Since the carbohydrate-free albumin gives a value of 4.1 when extrapolated to infinite dilution, we have tentatively assigned this value to both preparations. A small amount (approximately 2 to 5%) of the carbohydrate-containing preparation had a much larger sedimentation constant, of the order of 17×10^{-13}. The diffusion constant of these preparations and estimates of molecular weight and shape from diffusion and sedimentation measurements will be reported elsewhere.

I am indebted to Dr. J. L. Oncley for carrying out the ultracentrifugal measurements, to Dr. J. M. Newell for carrying out the electrophoretic measurements, and to Professor Edwin J. Cohn for suggestions and advice throughout this investigation.

Summary

1. A homogeneous crystalline serum albumin containing 5.5% carbohydrate has been separated from horse serum.

2. The carbohydrate-containing serum albumin was found to differ from carbohydrate-free serum albumin in crystalline form, nitrogen content, optical rotation and electrophoretic mobility.

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

Preparation and Properties of Serum and Plasma Proteins. III. Size and Charge of Proteins Separating upon Equilibration across Membranes with Ethanol–Water Mixtures of Controlled pH, Ionic Strength and Temperature

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The proteins of a tissue or body fluid are but rarely homogeneous. They generally differ both in molecular size and in electrical charge as well as in other chemical and physico-chemical properties. Most physico-chemical properties, such as solubility, diffusion, sedimentation, penetration through membranes and osmotic pressure, are functions of both molecular size and net charge. The value of these characteristics for the analysis of proteins and protein mixtures has been fully recognized.[1-8] It would appear of equal value to have available protein preparations homogeneous with respect both to size and charge, wherever these molecules are employed for physiological, clinical or industrial purposes.

The proteins of plasma or serum may be analyzed in terms of the size revealed by sedimentation during ultracentrifugation into the main albumin component of molecular weight close to 70,000, the various globulin components of molecular weight 140,000 to 160,000, a small amount of globulin component of a much greater molecular weight, some close to 900,000, and fibrinogen. There are molecules present in serum in small amounts, such as haemocuprein,[9] reported to have much smaller molecular weights than the other crystallizable albumins. This may be the case also of certain of the enzymes that have not yet been isolated in sufficiently pure form to be studied. None the less, ultracentrifugal analyses of serum reveal three main protein components.

Mobility during electrophoresis is a function of both size and net charge. Electrophoretic analysis reveals the main albumin component, at least three globulin components designated α, β and γ by Tiselius,[3] and fibrinogen, although many more diverse molecules are present, some simple, some conjugate proteins, the albumins and pseudoglobulins water-soluble, the euglobulins soluble only in salt solutions.

It has recently seemed of importance to standardize a method, capable of being employed for large-scale preparations, for the separation of plasma into as many as possible of its component proteins. Basing procedure on the knowledge gained from the isoelectric and neutral salt precipitation methods (for references see earlier paper

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

(2) A. S. McFarlane, *Biochem. J.*, **29**, 407, 660, 1175, 1209 (1935).

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

(4) E. Stenhagen, *ibid.*, **32**, 714 (1938).

(5) R. A. Kekwick, *ibid.*, **32**, 552 (1938); **33**, 1122 (1939).

(6) Longsworth and MacInnes, *Chem. Rev.*, **24**, 271 (1939); MacInnes and Longsworth, *Science*, **89**, 438 (1939); Longsworth and MacInnes, *J. Exptl. Med.*, **71**, 77 (1940).

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

(8) 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).

