7. The properties of adsorbed films vary according to the type of interface involved. The five interfaces used in this work may be classified as belonging to two different types, the members of either of which are much alike in their effects on films adsorbed upon them. In one group are the solution–air and the solution–paraffin interfaces, and in the other the solution–talc, the solution–stibnite and the solution–graphite interfaces.

8. The pressure–area relations and the wetting characteristics of films of adsorbed organic molecules are highly dependent on the type of polar group in the molecules.

9. A moderate change in the length of the hydrocarbon chain in the adsorbed organic molecule makes little difference in the pressure–area relations, but a great deal in the concentration of the organic compound necessary to give an adsorbed film of any certain film pressure.

10. That the films investigated in this work are monolayers, seems evident. Thus the lowest areas found per molecule are considerably larger than the minimum area in monolayers on the surface of water, which is 20 Å.[2] for *n*-long chain paraffin derivatives, and should be somewhat less than this for molecules as small as those of acetic acid.

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[CONTRIBUTION FROM THE DEPARTMENT OF PHYSICAL CHEMISTRY, HARVARD MEDICAL SCHOOL]

Preparation and Properties of Serum and Plasma Proteins. I. Size and Charge of Proteins Separating upon Equilibration across Membranes with Ammonium Sulfate Solutions of Controlled *p*H, Ionic Strength and Temperature

BY E. J. COHN, T. L. MCMEEKIN, J. L. ONCLEY, J. M. NEWELL AND W. L. HUGHES

Ever since the beginning of the last century it has been known that blood plasma or serum is a complex system containing a large number of protein components. Certain of these are water-soluble albumins, others are classified as globulins, soluble in neutral salt solutions, but not in water. Separation of globulins from albumins may be effected by dialysis and electrodialysis at reactions close to the isoelectric points of the respective proteins, or by "salting out" methods, ammonium or sodium sulfate or sodium or potassium phosphate being most generally used.[1] Water-insoluble, salt-soluble globulins are largely precipitated by half-saturated ammonium sulfate, whereas the water-soluble albumins require far higher concentrations of neutral salts for precipitation. The ratio of albumins to globulins reported is often the ratio of proteins remaining in solution to those precipitated at half saturation with ammonium sulfate under certain standardized conditions, or some method based on this relation.

The larger part of the protein precipitated by half-saturated ammonium sulfate is not true globulin (euglobulin) in the sense that it is insoluble in water. In the accumulated literature regarding plasma and serum proteins part is, or appears to be, water-soluble pseudoglobulin. Unlike the true serum globulins, the pseudoglobulins cannot be purified by isoelectric precipitation from salt-free solution, and unlike the hemoglobins, haemocuprein and the serum albumins, they have not thus far been prepared in a crystalline state. Like the true globulins and the albumins, pseudoglobulins can, however, be concentrated and purified by repeated reprecipitations by neutral salts.

The solubility of any purified protein in concentrated salt solution is given by the relation $\log S = \beta - K_s C$, where S is the solubility of the protein; C, the concentration of the neutral salt sometimes expressed as moles per liter, sometimes as moles per 1000 g. of water; K_s, the salting-out constant, a function of the protein and of the neutral salt but independent over wide ranges of *p*H and temperature; and β, a constant relating change in solubility to change in *p*H.[2] If two proteins of widely different properties are studied, analysis of their solubilities may be effected by means of the above equation. Whereas the solubility of the proteins in serum has been carefully

(1) Much of the literature on this subject not specifically referred to in this communication is considered in references (2–13)(17)(19)(22) and in the recent studies of H. Wu, *Chinese J. Physiol.*, **7**, 125 (1933); L. F. Hewitt, *Biochem. J.*, **28**, 2080 (1934); **30**, 2229 (1936); **31**, 360 (1937); Goettsch and Kendall, *J. Biol. Chem.*, **109**, 221 (1935); and R. A. Kekwick, *Biochem. J.*, **32**, 552 (1938).

(2) E. J. Cohn, *Physiol. Rev.*, **5**, 349 (1925); *Ann. Rev. Biochem.*, **4**, 93 (1935); *Harvey Lectures*, 124 (1938–1939).

investigated in phosphate buffers of known pH, temperature and ionic strength,[3] the large number of protein components in the system has rendered analysis of the resultant "salting out" curve difficult.

More recently a method of analyzing plasma or serum proteins in terms of their electrophoretic mobilities has been perfected by Tiselius.[4] The optical systems developed[5] permit analysis in terms of the major components migrating in an electric field with different velocities in a buffer solution of known pH, ionic strength and temperature. The temperature of maximum density of water, 4°, has proved most advantageous.[4] Although other buffers have also been employed, there is an accumulation of mobility studies upon plasma and serum components in phosphate buffers. They include studies upon the serum and plasma of different species, as well as of human plasma, which has been studied under a wide variety of pathological conditions,[6–10] and reveal fibrinogen, globulins moving with at least three velocities and designated by Tiselius as α, β and γ, and a still faster moving albumin component. A schlieren diagram of the electrophoretic pattern of horse serum is given in Fig. 1.

