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.

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-

(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).

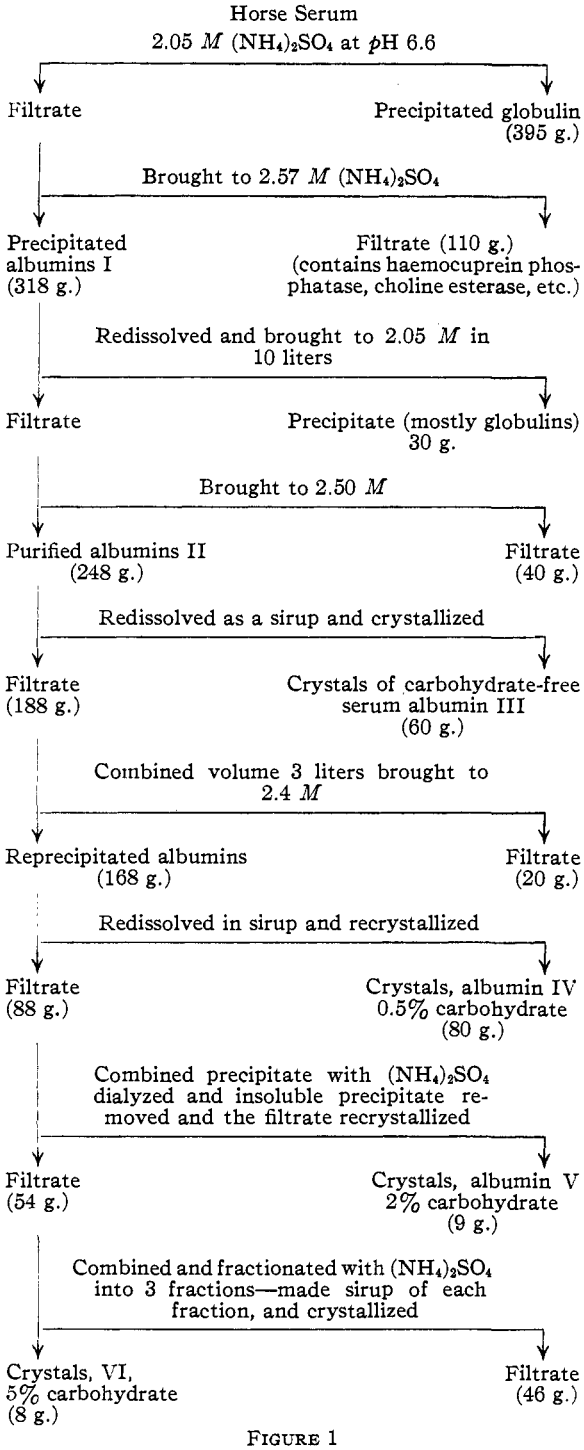

FIGURE 1

tate (II in Fig. 1) collected. The filtrate, containing 40 g. of protein and most of the remaining pigment, was completely precipitated with ammonium sulfate.

The precipitate formed between 2.05 and 2.5 *M* ammonium sulfate (II in Fig. 1) was employed in the subsequent fractional crystallizations. It was dissolved in water to form a sirup and crystallized by adding a saturated solution of ammonium sulfate with a pipet until near the point of precipitation and allowed to stand until a crop of crystals had formed. The crystals were examined for crystalline form and tested for carbohydrate. On adding more ammonium sulfate to the filtrate further crops of crystals were obtained. In this manner 60 g. of carbohydrate-free crystalline albumin was collected (III in Fig. 1). The filtrates from the carbohydrate-free albumin were combined, making a volume of 3 liters, and precipitated by adding ammonium sulfate through a rotating membrane until the concentration was 2.4 *M*. The precipitate was removed and used for further purification while the filtrate, containing 20 g. of material, was stored.