(9) Mann and Keilin, *Proc. Roy. Soc.* (London), **B126**, 303 (1938).

in this series[14]) that have been generally employed in our laboratory,[10-14] we have developed further the low temperature alcohol precipitation methods of Mellanby,[15] of Hardy and Gardiner[16] and of many subsequent investigators, some concerned with the preparation of antibodies. We have prepared (a) a γ-globulin fraction approaching homogeneity with respect to electrophoretic mobility and containing no albumin component and also (b) an albumin fraction which approaches homogeneity electrophoretically and in the ultracentrifuge. The albumin is free of γ-globulin and consists of molecules of less than half the size of the γ-globulin, though its net charge is far greater. Our diverse studies of the very different properties of these two protein fractions, which together represent close to 60% of the original plasma, suggest how different are the functions that each is capable of subserving. Preliminary experiments on the two purified fractions have suggested each is immunologically independent of the other.[17]

In addition to the albumins and γ-globulins, our procedure yields one fraction consisting largely of α- and β-globulins, and another which contains the fibrinogen and fibrin. Neither of these is, however, thus far homogeneous with respect to both size and shape, although they may subsequently be rendered so. They may, however, be found useful for certain purposes since their properties are also totally different from those of the albumin and γ-globulin fractions.

Method of Ethanol–Water Fractionation.— When ethanol–water mixtures are carefully added to proteins at sufficiently low temperature, denaturation may be prevented. Under these conditions, proteins such as crystallized egg albumin have been maintained in ethanol–water mixtures at $-5°$ for protracted periods of time, the ethanol removed before the temperature was raised and the protein recrystallized.[18] It therefore appeared probable that the very clear separations that have been obtained by adding ammonium sulfate to human, horse or bovine proteins through rotating cellophane membranes could also be attained by ethanol fractionation if carried out at $-5°$. Following thus the procedure now generally employed in our laboratory in order to avoid local excesses of reagents during their addition to protein solutions[11,13] alcohol was added to the plasma by diffusion through a rotating cellophane membrane. The gradient of alcohol within and without the membrane was always maintained as small as convenient. If plasma is equilibrated with pure ethanol, even through a cellophane membrane, denaturation occurs at its surface. In practice, if it was desired to introduce 10% ethanol into the plasma, the solution of ethanol within the membrane never exceeded 40% and could be smaller.

The equilibration of plasma with ethanol through cellophane membranes has the additional advantage that the diffusible, alcohol–water soluble components of plasma can be largely removed during the process of fractionation. These include small organic molecules such as sugars, such lipoids as dissolve in the alcohol–water mixtures employed, and most electrolytes.

Because of the loss of electrolytes from the plasma through the membrane, the salt content becomes so low that clean-cut separations of the protein fractions are not achieved unless this loss is compensated by adding salt to the alcohol–water mixture within the cellophane membrane. The initial ionic strength of the plasma is approximately 0.15 (0.9% sodium chloride). Solutions with which the red blood corpuscles were washed, and the plasma diluted were also of this ionic strength. It was also found desirable to add salt to certain of the alcohol–water mixtures with which the plasma was equilibrated. In the early stages of fractionation sodium chloride was used, but in later stages the *p*H at which the specific fractions separated was controlled by using acetate buffers.

The alcohol concentration in the plasma was estimated by density determinations upon the alcohol–water mixture with which the plasma was in equilibrium, correction being made for the dissolved electrolytes when this was not negligible. The density determinations were carried out at 25° and the volume per cent. ethanol refers to that temperature, whereas the mole fraction of ethanol is independent of temperature and defines the systems at whatever temperature separations are made.

The temperature in the initial fractionations was maintained at 0°. When the concentration

(10) Ferry and Green, *J. Biol. Chem.*, **81**, 175 (1929).
(11) Green, Cohn and Blanchard, *ibid.*, **109**, 631 (1935).
(12) A. A. Green, THIS JOURNAL, **60**, 1108 (1938).
(13) T. L. McMeekin, *ibid.*, **61**, 2884 (1939); **62**, 3393 (1940).
(14) Cohn, McMeekin, Oncley, Newell and Hughes, *ibid.*, **62**, 3386 (1940).
(15) J. Mellanby, *Proc. Roy. Soc.*, Series B, **80**, 399 (1908).
(16) Hardy and Gardiner, *J. Physiol.*, **40**, lxviii (1910).
(17) C. A. Janeway, personal communication.
(18) Ferry, Cohn and Newman, THIS JOURNAL, **58**, 2370 (1936).

of alcohol was sufficient to enable fractionation to be carried out at a lower temperature and preliminary experiments showed that a change of temperature produced desirable fractionation of proteins, the dialyses were continued at $-5°$. They could, of course, also be carried out at other temperatures and further refinements in the process may render this desirable.