Analysis in the ultracentrifuge, developed by Svedberg, reveals three main components of serum which sediment with different velocities, an albumin and two globulins. The sedimentation constants for these horse serum proteins given by Svedberg and Pedersen[11] are, respectively, $s_{20} \times 10^{13} = 4.46$, 7.1 and 19.3. Estimates of the molecular weights derived from these sedimentation constants and from diffusion studies are 70,000, 147,000 and 910,000. The first represents the albumin and the second, most of the globulin,[12] while the third represents a small proportion of the globulin, important in immunity.[13]

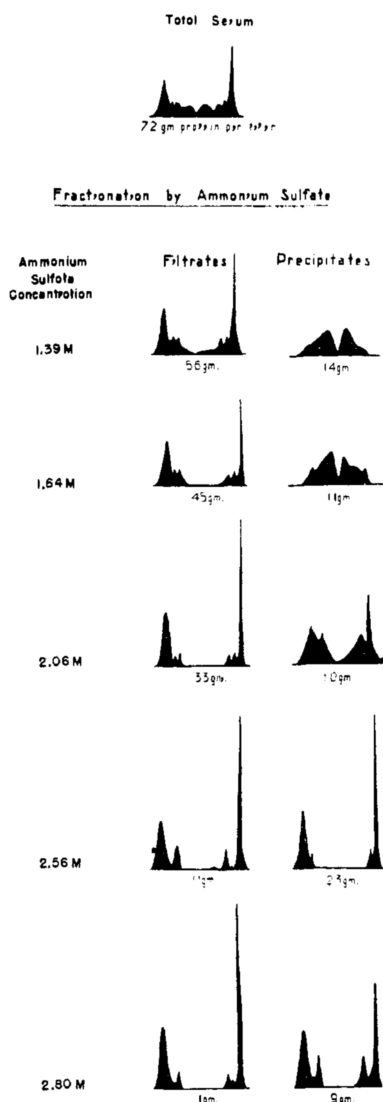

Fig. 1.—Normal horse serum: electrophoretic studies on 2% protein solutions in phosphate buffer of ionic strength 0.1 at pH 7.4.

These recent important developments thus permit far greater insight into the molecular species of protein systems than was hitherto possible. It seemed important to employ them in the accurate control of the fractionation procedures that have been developed during the last century for the separation from serum and the subsequent purification of proteins by isoelectric and neutral salt precipitation, by dialysis and electrodialysis. In this way it was hoped that many of the pro-

(3) Butler and Montgomery, *J. Biol. Chem.*, **99**, 173 (1932); D. M. Kydd, *ibid.*, **107**, 747 (1934); Butler, Blatt and Southgate, *ibid.*, **109**, 755 (1935).

(4) A. Tiselius, *Nova Acta Soc. Sci. Upsal.*, **4**, 7 (1930); *Biochem. J.*, **31**, 313, 1464 (1937); *Trans. Faraday Soc.*, **33**, 524 (1937).

(5) J. St. L. Philpot, *Nature*, **141**, 283 (1938); H. Svensson, *Kolloid-Z.*, **87**, 181 (1939); L. G. Longsworth, THIS JOURNAL, **61**, 529 (1939).

(6) E. Stenhagen, *Biochem. J.*, **32**, 714 (1938).

(7) G. Blix, *Z. ges. exptl. Med.*, **105**, 595 (1939).

(8) Longsworth and MacInnes, *Chem. Rev.*, **24**, 271 (1939); *J. Exptl. Med.*, **71**, 77 (1940); Longsworth, Shedlovsky and MacInnes, *ibid.*, **70**, 399 (1939).

(9) R. A. Kekwick, *Biochem. J.*, **33**, 1122 (1939).

(10) J. A. Luetscher, Jr., THIS JOURNAL, **61**, 2888 (1939); *J. Clin. Investigation*, **19**, 313 (1940).

(11) Svedberg and Pedersen, "The Ultracentrifuge," Oxford University Press, London, 1940.

(12) P. v. Mutzenbecher, *Biochem. Z.*, **266**, 226, 250, 259 (1933); A. S. McFarlane, *Biochem. J.*, **29**, 407, 660, 1175, 1209 (1935); R. A. Kekwick, *ibid.*, **32**, 552 (1938).

