and the compressibilities may be computed at any pressure and temperature within the range of our observations.

Our data enable us to compute $(\partial P/\partial T)_V$, $(\partial E/\partial V)_T$ and the volume changes on mixing for the various solutions, and to examine the variation of these quantities when the temperature is changed at constant volume. In the light of the results of this analysis, we suggest a correlation between the effect of temperature and of mixing on the molecular distribution in the liquids on the one hand and the internal pressures and the volume changes on mixing on the other.

The attractive pressures of the solutions were calculated in the same way as those of the pure liquids and were reproduced by the formula $P_A = a'/V^{2.737}$. An equation was developed whereby the constants a' for the solutions were represented as a function of the concentration in terms of constants for the pure components and the generalized volume fractions y_1 and y_2 which contain an empirical constant, U_2/U_1.

Although the volume changes on mixing were well represented as functions of the ordinary volume fractions of the components by equations of a conventional type, we also examined several equations expressing this quantity in terms of significant properties of the solutions and the fractions y_1 and y_2. These equations reproduced the volume changes on mixing fairly well. One of them had the form of the equations developed for regular solutions and another gave a value of the empirical constant, U_2/U_1, which was independent of temperature and pressure and was approximately equal to the corresponding constant obtained from the attractive pressure–composition equation.

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

Serum Albumin. I. The Preparation and Properties of Crystalline Horse Serum Albumin of Constant Solubility

BY T. L. McMEEKIN

Since the crystallization of serum albumin by Gürber in 1894,[1] many investigators have doubted its homogeneity. Fractional crystallization from salt solutions has yielded products that vary widely in solubility,[2] chemical composition,[3,4] and dielectric properties.[5] Despite this evidence of inhomogeneity, the recognition and separation of constituent proteins has proved difficult. Perhaps the most important advance in its purification was the separation of carbohydrate-containing globulins[6] from the albumin fraction and the recognition that serum albumin does not contain carbohydrate.[3,4]

Serum albumin has been reported to be homogeneous in respect to size[7] and electrophoretic mobility.[8] Ultracentrifugal studies have shown it to have a molecular weight of 67,500,[7] independent of *p*H between 4 and 9. In solutions more acid than *p*H 4 it was decomposed into smaller molecules, the process being found to be reversible.

This paper first describes our present procedure for the separation of the serum proteins and the preparation of crystalline carbohydrate-free serum albumin homogeneous with respect to electrophoresis at neutral reactions.[9] This highly purified serum albumin can be separated further into two crystalline fractions differing in chemical composition and electrophoretic mobility in acid but not in neutral solution.[9] The separation of serum albumin into two fractions is based on the discovery made in this Laboratory by J. D. Ferry,[10] that a salt-free concentrated solution of serum albumin crystallizes when brought to *p*H 4.0 with sulfuric acid.[11] The solubility of the

(1) Gürber, *Würzburger physiol. med. Ges.*, **113** (1894).
(2) Sörensen, *Compt. rend. trav. Lab. Carlsberg*, **18**, No. 5, 1 (1930).
(3) Sörensen and Haugaard, *ibid.*, **19**, No. 12 (1933).
(4) Hewitt, *Biochem. J.*, **30**, 2229 (1936).
(5) Ferry and Oncley, THIS JOURNAL, **60**, 1123 (1938).
(6) Hewitt, *Biochem. J.*, **32**, 26 (1938).
(7) Svedberg and Sjogren, THIS JOURNAL, **50**, 3318 (1928); **52**, 2855 (1930).
(8) Tiselius, *Biochem. J.*, **31**, 1464 (1937).
(9) I am indebted to Dr. John A. Luetscher, Jr., for these measurements. See also the second paper in this series, THIS JOURNAL, **61**, 2888 (1939).
(10) Reported by Ferry to the Division of Biological Chemistry at the 96th meeting of the American Chemical Society, Milwaukee, Wis., September 7, 1938.
(11) Piettre, *Compt. rend. Acad. Sci.*, **188**, 463 (1929), reports that among other proteins serum albumin crystallizes from aqueous solution following removal of globulin with acetone, and of acetone by evaporation over sulfuric acid *in vacuo*.

albumin sulfate, and of the regenerated protein, has been found to be essentially independent of the amount of saturating body and certain of its properties have been investigated and are reported.

Fractionation of Serum Proteins.—The proteins of serum were divided into three fractions depending on solubility in salt as a preliminary to further purification. These were: (a) the fraction insoluble in 1.6 *M* ammonium sulfate (0.4 saturated); (b) that soluble in 1.6 *M* but insoluble in 2.1 *M* ammonium sulfate; and (c) that soluble in 2.1 *M* ammonium sulfate but insoluble in 2.8 *M* ammonium sulfate. These fractions were stored in closed containers at 4°.