Fractionation was controlled by effecting separations at various temperatures and with various alcohol–water mixtures and studying both the proteins precipitated and those remaining in solution by measurement of electrophoretic mobility and sedimentation velocity. Electrophoretic mobilities were generally carried out with 1% protein in the Tiselius apparatus at close to 4° in phosphate buffers of *p*H 7.7 and ionic strength 0.2, since a large amount of accumulated information was available regarding human, horse and bovine serum proteins under these conditions.[3-8,13,14] Photographs were taken at varying times, and electrophoretic mobilities under these conditions were estimated from the change in position of the peaks of the schlieren diagrams, account being taken of the differences in the ascending and descending boundaries. The concentration of the protein moving with each mobility could be calculated from the area under the skewed probability curves. Sedimentation constants were determined in the air-driven ultracentrifuge, also 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 ionic strength 0.2 are given in the accompanying figures and tables, and have been corrected to the density and viscosity of water at 20°. Molecular weights can be calculated only for proteins for which sedimentation and diffusion constants have been measured as a function of protein concentration, so that calculations can be made at infinite dilution. Such measurements on the pure fractions will be reported subsequently.

Fibrinogen, which is precipitated from plasma by a low concentration of neutral salts, is also precipitated by a low concentration of ethanol. Filtrates from 10% ethanol–water mixtures contain but small amounts of fibrinogen. The fraction separating from 10 to 15% ethanol at 0° contains less fibrinogen but more globulin, moving with the mobility characteristic of the γ fraction. It is largely water-soluble, provided too much salt has not been removed during the equilibrations, but is rich in euglobulin. At 15% ethanol the system can be brought to $-5°$ and in certain experiments the fractions separating at 15% ethanol with change in temperature from 0 to $-5°$ have been separated and studied. In other experiments the temperature has been maintained at 0° until the ethanol was brought to 20% and the fraction separating at 20% ethanol from 0 to $-5°$ collected. The general principle that we have employed is to determine those conditions of temperature, alcohol concentration, *p*H and ionic strength which yield the most effective separations of the components of serum and plasma as indicated by electrophoretic mobility and sedimentation constant, as well as by other chemical and physical chemical criteria.

Examples of procedures that have thus far been found useful in the preparation of human, horse and bovine γ-globulin and albumin are given in detail as illustrations of the general method which can, of course, be modified to some extent with the species of serum or plasma, the fraction sought in the condition of greatest purity, and perhaps also with the scale of preparation. The method of fractionation by equilibration through membranes with alcohol–water mixtures or mixtures of related substances of controlled *p*H, ionic strength and temperature, appears to be applicable to the preparation of protein fractions for many purposes, some industrial and some clinical.[19]

Fractionation of Beef Plasma.—Beef blood was collected in clean glass containers and mixed immediately with enough citrate to prevent clotting of blood. The blood was chilled as soon as possible at 0°, the red blood corpuscles separated by centrifugation and the plasma siphoned off. Two liters of plasma were diluted in both Preparations III and IV (but not in I and II) with an equal volume of 0.9% sodium chloride solution. This was done in part to facilitate comparison with the separations that had been effected by means of ammonium sulfate, in part because earlier experiments suggested that fractionation of undiluted plasma with ethanol was less satisfactory. It remains possible, however, that this end could be accomplished with a less great dilution.