(13) M. Heidelberger, *Cold Spring Harbor Symp. Quant. Biol.*, **6**, 369 (1938); *Chem. Rev.*, **24**, 323 (1939); Kabat and Pedersen, *Science*, **87**, 372 (1938); E. A. Kabat, *J. Exptl. Med.*, **69**, 103 (1939).

tein components of serum, some albumin, some euglobulin, some pseudoglobulin, some antibodies, some hormones and some enzymes, each homogeneous with respect to size and net charge, could be prepared in states of purity in sufficient amounts to render possible their further investigation as chemical individuals.

I. Fractionation by Ammonium Sulfate

The most frequently employed procedure in the preparation of serum proteins is precipitation by ammonium sulfate. In order to determine the limits in ammonium sulfate concentration for precipitation of each of the proteins revealed by electrophoretic mobility, varying amounts of salt were dialyzed into serum[14] through cellophane membranes according to a method previously described.[15-17] In this method water is extracted by salt solution and precautions were therefore taken, after the separation by centrifugation or filtration of each precipitate, to wash the precipitate with small aliquots of ammonium sulfate of the concentration at which separation was effected in order to avoid gross occlusions, adding the washings to the solution and thus restoring the volume to that to which the serum proteins were originally diluted for the fractionation. In the experiments reported, the volume was maintained at twice that of the original serum, since separations appear to be slightly more effective if the proteins are in a somewhat less concentrated solution than serum. Experiments have been carried out in which the temperature has been at 0 and 25°. In those reported to illustrate procedure, room temperature was employed and the reaction was maintained near *p*H 6.

The electrophoretic patterns of the precipitates and filtrates separated are reported in Fig. 1. The protein was dialyzed against either a large volume or repeated changes of the buffer solution employed in electrophoresis, and brought to one (or in earlier series to two) per cent., as judged by refractive index, for electrophoresis. As a result, the areas under each schlieren diagram are the same, the peaks in the diagrams of the ascending column, on the right, always being higher than those of the descending columns. These results could only be employed in the calculation of protein concentrations and mobilities if corrected by some method such as that of Longsworth and MacInnes.[18] In practice, when we wished to measure mobilities accurately, far more dilute protein solutions of approximately 0.2% were employed, in which case the ascending and descending boundaries were more nearly symmetrical, and the corrections in the calculation of mobilities become negligible. More concentrated solutions are preferable, however, if it is desired to discover small amounts of protein impurities moving with different mobilities than the main component.

The amount of protein precipitated and that remaining in solution, reported in Fig. 1, refer to grams of original serum. Thus, 14 g. of the total 72 g. protein per liter of serum was precipitated at one-third saturation with ammonium sulfate; the concentration so often employed in immunological studies. This precipitate appears to consist almost completely of γ-globulin. Protein moving with this mobility—as well as β-globulin—is also precipitated from 0.34 to 0.40 saturated ammonium sulfate, which is therefore a less pure precipitate than the first. The filtrate at the higher concentration is, however, almost completely free of the γ component.

The fraction from 0.40 to 0.50 saturated ammonium sulfate, which is also often employed in the preparation of antitoxins, is thus largely free of γ-globulin and consists almost completely of α- and β-globulins, together with a trace of albumin. This is the fraction that we have employed in the further purification of proteins with the mobility of the α- and β-globulins of the whole serum.

The fraction precipitating from horse serum from 0.50 to 0.62 saturated ammonium sulfate is largely albumin and largely crystalline if the salt is added with sufficient care through a cellophane membrane, and has been the starting material for the isolation both of carbohydrate-free crystalline albumin[17] and for that of the 5-5% carbohydrate-containing crystalline albumin.[19] The electrophoretic pattern in Fig. 1 also clearly reveals protein of mobility close to that of α-globulin in the precipitates beyond half-saturated ammonium sulfate. The filtrate at 0.62 saturation and the precipitate of 0.68 saturation contain, moreover, appreciable amounts of material with a mobility closer to that of β-globulin. The nature of these

(14) For which we are indebted to Dr. E. S. A. Robinson of the Massachusetts State Antitoxin Laboratory.

(15) H. Theorell, *Biochem. Z.*, **268**, 46 (1934).

(16) Green, Cohn and Blanchard, *J. Biol. Chem.*, **109**, 631 (1935); V. E. Morgan, *ibid.*, **112**, 557 (1936).

(17) T. L. McMeekin, THIS JOURNAL, **61**, 2884 (1939).

(18) Longsworth and MacInnes, *ibid.*, **62**, 705 (1940).

(19) T. L. McMeekin, *ibid.*, **62**, 3393 (1940).

proteins we shall consider subsequently. The danger inherent in completely characterizing proteins in terms either of their solubilities in concentrated salt solution or of their mobilities, without taking account of their other properties, cannot, however, be overemphasized. Results giving the per cent. protein precipitated from horse serum by ammonium sulfate and its probable nature are summarized in Table I.

