristics of such molecules should in turn increase our insight into the nature of their functions and interactions.

The γ-globulin fraction contains molecules which have not thus far been separated as euglobulins by exhaustive dialysis in the neighborhood of their isoelectric points and which may therefore be called pseudoglobulins. The γ-pseudoglobulin of horse serum has been very carefully studied and found to have an isoelectric point of 6.3, an electrophoretic mobility at 0°C. in phosphate buffer of 0.2 ionic strength at pH 7.7 of 1.9×10^{-5}, a sedi-

[5] The so-called "mid piece" and "end piece" of guinea pig serum have recently been purified and characterized by L. Pillemer of Western Reserve University, working in this laboratory.

mentation constant, $s_{20,w}$, of 6.2×10^{-13}, a diffusion constant, $D_{20,w}$, of 4.1×10^{-7}, and a partial specific volume of 0.730. Its molecular weight is thus calculated to be 142,000 (9). This fraction of γ-globulin, though it has a very small net charge, has proved to have the greatest influence in increasing the dielectric constant of solutions of any of the plasma proteins thus far investigated (14, 7, 58). Its electrical asymmetry is thus very great and this should result in strong interactions with ions and with other proteins.

IV. THE β-GLOBULIN COMPONENTS OF PLASMA

Globulins of electrophoretic mobility greater than γ-globulin and fibrinogen have been termed α- and β-globulins by Tiselius. In plasma or serum, β-globulins reveal a double peak (figure 2), suggesting the presence of two or more groups of molecules. Upon separation of the serum and reprecipitation, this double peak often appears to vanish. Purified globulins have, however, been prepared with mobilities intermediate between those characteristic of the β- and γ-globulins.

Whereas γ-globulin preparations are often nearly colorless, β-globulin fractions are often highly colored, some blue (21), some deep yellow (75), some rich in lipoids, and some rich in other organic molecules associated with this group of proteins. Their concentration in sera increases in lipoid nephrosis, cirrhosis of the liver, and other pathological conditions (24, 72, 2, 41, 42, 44, 40).

β-Globulins are precipitable by neutral salts (9) or by alcohols (8) at concentrations greater than those necessary for the precipitation of γ-globulins. Their electrophoretic mobilities at neutral reaction reflect greater net charge and more acid isoelectric points. In terms of equation 1 it does not follow that the "salting out" constant, K_s, is smaller for β- than for γ-globulins, although this may be the case. More probably, the solubility coefficient, β, is greater. Separation of globulins of more acid isoelectric points can thus be accomplished either by increasing the concentration of the precipitant or by bringing the acidity closer to the isoelectric point of each protein.

These generalizations apply equally to albumins, pseudoglobulins, and euglobulins. Euglobulins are, as we have seen, precipitable near their isoelectric point in the absence of salt. Such separations are, however, often carried out more satisfactorily after preliminary fractionation, so as to reduce interactions between proteins of very different isoelectric point. Evidence that there are euglobulins in serum differing in isoelectric points has come not only from electrophoretic but also from solubility (13, 65, 21, 23) and immunological (18, 34, 36, 27, 33, 43, 60) studies. Most proteins isoelectric at neutral reactions are, as we have seen, part of the

γ-globulin fraction. On the other hand, the one component of complement (see footnote 5), prothrombin, and the very characteristic blue-green, jelly-like euglobulin, with an isoelectric point close to pH 5 (described by Green as P_1), appear to move in the electric field "with the β-globulin," as does a globulin component with an isoelectric point near pH 6 (42).