(19) The use of fractions purified in this way for transfusions is being investigated in collaboration with Drs. Soma Weiss, C. A. Janeway and P. B. Beeson of the Peter Bent Brigham Hospital and for antibody preparations in collaboration with Drs. E. S. A. Robinson and G. Edsall of the Massachusetts State Antitoxin Laboratory. The fractionation of convalescent, as well as normal, sera by these methods will also be standardized. Among other industrial uses are the modification of the properties of systems containing other proteins by their combination, either physically or chemically, with plasma fractions (see for instance 20 and 21), as well as the chemical modification and the controlled recombination of the various plasma proteins.

(20) Burrows and Cohn, *J. Biol. Chem.*, **36**, 587 (1918).

(21) Cohn and Henderson, *Science*, **48**, 501 (1918).

Separation of Fibrinogen.—In both Preparations III and IV the first precipitates were separated when the fraction of ethanol was 10% by volume. These precipitates consisted largely of fibrinogen and fibrin. They contained only small amounts of serum globulin.

Separation of γ-Globulin.—In Preparation III the volume fraction of alcohol was next brought to 20%,[22] the temperature being maintained at 0°. The alcohol concentration within the membrane never exceeded 50% by volume. When the alcohol–water mixture of the plasma had reached 20%, the precipitate was removed by centrifugation in a brine-cooled centrifuge at −5° and washed in an alcohol–water mixture of the same composition as the solution from which it had separated. The washed precipitate was then mixed with a further small amount of this ethanol–water mixture, frozen in a thin layer on the sides of the large centrifuge bottle in which it had been separated from the plasma, and dried by the method of Flosdorf and Mudd.[23] The dried, pure white, readily soluble protein was ground with pestle and mortar and dried further in a vacuum desiccator. Whereas the moisture of precipitates not well subdivided was occasionally as high as 10%, after powdering and further drying in a vacuum desiccator it was generally under 2%. The electrophoretic analysis of this precipitate dissolved in phosphate buffer at *p*H 7.7, of ionic strength 0.2 and measured in the Tiselius apparatus indicated that the product was very largely, if not entirely, γ-globulin, with no more than a trace of faster moving components (Fig. 1).

The concentration of ethanol in the system was now sufficiently high to permit fractionation to be carried out at a lower temperature. When the plasma was brought to −5° without any other change in conditions, a further precipitate separated. This was removed as before and after being frozen and dried yielded an additional precipitate which, upon electrophoretic analysis, was proved to be at least as completely γ-globulin as that previously removed (Fig. 1).

In order to determine more precisely the optimum conditions of temperature and alcohol concentration for this separation, a precipitate was removed in Preparation IV at 15% ethanol at 0°. The temperature in this experiment was reduced from 0 to −5° at 15% ethanol. The fraction from 15 to 20% ethanol at −5° was then collected separately. The very soluble globulins separating at 0 to −5° from 10 to 20% ethanol amounted to slightly more than a fourth of the total protein. No essential improvement in the purity of any one fraction was conclusively demonstrated, but the fraction from 10 to 15% at 0°[24] appeared to be less pure than the fraction separating from 15% ethanol at −5° and the fractions separating up to 20%. The further separations of these γ-globulin fractions into various euglobulins and γ-pseudoglobulin will be reported subsequently.

In Preparation IV two fractions were removed between 20 and 30%, the material separating at 25% and 30% being collected separately. The amount precipitated by ethanol from 20 to 30% is far smaller in amount than that separated from 10 to 20%, although electrophoretic analyses revealed that this material is also largely γ-globulin (Fig. 1). The electrophoretic mobility of the protein in this fraction was 1.6×10^{-5} in phosphate buffer of ionic strength 0.2 and *p*H 7.7.