The material precipitated at 2.4 *M* ammonium sulfate was dissolved in water to make a sirup and a saturated solution of ammonium sulfate was added to the point of crystallization. The crystals at this stage consisted of long needles, rather than the well-formed hexagonal rods obtained in the earlier crops of crystals, and contained 2.0% carbohydrate. The amount of carbohydrate was, however, slowly reduced with each recrystallization, and the protein gave no indication of having a constant carbohydrate content. A yield of crystalline material, amounting to 80 g., and containing 0.5% carbohydrate, was in this way separated (IV in Fig. 1). At least part of the crystalline albumin in this fraction could probably have been rendered carbohydrate-free.

The filtrates from this crystalline material were again combined and completely precipitated by adding ammonium sulfate through a rotating membrane. The precipitate was dialyzed free from salt and a small amount of water-insoluble material removed by centrifugation. Ammonium sulfate was then added until crystallization took place. Nine grams of the characteristic serum albumin needles containing 2% carbohydrate was obtained (V in Fig. 1). The filtrate, containing 54 g. of protein in 1800 cc., was divided into three fractions by adding ammonium sulfate through a membrane, one precipitating at 2.1 *M* ammonium sulfate, the second between 2.1 and 2.25 *M* ammonium sulfate, and the third precipitating between 2.25 and 2.8 *M* ammonium sulfate. Each of these fractions was dissolved in a small quantity of water and a saturated solution of ammonium sulfate added to the point of turbidity. After standing at room temperature for four days, the first and third fractions had well-formed, symmetrical crystals which were strikingly different in appearance from the rod-shaped carbohydrate-free albumin crystals. The crystals were removed by centrifugation and were sufficiently insoluble in 2.0 *M* ammonium sulfate to be washed with this salt solution. Eight grams of crystalline material was obtained from the combined fractions. The carbohydrate content was 5.0% (VI in Fig. 1).

Electrophoretic mobilities showed that there were at least two components at this stage. On recrystallizing, the carbohydrate content was increased to 5.2%. Two components were, however, still revealed by electrophoresis.

A third crystallization yielded 4.0 g. of the very homogeneous crystalline product, shown in Fig. 2, which was also homogeneous with respect to electrophoretic mobility and sedimentation velocity in the ultracentrifuge. The carbohydrate content was 5.5% and remained constant on

recrystallization. The method of Sörensen and Haugaard[1] was used for the determination of carbohydrate.

Properties of the Carbohydrate-Containing Serum Albumin.—The carbohydrate-containing crystalline albumin is readily distinguished from the carbohydrate-free albumin even by difference in appearance. The carbohydrate-containing albumin crystals were perfectly symmetrical hexagonal disks (Fig. 2), while the carbohydrate-free albumin crystals are rod-shaped.

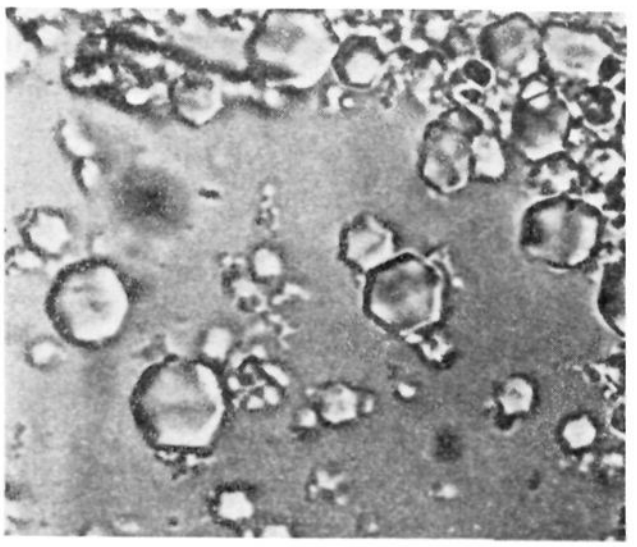

Fig. 2.—Microphotograph of carbohydrate-containing serum albumin crystals.

The carbohydrate-containing albumin is easily recrystallized by adding ammonium sulfate to the point of turbidity and allowing the solution to stand but the best method for recrystallization, appears to be to place the protein solution inside of a cellophane membrane and rotate in a 2.0 *M* solution of ammonium sulfate. Although originally precipitated from serum at higher ammonium sulfate concentrations, the purified crystals are quite insoluble at this concentration.