Those proteins characterized in the electrophoretic analysis of whole serum or plasma as β- or γ-globulins would thus appear to consist of various euglobulins and pseudoglobulins, of slightly different isoelectric point and acid- and base-combining capacities. Some of these are presumably present in but small amount, and as in the case of the γ-globulin, some may have molecular weights that are both higher and lower than is characteristic of most of the β-globulin. A fair amount of the β-globulin of horse serum, upon purification, appears to have a lower sedimentation constant in the presence of high protein concentration, presumably owing to dissociation of β-globulin into components of smaller molecular weights (45, 73, 9). The sedimentation constants and molecular weights of most of the globulin fractions would appear, however, to be closely similar (55, 45, 35), but this may not be true of their shapes (12, 56), phosphorus or carbohydrate contents, dielectric dispersion curves, or other chemical or physicochemical properties which reflect in more detail the fine structure of molecules upon which their physiological functions presumably depend.

V. THE α-GLOBULIN COMPONENTS OF PLASMA

The chemical separation of α- and β-globulins from γ-globulins is far simpler than the separation of α- and β-globulins from each other. α-Globulin is generally, although not always, represented by a single peak, apparently moving with uniform electrophoretic mobility in serum or plasma. Precipitable by higher concentrations of ammonium sulfate than β- or γ-globulin (9), α-globulin consists, in the main, of water-soluble pseudoglobulins, isoelectric near pH 5. There are euglobulins, however, which move with the electrophoretic mobility of the α-globulin fraction; among them is another component of complement, the so-called "end piece", which has been separated in a state of considerable purity, has an electrophoretic mobility of 4.2×10^{-5}, and contains 10.3 per cent of carbohydrate.[5] The experimental conditions which it was necessary to achieve in order that this component, previously believed to be pseudoglobulin in nature, would separate as a euglobulin suggest that other substances associated with the α-globulin may also ultimately be isolated as euglobulins. It is possible that the so-called mucoglobulin (28), as well as certain other carbohydrate-rich globulins (71, 35), and perhaps also the crystals reported by Jameson (30, 31), may have been α-globulins.

The α- and β-globulin fractions of plasma are often more labile than

either the γ-globulin or the albumin fractions. They also contain a large proportion of the chromogenic groups associated with serum proteins. It is not impossible that their lability is related to changes in these associated groups.

α-Globulin preparations, uniform electrophoretically, have revealed diverse sedimentation constants suggesting components of different molecular weights (43, 60, 9). In how far these components represented β-globulin or albumin rich in carbohydrate impurities remains uncertain, but in the case of both fractionated horse and bovine serum proteins, ultracentrifugal analyses suggested far higher concentrations of molecules of the size of the albumins than electrophoretic analyses indicated were present.

The functions of the α- and β-globulins are presumably different from those of either the γ-globulins or the albumins. They are different immunologically and most antibodies have been associated with the γ fraction, which increases in amount with immunity. In febrile conditions, on the other hand, it is the α-globulin fraction which largely increases (44, 41, 40). In lipoid nephrosis, in which fibrinogen increases in the plasma, so also do the α- and β-globulins, whereas the γ-globulin and albumins are greatly diminished. Conversely, albumin, and to some extent γ-globulin, are found in lymph (57) and perhaps in other body fluids (42) in larger amounts than α- and β-globulins, which would seem to leave the blood less readily (44, 41, 40). More exact characterization of these various plasma proteins, under varying physicochemical conditions, and their availability as purified proteins in adequate amounts for physiological and clinical investigation should greatly increase our knowledge, not only of the nature and the function of blood proteins, but also of kidney and tissue permeability.

VI. THE ALBUMIN COMPONENTS OF PLASMA

Albumins are by definition soluble in water. There are, however, many water-soluble proteins in serum and plasma which are far more closely related to the globulins than to the albumins and which we have termed pseudoglobulins. They move in the electric field with the mobility characteristic of globulins and have sedimentation constants characteristic of globulins but are often completely "salted out" only at concentrations which effect the precipitation of albumins.

Serum albumins are precipitated by neutral salts or by organic solvents at higher concentrations than are most globulins. One of the conventional methods for their preparation is to half-saturate plasma with ammonium sulfate, and to purify the albumin from the filtrate. Whereas very little albumin is precipitated in neutral solution by half-saturation with ammonium sulfate, that is to say, from a 2 molal solution of ammonium

sulfate at ordinary temperatures, the filtrate always contains globulin, some rich in carbohydrate (28, 76, 66) and some closely related to the α- and β-globulins.