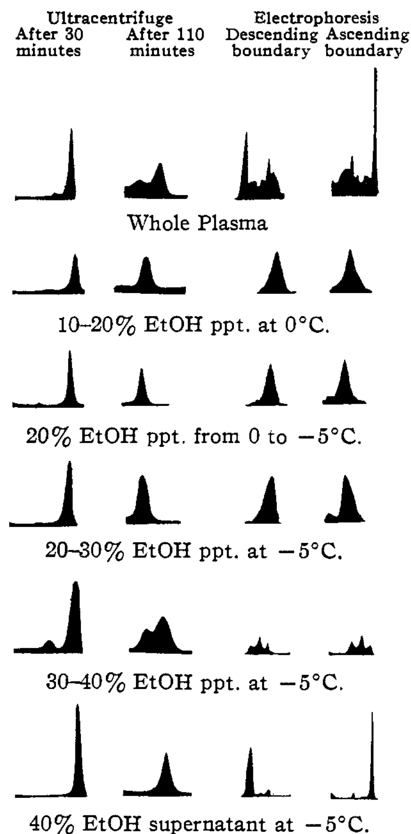

Fig. 1.—Ultracentrifugal and electrophoretic schlieren patterns of proteins from bovine plasma.

In the ultracentrifuge all of the γ-globulin fractions revealed a main component of $s_{20°,w}^{1\%} = 6.4 \times 10^{-13}$. In addition, all of these fractions showed at least a trace of a much heavier component sedimenting with a constant estimated to be $s_{20°,w}^{1\%} = 17 \times 10^{-13}$. This is probably the protein[1,8] of molecular weight 900,000. Further investigations on the isolation of high molecular weight components, especially in anti-pneumococcus horse serum, are in progress.

Separation of α- and β-Globulin.—The filtrate from 30% ethanol was re-equilibrated with a higher concentration of ethanol through a rotating cellophane membrane until the concentration in the plasma had been brought to 40%, the temperature being maintained at −5°. Whereas the plasma becomes densely opalescent when the alcohol concentration is increased over this range, the precipitate did not separate readily. The salt concentration in the plasma had, however, greatly diminished as a result of the repeated equilibrations with large volumes of ethanol and water.

(22) In this case it was not found necessary, but occasionally it has proved desirable, in order to assure the attainment of equilibrium, to make the final equilibration by placing the desired per cent. of ethanol in the membrane.

(23) Flosdorf and Mudd, *J. Immunol.*, **29**, 389 (1935).

(24) It is possible that the removal of a smaller fraction at 0° from 10 to 12 or 13% might suffice to remove the less uniform material.

The 40% ethanol–water mixture within the membrane through which the plasma had been equilibrated was accordingly replaced by a solution containing acetate buffer in 40% ethanol. This acetate buffer was constituted of approximately 10 cc. of normal acetic acid (in Preparation III, and 11 cc. in Preparation IV) and sufficient buffer containing sodium acetate and acetic acid in the molar ratio of 7.1 to 1 to bring the ionic strength, both within and without the membrane, to 0.05. The addition of the acetate buffer sufficed to effect an adjustment in the *p*H and to bring about separation of the protein, which could be centrifuged as were previous precipitates in a brine-cooled International centrifuge at $-5°$. Unlike the previous precipitates, however, which upon freezing and drying were white and friable, this fraction was deeply colored and readily formed gels which were far more difficult to dry and which, upon drying, were less readily redissolved. Upon electrophoretic analysis these fractions from 30 to 40% at $-5°$ appeared to consist mainly of α- and β-globulins with traces only of albumin and γ-globulin (Fig. 1).

Separation of Albumin.—The filtrate at 40% ethanol in the case of Preparation III consisted of albumin containing but small amounts of more slowly moving components, and represented approximately one-third of the plasma proteins.[25] The electrophoretic and ultracentrifugal analyses of this albumin are given in Fig. 1, and gave a mobility of 5.2×10^{-5} in phosphate buffer at ionic strength 0.2, *p*H 7.7 and a sedimentation constant $s^{1\%}_{20°,w} = 4.0 \times 10^{-13}$. The traces of slower moving components revealed in the electrophoretic analysis were not readily detected in the ultracentrifugal analysis, since a small amount of globulin would be difficult to detect in the presence of so much albumin.