TABLE I

SERUM PROTEINS OF THE HORSE

Concentration of ammonium sulfate Moles per liter	% of satn.	Type of protein precipitated	% of total protein pptd.	% of protein pptd. Water soluble	Water in-soluble
1.39	0.34	Largely γ-globulin	20	71	29
1.64	.40	γ-, β-, α-Globulin	15	67	33
2.05	.50	β-, α-Globulin, mucoglobulin	14	94	6
2.57	.62	Largely crystalline albumins	32	98	2
2.80	.68	Crystalline albumins, haemocuprein, choline esterase, glycoprotein, phosphatase	14	99	1

Although the order of precipitation is essentially the same for other species, as we shall demonstrate in subsequent communications, the per cent. of the various components and the concentrations of salt for their precipitation, vary appreciably.

II. Fractionation by Dialysis

The classical nomenclature distinguishes euglobulins from pseudoglobulins, and the precipitates separating at the various ammonium sulfate concentrations, whose electrophoretic patterns are given in Fig. 1, can be further fractionated by dialysis. The per cent. insoluble in water after simple dialysis in thin cellophane tubing against cold, running, distilled water, is given in the last column of Table I. Almost a third of all the proteins precipitating up to 0.40 saturated ammonium sulfate precipitated on dialysis. Far more precipitated, as we shall see, on being separated (a) from the small remaining amounts of salt by electrodialysis, and (b) from the solvent action of the various proteins upon each other.

The electrophoretic patterns of the euglobulins and pseudoglobulins precipitated at 0.34 and 0.40 saturated ammonium sulfate are reported in Fig. 2. Whereas the total globulin separating at the lower concentration appeared to superficial analysis (Fig. 1) to consist almost completely of γ-globulin, the euglobulin derived from it revealed at least three distinct peaks previously masked by the larger amount of γ-pseudoglobulin present. The euglobulins in Fig. 2 have been further purified by precipitation at their isoelectric points by A. A. Green.[20] Methods that have yielded the α-, β- and γ-globulins approaching homogeneity with respect to mobility and size may now be considered.

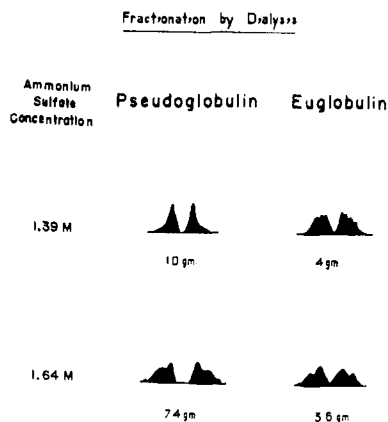

Fig. 2.—Globulins of normal horse serum: electrophoretic studies on 2% protein in phosphate buffer of ionic strength 0.2 at pH 7.7.

III. Purification of γ-Globulin

For the separation of γ-globulin,[21] the precipitate insoluble at 0.34 saturated ammonium sulfate (1.39 M) was purified by dialysis and repeated precipitation with ammonium sulfate. The protein soluble in 0.40 saturated (1.64 M) but insoluble in 0.50 saturated (2.05 M) ammonium sulfate was used for the further fractionation of the α- and β-globulins and the filtrate from 0.50 saturated ammonium sulfate for crystallization of albumins.

Each liter of serum was made to a volume of two liters by adding 580 cc. of distilled water, 400 cc. of saturated ammonium sulfate and 20 cc. of normal sulfuric acid, giving a protein solution of approximately 3.5% and a pH near 6.0. Ammonium sulfate was slowly added to the serum through a cellophane membrane until the concentration of salt was 0.34 saturated (1.39 M). After standing for twenty-four hours, the precipitate was removed by filtration on a Büchner funnel and washed thoroughly with a 0.34 saturated ammonium sulfate solution. This precipitate served as the starting material for various γ-globulin

(20) A. A. Green, THIS JOURNAL, 60, 1108 (1938).

(21) We are also indebted to Dr. A. A. Green for several preparations of γ-pseudoglobulin fractionated in a somewhat different manner.

purifications. Details of one such purification are given as an example of procedure.

The precipitate at 0.34 saturation from 24 liters of serum contained 360 g. of protein in Preparation IV. On dialysis and dilution, 140 g. of protein precipitated, leaving 220 g. of protein in a volume of 15,420 cc. of solution. Ammonium sulfate was added gradually until the concentration was 1.22 *M*. After the removal of the precipitate the fraction precipitating at this concentration contained 40 g. of protein. More ammonium sulfate was added to the filtrate and the precipitate of 102 g. was removed at 1.47 *M* and 51 g. at 1.86 *M*, leaving a small fraction in the filtrate.