The further purification of the serum albumins is accomplished either by increasing the concentration of the precipitant or by increasing the acidity. Serum albumin is isoelectric near pH 4.8. This is true at least for the albumin of human, horse, and bovine serum or plasma. The albumins in the blood are thus far from their isoelectric point, are combined with more base than are the globulins, and therefore would have a greater electrophoretic mobility even were they of the same molecular weight as the globulins.

The molecular weight of most albumins is in the neighborhood of 70,000, or approximately half of that of most of the globulins, and this also would lead to greater electrophoretic mobilities, as well as to greater osmotic pressures per gram of protein. Although albumins have a greater net charge at neutral reactions, as well as a greater number of charged groups in the isoelectric condition, these are arranged with far greater symmetry. As a result, albumins have the smallest electric moments thus far observed for any proteins. They should thus interact less with other proteins, as well as with salts. Albumin solutions are also more limpid and exhibit the Tyndall effect to but a negligible extent. They are, moreover, far less readily denatured than are most other proteins.[6]

Immunologically, also, the albumins may be contrasted with the globulins. The purified albumins may be sharply differentiated from the γ-globulins, the chief antibodies of the blood stream, by serological methods (32, 28).

Although albumins can thus be contrasted with globulins, the albumins of different species and indeed the albumins of the same species are not homogeneous. This may be shown chemically or immunologically. Thus the purified albumins of man, horse, and cow seem to be immunologically distinct from one another (32). The albumins of the horse crystallize readily from serum, while those of man crystallize only after being separated from α- and β-globulins and lipoids. None the less, the electrophoretic mobilities, sedimentation constants, and many other properties of the albumins of different species are closely comparable (table 2).

Different albumins may be demonstrated to be present in any of these species. The readily crystallizable horse serum albumins have been the most studied (69, 70, 7, 14, 28, 35, 46, 58, 66). Albumin may be crystallized

[6] Indeed, it is possible to purify albumins of certain other protein impurities by permitting the latter to denature in 15 or 20 per cent ethanol at pH 4.8 at temperatures slightly above zero, separating the precipitate, and, in the case of human protein fractions, crystallizing the albumin.

if it is carbohydrate-free or if it contains as much as 5.5 per cent of carbohydrate (46). Among the crystalbumins, a further separation can be made, in that a portion thereof may be crystallized from salt-free solution as a sulfate[7] (46), although the crystal forms of the insoluble and soluble sulfates appear to be identical. Another type of crystalline albumin, hemocuprein, containing copper, has been isolated by Mann and Keilin (47) and confirmed in our laboratory. Its function remains to be discovered; it is not respiratory and may well be enzymatic. On the other hand, the choline esterase and phosphatase of serum, the lipase and iodine-containing proteins, as well as other hormones and enzymes which separate with the albumin fraction, remain to be crystallized.

The electrophoretic mobilities of all of the serum albumins are not identical. That of hemocuprein has not been reported, although its molecular weight is said to be smaller than that generally ascribed to serum albumin. Whereas the sedimentation constant of the crystalline albumin containing 5.5 per cent of carbohydrate appears to be very nearly that of carbohydrate-free albumin, its electrophoretic mobility has been demonstrated to be appreciably smaller at pH 7.7, that is to say, at the reaction at which all of the electrophoretic mobilities thus far reported in this communication were determined.

It is probable that other crystalline serum albumins will in time be isolated and their properties and functions accurately determined. It has long been known that even the beautifully crystalline serum albumin of the horse does not consist of a single chemical individual as judged by solubility (69, 70) or by dielectric studies (14, 7, 58), even though homogeneous as to molecular weight or electrophoretic mobility at neutral reactions.