The drying of the albumin from the 40% ethanol solution was accomplished by first concentrating the filtrate at low temperature under reduced pressure to a sirup containing more than 20% protein and from which the ethanol had been almost completely removed by evaporation. Upon freezing and drying thin layers of the sirup, a slightly colored, friable powder was obtained. The albumin precipitated in this manner from the filtrate had a characteristic odor which, however, could be removed by Soxhlet extraction with either ethyl or petroleum ether. The resulting powder was far whiter. Experiments are in progress to separate the albumin fraction also as a precipitate by adjustment of the temperature, *p*H and ionic strength so as to yield a still purer, whiter and more readily concentrated precipitate.[26]

(25) In the previous preparation in which the *p*H had been brought to a somewhat more acid reaction the amount of α- and β-globulin appeared to have been sensibly reduced and it is possible that a slightly greater acidity at this stage, whereas it would increase the separation of albumin in 40% ethanol and thus diminish its yield, would result in an even purer product than has thus far been obtained.

(26) Equilibration of that portion of plasma soluble in 40% ethanol at $-5°$ with sufficient acetic acid dissolved in this solvent to bring the *p*H of the protein precipitate—when re-dissolved in water—close to *p*H 5.5, removes α- and β-globulin. The albumins remaining in solution may be precipitated (1) by the further addition of sufficient acetic acid to bring the *p*H of the protein, when redissolved in water, to *p*H 5.0, and (2) by further reducing the temperature to $-15°$. These bovine albumin fractions are being further subfractionated to yield the various albumin components of plasma.[18]

We should like to express our appreciation to Dr. Ingersoll, Dr. Perlmann, Mr. Gordon, Mr. Weare and Mr. Blum for their aid in the drying and the analysis of these preparations.

Summary

Plasma can readily be separated into four fractions by equilibration through membranes with ethanol–water mixtures of controlled *p*H, ionic strength and temperature. The conditions for effective separation vary somewhat with the nature of the species and the scale of preparation, and are illustrated by a study of bovine plasma.

The precipitate separating from bovine plasma at 15% ethanol and 0° consists largely of fibrinogen and fibrin and small amounts of γ-globulin. The fraction (II) that precipitates when the filtrate at 15% ethanol is brought to $-5°$ and the ethanol concentration increased to 20 or even 25% appears to be in large part γ-globulin. Fraction III, separating from 30 to 40% ethanol, is a mixture, largely of the α- and β-globulins; Fraction IV of bovine plasma remaining in solution at 40% ethanol at $-5°$ in the presence of acetate buffer of ionic strength 0.05 and at a *p*H close to 5.5, appears to consist almost entirely of albumin.

This procedure thus segregates fibrinogen and various impurities in Fraction I, α- and β-globulins and traces of γ-globulin and of albumin in Fraction III. It yields two fractions, II of γ-globulin and IV of albumin, consisting almost completely of proteins approaching homogenity with respect both to size and net charge.

The electrophoretic mobility of the bovine γ-globulin at 4° in phosphate buffer of ionic strength 0.2 and *p*H 7.7 is 1.6×10^{-5}. The sedimentation constant of the main component is $s^{1\%}_{20°,w} = 6.4 \times 10^{-13}$, although a small amount of a heavier component of $s^{1\%}_{20°,w} = 17 \times 10^{-13}$ is also present. The sedimentation constant of bovine albumin $s^{1\%}_{20°,w}$ is 4.0×10^{-13} and its electrophoretic mobility 5.2×10^{-5} in phosphate buffer of ionic strength 0.2 and *p*H 7.7.

The molecular weight of the albumin is but half that of the γ-globulin, but its net charge is half again as great. It appears certain that these totally different fractions of such different properties may subserve quite different purposes in physiology and medicine, and also in industry.

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