Only the first two fractions were further purified by reprecipitation with ammonium sulfate. The first fraction was twice reprecipitated at 1.22 *M* ammonium sulfate, the yield being 20 g. The middle fraction was also twice reprecipitated at approximately 1.22 *M* ammonium sulfate, yielding 50 g. of insoluble material. These fractions had identical electrophoretic patterns and were combined and again precipitated at 1.22 *M* ammonium sulfate, yielding 47 g. They still contained a trace of protein moving faster than γ-globulin. Ten grams of this material was removed and the remainder once more precipitated at 1.22 *M* ammonium sulfate, yielding 34 g. of protein. Its electrophoretic pattern is reported in Fig. 3 and indicated only proteins of a single mobility. More electrophoretically pure γ-globulin presumably could have been obtained by reworking the discarded fractions.

Neither electrophoretic nor ultracentrifugal studies yield sufficient criteria for the purity of γ-globulin preparations. Indeed, fractions obtained by the above procedure, including dialysis, appeared homogeneous both in the Tiselius apparatus and in the ultracentrifuge, had the characteristic electrophoretic mobility and sedimentation constant of γ-globulin, but could be further separated into eu- and pseudoglobulins. "Electrophoretically pure" γ-globulin was therefore next electrodialyzed in the cold against flowing, distilled water, in a Brintzinger type electrodialyzer. If the protein was not too concentrated, a small precipitate generally separated as the conductivity diminished. When the conductivity had been reduced to a minimum, close to that of good conductivity water, the protein was removed from the electrodialyzer and diluted with cold conductivity water. Electrodialysis was carried out on concentrated solutions containing from 4 to 8% protein and diluted from two- to fourfold in order to bring about the maximum separation of euglobulin. Upon standing in an ice-bath at 0°, this generally separated as a gelatinous precipitate. The precipitate was soluble in neutral salt solutions and studies upon it will be reported elsewhere. The clear supernatant solution of γ-pseudoglobulin was again concentrated either in the negative pressure dialyzer or by reprecipitation with ammonium sulfate added through a cellophane membrane. Upon redialyzing and re-electrodialyzing, small additional precipitations of euglobulin were often obtained. The pseudoglobulin solution becomes more nearly neutral as euglobulin is removed and has generally been obtained in the neighborhood of *p*H 6.3 to 6.4.

The dielectric constant increment of a 1% solution of γ-pseudoglobulin at 0° has been found to be close to 1.1 at this *p*H, but "electrophoretically pure" preparations that still contain euglobulin have generally had appreciably lower dielectric constants, in one case as low as 0.75. Both the *p*H and the dielectric constant increment[22] of the γ-pseudoglobulin are thus important characteristics supplementing the electrophoretic mobility and the sedimentation constant of this protein. Its titration curve and its dielectric constant increment as a function of *p*H have been investigated.

IV. Purification of α- and β-Globulins

The portion of the serum proteins soluble at 0.40 saturated but precipitated at 0.50 saturated ammonium sulfate was refractionated in order to purify α- and β-globulins. After a preliminary experiment, 192 g. of this material was dialyzed, yielding a small amount of insoluble, and 167 g. of soluble, protein. The soluble fraction, in a volume of 12 liters, was reprecipitated by slowly adding ammonium sulfate through a cellophane membrane, the portions precipitating being removed successively at 1.78, 1.83, 2.04 and 2.20 *M* ammonium sulfate. After dialysis the fraction precipitating from 1.64–1.78 *M*, A, contained 81 g. of soluble and 5 g. of insoluble material; that precipitating from 1.78–1.83 *M*, B, contained 25 g. of soluble and 3 g. of insoluble material; that precipitating from 1.83–2.04 *M*, C, contained 35 g. of soluble and 1.4 g. of insoluble material; and that precipitating from 2.04–2.2 *M*, D, contained 10 g. of soluble protein and only traces of insoluble material. Electrophoretic studies indicated that fraction A contained more β-globulin and fraction C more α-globulin, whereas fraction B contained α- and β-globulin in almost equal amounts.

α-Globulin.—Fraction C and the corresponding material from preliminary experiments containing a large propor-

(22) Ferry and Oncley, THIS JOURNAL, **60**, 1123 (1938), studied pseudoglobulin that had not been purified to the extent here described.

tion of α-globulin was dissolved in a volume of 2 liters and ammonium sulfate was added by dialysis through a cellophane membrane until the concentration was 1.85 *M*. The *p*H was kept near 6.0. The precipitate at 1.85 *M* was dialyzed and gave 15 g. of soluble and 1 g. of insoluble material. The soluble portion was found to be composed of approximately equal amounts of α- and β-globulin when studied by electrophoresis. The filtrate from the precipitate at 1.85 *M* ammonium sulfate was precipitated at 2.05 *M* ammonium sulfate, dialyzed and diluted with the separation of 1 g. of insoluble material. The solution was adjusted to *p*H 5.0 and a blue-green precipitate of 3 g. separated on further dialysis (presumably the P III of Green[20]), leaving 20 g. of protein in solution. Electrophoretic studies on the soluble portion of this fraction indicated that it was largely α-globulin.