The crystallization of salt-free albumin sulfate was carried out near pH 4 (46).[7] At this reaction not only horse but also bovine and human albumin revealed components of different electrophoretic mobility, the faster moving albumin constituting nearly two-thirds of the total (42). In certain pathological conditions in man (among them lipoid nephrosis and cirrhosis of the liver), however, electrophoretic analysis of both serum and urinary albumins showed a greater loss of the faster moving albumin from the blood, leaving the slower component preponderant. Studies of the dielectric properties of the albumin separated from the urine of these conditions yielded a fraction with a dielectric increment far greater than that generally ascribed to serum albumin (17). Since the ratio of albumin components appeared to be the same in blood and in urine, differential per-

[7] An observation reported by J. D. Ferry at the Division of Biological Chemistry at the Ninety-sixth Meeting of the American Chemical Society, held at Milwaukee, September 7, 1938.

meability for excretion was not assumed, but rather a differential production of the two albumins in the body (42).

Clearly, further studies are necessary and possible not only on the loss under pathological conditions from the blood to the urine or the tissues of various proteins, but of their various functions in the blood. The albumins and γ-globulins would appear to be lost more readily than the α- or β-globulins. On the other hand, the influence of the albumins on water balance must be greater than that of the other proteins. Whereas the albumins represent over 60 per cent of the proteins of normal human plasma, they give rise to an even greater proportion of the blood osmotic pressure by virtue of their smaller size and larger net charge. These properties and their lower viscosity, more symmetrical shape, and charge distribution, and their far greater solubility and stability in solution separate them in property and function from the more labile, asymmetric, and viscous globulin fractions.

The preparation of large amounts of relatively pure albumin is now possible. Although knowledge of the conditions for the crystallization of various serum albumins, especially those of horse serum (28, 35, 46; footnote 7), from concentrated solutions of sulfates or phosphates has been greatly extended in recent years, this method is not susceptible to large-scale preparation. The method of fractionating serum or plasma proteins by equilibration across membranes with ethanol–water mixtures of controlled pH, ionic strength, and temperature, which also yields fibrinogen and γ-globulin fractions approaching homogeneity with respect to both size and net charge, is especially advantageous in the separation and purification of the albumins (8). Schlieren diagrams of these fractions separated from plasma are illustrated in figure 4. Whereas fibrinogen and all the γ-globulins, as well as a fair amount of the α- and β-globulins, are largely precipitated by 40 per cent ethanol at -5°C., the solubility of these is further reduced if the acidity is increased to approximately pH 5.5, preferably by acetate or carbonate buffers.[8] The albumin remaining in solution in 40 per cent ethanol at pH 5.5 at -5°C. is largely precipitated from this solvent at pH 4.4–4.8, as is also the small amount remaining in solution at this temperature by concentration at -15°C. Albumin, both human and bovine, has been prepared by this method and is pure both electrophoretically and in the ultracentrifuge, and there is practically no

[8] Equilibration with carbonate buffers, which we are further developing in collaboration with Dr. R. M. Ferry, has the advantage that, at constant carbon dioxide pressure, increase in acidity is produced by diminution of free base. Its removal from the protein solution by dialysis through membranes is favored by the Donnan equilibrium, whereas the introduction of other acids to the protein phase by diffusion across membranes is impeded by the Donnan equilibrium.

upper limit to the amounts of this material (as of fibrinogen and the γ-globulins) that can readily be made available.

The albumin precipitable from 40 per cent ethanol is readily dried and is a colorless white powder, free of reducing substances and indeed of other organic and inorganic molecules for which we have thus far tested. It readily dissolves in water, yielding clear solutions even at concentrations greater than 25 per cent. In our experience no precipitate appears in such solutions, even after they have stood for protracted periods of time. Indeed, albumin purified in this way appears to be stable for short periods of time even in 20 per cent ethanol at room temperature.[6]

Albumin that appears to be uniform both electrophoretically and ultracentrifugally can be further fractionated by ethanol–water mixtures. Thus, a fraction of the albumin which contains traces of globulin has a high heat of solution and dissolves as an oil, with increase in temperature, and is insoluble in 25 per cent ethanol at pH 4.8 and 0°C.; another fraction is soluble even in 20 per cent ethanol at -5°C., but insoluble in 40 per cent ethanol at -5° and -15°C. The fractionation of albumins in alcohol–water mixtures by change in temperature is the more interesting because the heat of solution in these solvents is opposite in sign[9] to that in the concentrated ammonium sulfate solutions in which albumins have heretofore generally been fractionated and crystallized. Human albumin is readily crystallized from ammonium sulfate after such fractionation.