Solutions of the carbohydrate-containing albumin were not coagulated by heating in the presence of neutral salt or buffer at *p*H 4.8, thus resembling the seroglycoid of Hewitt. The solutions were, however, precipitated by 2% trichloroacetic acid, differing from seroglycoid in this respect.

The nitrogen content of the carbohydrate-containing albumin was estimated to be 15.1% by the micro-Kjeldahl method, while the unfractionated carbohydrate-free albumin we have reported to contain 16.1% nitrogen and the albumin prepared from the crystalline sulfate 16.8%.[4]

The optical rotation of the carbohydrate-containing albumin in a 3% solution was $[\alpha]^{20}D$ −47°, that of the carbohydrate-free albumin −57°.

The crystallized carbohydrate-containing albumin was electrophoretically homogeneous at *p*H 7.7 and moved with a velocity nearer to that of α-globulin (Fig. 3) than of carbohydrate-free serum albumin, and also at quite different mobilities than reported by Kekwick for his carbohydrate-containing albumin. The mobilities of the crystalline carbohydrate-free and carbohydrate-containing albumins are given in Table I. To confirm this difference in mobility, preparations of crystalline serum albumin with and without carbohydrate, each of which revealed a single boundary, were mixed and revealed two boundaries with a ratio of mobilities of 0.87, very close to that calculated from the results of the independent determinations.

TABLE I

CONSTANTS DEFINING THE PROPERTIES OF ALBUMINS CRYSTALLIZED FROM HORSE SERUM

	Nitrogen, %	Optical rotation, $[\alpha]^{20}D$	Sedimentation constant $s_{20°,w} \times 10^{13}$	Electromobility *p*H, 7.7; $\Gamma/2 = 0.2$ $\mu \times 10^5$
Crystalline unfractionated carbohydrate-free albumin	16.1	−57	4.1	5.3
Crystalline albumin separated as sulfate	16.8		(4.1)	
Crystalline haemoprein	14.4[8]			
Crystalline carbohydrate-containing albumin	15.1	−47	(4.1)	4.5

The carbohydrate-containing albumin was also practically homogeneous in the ultracentrifuge (Fig. 3). A 1% solution gave a sedimentation constant reduced to water at 20° of $3.6_6 \times 10^{-13}$ and the carbohydrate-free albumin gave a value of 3.8_5, the difference of 6% being not much larger than the probable error for a single determination.

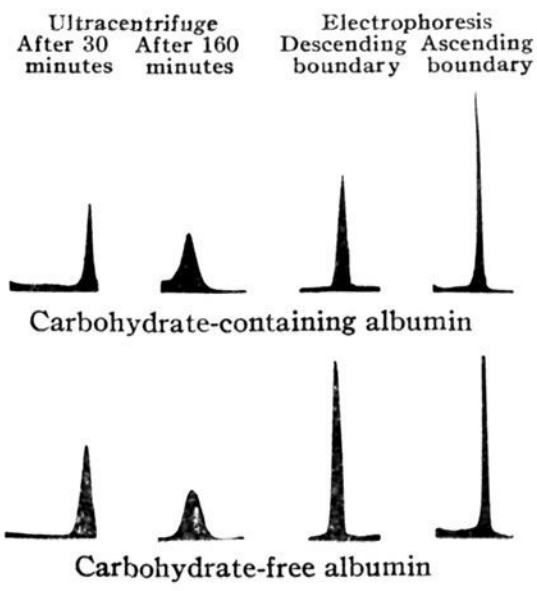

Fig. 3.—Ultracentrifugal and electrophoretic schlieren patterns of crystalline albumins from horse serum.

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

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

BY E. J. COHN, J. A. LUETSCHER, JR., J. L. ONCLEY, S. H. ARMSTRONG, JR., AND B. D. DAVIS

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 Kellin, *Proc. Roy. Soc.* (London), **B126**, 303 (1938).