This fraction was further purified by diluting to a volume of one liter and equilibrating with ammonium sulfate until the concentration was 1.88 *M*. The insoluble portion was removed by filtration. After dialysis of this precipitate 0.5 g. was insoluble and 5 g. remained in solution. The soluble portion contained approximately equal amounts of α- and β-globulins. The filtrate at 1.88 *M* ammonium sulfate was precipitated by slowly adding ammonium sulfate to a concentration of 2.10 *M*. After dialysis and dilution, the soluble portion, amounting to 14 g. was principally α-globulin as judged by electrophoresis (Fig. 3). Several further fractionations, with slight changes in concentration of ammonium sulfate and protein, did not essentially change the electrophoretic pattern.

β-Globulin.—Fractions A derived from several experiments were precipitated in a volume of 3 liters by slowly adding ammonium sulfate. A precipitate removed at 1.60 *M* ammonium sulfate contained only 6 g., which was largely insoluble on dialysis. Further ammonium sulfate was added to the filtrate until the concentration reached 1.70 *M*. The precipitate containing 50 g. of protein was dialyzed and diluted with the separation of 2 g. of insoluble material. On readjusting the *p*H to 5.0 and dialyzing, a further 5 g. of insoluble material separated, leaving 40 g. of protein in solution. The electrophoretic analysis indicated that this fraction contained largely β-globulin with some α-globulin. This material in a volume of 1400 cc. was again precipitated with ammonium sulfate. The precipitate, containing 14 g., was largely composed of β-globulin, while the filtrate, weighing 20 g., also contained considerable α-globulin.

V. Properties of Purified Fractions

All of the various fractions whose preparation has been described in detail have not yielded equally pure products. In the case of the albumins of the horse, where crystallization could be effected both from ammonium sulfate and for a part of it from aqueous sulfuric acid solutions at *p*H 4,[17] proteins could be obtained carbohydrate-free and carbohydrate-containing[19] which appeared to be chemical individuals.

Sedimentation constants were determined in an air-driven ultracentrifuge equipped with an optical system of the Philpot type for obtaining schlieren photographs. The sedimentation constants, $s^{1\%}_{20°, w}$, of 1% protein solutions in potassium chloride of 0.20 ionic strength, have been corrected to the density and viscosity of water at 20° but *not* to zero protein concentration. Electrophoretic mobilities were measured in the Tiselius apparatus for 1% protein solutions at about 4° in phosphate buffer of 0.20 ionic strength and *p*H 7.7, and have been expressed as mobilities at 0° (since the conductivity of the buffer was measured at 0°). The electrophoretic and sedimentation diagrams of the albumin are reported elsewhere.[19]

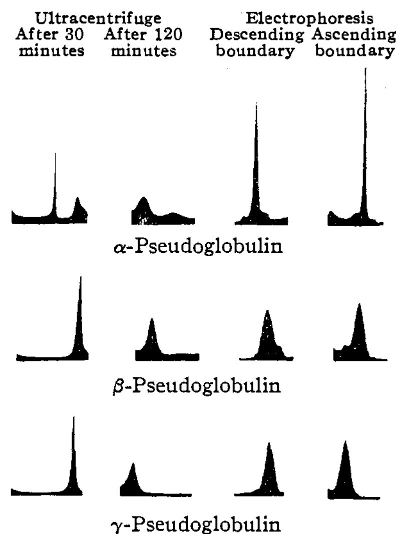

Fig. 3.—Ultracentrifugal and electrophoretic schlieren patterns of proteins from horse serum.

γ-Globulin.—Of the globulins, the γ fraction appears to have been prepared in the state of greatest purity. Our best preparations appear to be homogeneous both with respect to electrophoretic mobility and sedimentation velocity. Although titration curves of various preparations have proved identical as have dielectric dispersion curves, even our best preparations of γ-pseudoglobulin have thus far failed to rigorously satisfy the solubility criterion for a chemical individual.