The availability of bovine albumins is making possible a systematic study of the chemical modification of their free groups by reagents which are yielding proteins of new physical-chemical properties and new immunological behavior. Experiments which will be reported elsewhere are now under way in which amino groups are replaced by guanidine,[10] acetyl, ureide, or other groups and in which the other basic groups or the phenolic hydroxyl groups of the proteins are modified. Modification of carboxyl groups, although often leading to changed antigenicity, has seemed a less promising approach to present needs, since the preparation of proteins of modified antigenicity but of unmodified, or increased, rather than decreased, osmotic pressure at neutral reactions is our aim. Such modified

[9] The theoretical consideration of these differences, involving the theory of "salting out" and of electrostatic interaction, will be considered elsewhere.

[10] Treatment of the albumin of horse or bovine serum with methylisourea (for a considerable supply of which we are indebted to the American Cyanamid Company) has yielded euglobulins. Thus, W. L. Hughes and the author have succeeded in separating water-insoluble, salt-soluble globulin from albumin, the amino groups of which had been guanidinated. The immune properties had, however, not been greatly changed by this complete change in solubility behavior, nor probably had the net charge or electric moments of the molecules.

proteins derived from bovine plasma might prove especially suitable for transfusion after a period of careful clinical investigation.

Human albumin, as well as bovine albumin, can be prepared by the above procedures even from plasma or serum containing so much hemolyzed blood as to render it unsuitable for transfusion. Such purified albumin in solution, freed from the more labile and more readily denatured fibrinogen and globulins, should prove useful for a number of therapeutic purposes, particularly in the treatment of shock, due to acute loss of blood. There is good reason to believe that its marked osmotic effect will draw water into the blood vessels, raise the total blood volume, and thus relieve the condition, provided it is not too rapidly lost from the blood stream because of its smaller molecular weight. From a physicochemical point of view, human and bovine albumin are remarkably similar molecules and the latter could be used as a substitute for human albumin provided no clinical contradictions are demonstrated.

The multiplicity of functions performed by the plasma proteins remains larger than the number of pure proteins that have thus far been isolated from serum or plasma. The methods would appear to be available, however, for the purification of any protein for which there are adequate methods of bioassay. The procedures now being carried out on a large scale, with both the human and the bovine plasma, yield, in separate fractions, fibrinogen, three γ-globulins, prothrombin, α- and β-globulins, and the serum albumins. These developments, which permit the separation of molecules of closely similar properties, depend less on the discovery of new chemical reagents than upon the development of the theory and the more precise control of the conditions upon which purifications depend and on increasing knowledge of the physicochemical properties of the proteins.

SUMMARY

1. The diverse nature of the plasma proteins is discussed and the physical-chemical bases for their characterization defined.

2. The physiological functions of certain of the proteins are considered in relation to their chemical functions.

3. The large-scale preparation of the different proteins of human and of animal plasma in purified states renders possible not only chemical but also clinical investigations of their functions and possible therapeutic uses.

4. Although albumins may be lost from the circulation more readily than certain globulins, their smaller size and greater net charge lead to greater osmotic effects. The high osmotic pressure, low viscosity, and great stability of albumin solutions would appear to render them the most useful of the plasma proteins for the treatment of some, but not necessarily all, conditions associated with diminished plasma volume.

5. Comparative studies on the physical, chemical, and immunological properties of bovine, of chemically modified bovine, and of human albumins and globulins are considered.

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