The electrophoretic mobility of a 1% solution at 4° in phosphate buffer of ionic strength 0.2 and *p*H 7.7 is 1.9×10^{-5}. The sedimentation constant, $s^{1\%}_{20°, w}$, is 6.2×10^{-13}. The influence of concentration of purified preparations of this protein upon viscosity, diffusion and sedimentation constant, has been measured and will be reported elsewhere. The sedimentation constant at infinite dilution, $s^{0}_{20°, w}$ has been estimated to be 6.5×10^{-13}. On the basis of a diffusion con-

stant of 4.1×10^{-7}, and a partial specific volume of 0.730, a molecular weight of 142,000 may be calculated.[23] Investigations upon the physical-chemical and chemical properties of γ-pseudoglobulin will subsequently be reported.[24]

The **β-globulin** that has been studied also appears to consist largely of a pseudoglobulin homogeneous with respect to both electrophoretic mobility and sedimentation velocity. Indeed, the ultracentrifuge diagram in Fig. 3 reveals no molecules sedimenting faster than $s_{20°,w}^{1\%} = 6.3 \times 10^{-13}$ and only a few per cent. of slower-moving components. That the slower-moving component does not consist of any of the characterized albumins is demonstrated by the electrophoretic diagram in which there is no component moving with a mobility faster than that of the β-globulin, that value being 2.9×10^{-5}. On the other hand, this β-globulin preparation definitely contained a small amount, approximately 5%, of protein migrating electrophoretically with the velocity of γ-globulin. Since the sedimentation constants of β- and γ-globulins are so nearly alike this material is, of course, not revealed by the ultracentrifuge analysis.

The nature of the component of smaller sedimentation constant in the ultracentrifuge diagram is suggested by experiments at varying protein concentrations that have been carried out upon this β-globulin preparation. At higher protein concentration increasing amounts of the protein of lower sedimentation constant are observed, presumably due to such a dissociation of β-globulin as has been previously postulated. The nature of the equilibrium in such systems, which is further complicated in that new sedimentation constants appear at sufficiently high protein concentrations, is being further investigated.

α-Globulin.—The α-globulin preparation, also largely pseudoglobulin in nature, appeared to consist predominantly of molecules homogeneous with respect to net charge. Less than 10% of the protein moved with a slower mobility and none was detected moving with a higher mobility. The small amount of impurity was, according to this criterion, largely β-globulin, as indicated by this analysis. The mobility observed for the α-globulin was about 3.9×10^{-5}.

Ultracentrifuge analysis, however, revealed a far more complex system than had electrophoretic analysis (Fig. 3). Indeed, the preparation of α-pseudoglobulin revealed approximately equal quantities of protein sedimenting in a 1% solution with constants $s_{20°,w}^{1\%}$ of 17, 6.8 and 3.6×10^{-13}.[25] The slow-moving component might be the β-globulin component of small molecular weight described above, the carbohydrate-containing albumin of McMeekin which has closely the same electrophoretic mobility,[19] or another protein. The high concentration in this fraction of protein of sedimentation constant $s_{20°,w}^{1\%} = 17 \times 10^{-13}$ should prove useful for further purifications, and in this connection it should be noted that the sedimentation diagram of the carbohydrate-containing crystalline serum albumin of McMeekin[19] also contains traces of this component.

None of the globulins have thus far been prepared in a state comparable to that of our best crystalline albumins. Methodical investigation of the diverse molecules in serum and plasma are, however, yielding both purer proteins than have been available and a better understanding of the interactions in this complex system.

Summary

1. The normal serum of the horse has been fractionated by equilibration across cellophane membranes with ammonium sulfate solutions of known *p*H, concentration, volume and temperature.

2. The order in which the proteins are precipitated by ammonium sulfate has been related to the electrophoretic mobilities of the proteins in serum.

3. The γ-globulin precipitates with lower concentrations of salt than the β- and α-globulins. Albumins, with the highest mobility, require the highest salt concentrations for precipitation and crystallization. This rule depends upon the higher solubility of protein salts than of neutral proteins, but exceptions are found whenever proteins that are sufficiently unlike in size and shape as well as net charge are considered. Thus fibrinogen is precipitated by lower concentrations of

(23) We are indebted to Dr. J. W. Mehl for diffusion, and to Dr. S. H. Armstrong, Jr., for partial specific volume, measurements.

(24) The dielectric properties are reported by J. L. Oncley, *J. Phys. Chem.*, in press.

(25) A component of sedimentation constant 6.8×10^{-13} and also a smaller amount of a component of sedimentation constant $17\text{–}18 \times 10^{-13}$, were present in a preparation previously prepared in this Laboratory electrophoretically by Pappenheimer and analyzed ultracentrifugally by Lundgren, Pappenheimer and Williams, THIS JOURNAL, **61**, 533 (1939); Pappenheimer, Lundgren and Williams, *J. Exptl. Med.*, **71**, 247 (1940). This component of sedimentation constant 6.8×10^{-13} did not have the mobility of α-globulin. Most globulin preparations on which ultracentrifugal analysis have been reported in the literature have contained small amounts of the faster sedimenting component.

salt than γ-globulin, though it has a greater electrophoretic mobility.

4. Proteins of the same electrophoretic mobility have been further fractionated by dialysis into euglobulins and pseudoglobulins demonstrating the presence of many more components than are revealed by analyses of unfractionated serum.

5. The pseudoglobulin fractions have been further purified by repeated precipitations with ammonium sulfate until essentially free of proteins of different electrophoretic mobility and by electrodialysis until essentially free of euglobulin. Precipitation of the purified fractions generally occurred at lower salt concentration in the absence of other protein components.

6. Ultracentrifuge studies upon the various solutions have yielded further knowledge regarding the sedimentation constants of the protein components of different electrophoretic mobilities that are precipitable by varying concentrations of ammonium sulfate. Whereas preparations of α- and β-globulins often consist of components varying in molecular weight, γ-pseudoglobulin, like serum albumin, has been prepared homogeneous with respect both to size and to net charge.

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[CONTRIBUTION FROM THE DEPARTMENT OF PHYSICAL CHEMISTRY, HARVARD MEDICAL SCHOOL]

Preparation and Properties of Serum and Plasma Proteins. II. Crystallization of a Carbohydrate-Containing Albumin from Horse Serum

BY T. L. MCMEEKIN

The separation of a crystalline, carbohydrate-free albumin from horse serum has been repeatedly accomplished.[1-4] However, the yields of crystalline carbohydrate-free albumin have been small, leaving large quantities of carbohydrate-containing material in the filtrates. From such filtrates, Hewitt[5] has isolated a globulin fraction with a carbohydrate content of 4.6%, and a fraction which he called seroglycoid with 8.6% carbohydrate. Both of these carbohydrate rich fractions were amorphous and differed markedly from serum albumin. Kekwick[3] has separated crystalline horse serum albumin into a fraction containing 1.95% carbohydrate. The electrophoretic mobilities and molecular weights of these two fractions of varying carbohydrate content were found to be the same. Sörensen,[6] however, showed conclusively that albumins could be crystallized, differing markedly in solubility, and Ferry and Oncley,[7] confirming his work, also demonstrated differences in dielectric properties.

This paper describes the separation of a crystalline carbohydrate-containing albumin from horse serum, which is homogeneous in regard to electrophoretic mobility and size. The carbohydrate-containing albumin differs from the carbohydrate-free albumin of serum described in our previous communication[4] in crystalline form, in electrophoretic mobility and in other respects, leaving no doubt that they are distinct proteins.

(1) Sörensen and Haugaard, *Compt. rend. trav. lab. Carlsberg*, **19**, No. 12 (1933).
(2) L. F. Hewitt, *Biochem. J.*, **30**, 2229 (1936).
(3) R. A. Kekwick, *ibid.*, **32**, 552 (1938).
(4) T. L. McMeekin, THIS JOURNAL, **61**, 2884 (1939).
(5) L. F. Hewitt, *Biochem. J.*, **28**, 2080 (1934); *ibid.*, **31**, 360 (1937).
(6) S. P. L. Sörensen, *Compt. rend. trav. lab. Carlsberg*, **18**, No. 5 (1930).
(7) Ferry and Oncley, THIS JOURNAL, **60**, 1123 (1938).

Fractionation of Serum.—The globulins of serum were removed by diluting with an equal volume of water and adding ammonium sulfate through a rotating membrane[4] until the concentration was 2.05 *M*, the *p*H being near 6.6. The precipitated globulin was filtered on a Buchner funnel and washed with 2.05 *M* ammonium sulfate. The filtrate containing the albumin fraction (Precipitated Albumins in Fig. 1) was adjusted to the original volume with 2.05 *M* ammonium sulfate and the concentration of ammonium sulfate increased to 2.57 *M* by adding the required amount of ammonium sulfate through a rotating membrane. The precipitate formed between 2.05 and 2.57 *M* ammonium sulfate was largely crystalline and was used as a source for both carbohydrate-free and carbohydrate-containing crystalline serum albumins. The filtrate contained haemocuprein, choline esterase, phosphatase and other proteins.

Fractionation of Albumins.—The fraction of serum precipitated between 2.05 and 2.57 *M* ammonium sulfate derived from 11 liters of serum and containing 318 g. of protein was dissolved in water and diluted with 1.6 *M* ammonium sulfate to a volume of 10 liters and then adjusted to 2.05 *M* ammonium sulfate by adding the required amount of ammonium sulfate through a rotating membrane. The precipitate, amounting to 30 g., was filtered off. It contained globulin, largely euglobulin. The filtrate was brought to 2.5 *M* ammonium sulfate and the precipi-