Fresh serum[12] was diluted with an equal volume of water and the calculated amount of ammonium sulfate (either in the form of a solid or solution) to make the solution 1.6 molar was placed in a cellophane membrane and rotated until equilibrium with the salt had been reached on both sides of the membrane (Fig. 1), usually within forty-eight hours. The precipitate was allowed to settle and the supernatant solution siphoned off. Protein precipitated in this manner was granular, resulting in better separations than when precipitation was more rapid. Because of the granular nature of the precipitates, filtration was carried out on a Büchner funnel, using Schleicher and Schull No. 575 hardened filter paper. The resulting filter cake was washed free from mother liquor.

The filtrate was brought to 2.1 molar ammonium sulfate by adding the calculated quantity of solid ammonium sulfate in a membrane and rotating until equilibrium was reached. After removing the precipitate in the usual manner the filtrate was brought to 2.8 *M* ammonium sulfate by equilibrating with the calculated amount of solid ammonium sulfate in a cellophane membrane. The protein fraction that was soluble in 2.1 molar and insoluble in 2.8 molar ammonium sulfate contains serum albumin as well as many other substances. The yield from each liter of serum was approximately 20 g. of protein soluble in 2.1 *M* and insoluble in 2.8 *M* ammonium sulfate. No acid was added during this separation.

The fraction precipitated per liter of serum between 2.1 and 2.8 molar ammonium sulfate, was dissolved in 80 cc. of water and diluted to 800 cc. with 1.6 molar ammonium sulfate and the *p*H adjusted to 6.4. The solution was then equilibrated at 2.1 molar ammonium sulfate in a membrane. The globulin precipitate was removed and the filtrate was brought to 2.4 molar ammonium sulfate again by equilibrating in a membrane. The precipitate formed was largely crystalline and was removed by filtration. It contained about 10 g. of protein and was the starting material for further purification of albumin.

The precipitate insoluble in 2.4 *M* ammonium sulfate was dissolved in the smallest possible quantity of water, forming practically a sirup and the *p*H adjusted to 5.5. A saturated ammonium sulfate solution was added from a pipet with stirring until near the point of precipitation. On allowing the clear solution to stand overnight turbidity developed and well-shaped, large, long, hexagonal crystals of serum albumin separated.

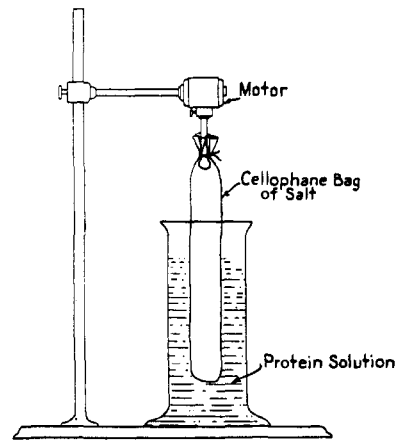

Fig. 1.—Diagram of the apparatus used for adding reagents to protein solutions.

The crystals were removed by centrifugation. Small amounts of globulin did not settle on centrifugation and were thus separated from the crystals. After centrifugation, the crystals were filtered on a Büchner funnel and washed with 2.0 *M* ammonium sulfate. Two further recrystallizations in this manner were usually found adequate for the removal of carbohydrate. Further quantities of carbohydrate-free crystalline albumin were prepared from the supernatant liquors by diluting with 1.6 *M* ammonium sulfate so that the protein concentration was about 1–2% and then gradually adding ammonium sulfate, 0.1 or 0.2 mole greater in concentration than the protein solution, through a rotating membrane.

The crystalline fractions were collected and recrystallized from concentrated solution. The yield of carbohydrate-free albumin as tested for by the Orcin method following the procedure of M. Sörensen and Haugaard[3] has thus far not been greater than 3 g. per liter of original serum.

The proteins soluble in 2.4 *M* ammonium sulfate at *p*H 6.4 and insoluble at 2.8 *M* ammonium sulfate contain besides serum albumin, a considerable amount of globulin, as has been shown by Hewitt,[6] most of the pigments of serum, and choline esterase.[13] Haemocuprein, the copper-containing albumin,[14] was crystallized from this fraction with ammonium sulfate.

Solubility in Ammonium Sulfate of Carbohydrate-free Crystalline Serum Albumin.—The solubility of serum albumin in ammonium sulfate even after repeated recrystallization from this solvent has been shown by Sörensen[2] to depend largely upon the amount of saturating body with which it is in equilibrium, increasing with increase in the total protein in the system. This phenomenon might be expected if more than one protein component were present; that is to say, if the protein had not been

(12) We are indebted to the Massachusetts Antitoxin Laboratory for the horse serum albumin employed in these investigations. In several preparations of the albumin fraction, large amounts of the filtrate from *anti*-pneumococcus serum were employed from which the immune proteins had been removed by 1.6 *M* ammonium sulfate.

(13) McMeekin, *J. Biol. Chem.*, **128**, LXVI (1939).

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

isolated as a chemical individual, and is characteristic also of other proteins.[15] Mellanby[16] demonstrated that for the serum globulins that he studied a definite ratio obtained between solubility and the amount of total protein in the system. A comparable relation obtained for the various serum proteinsstudied by Sörensen[2,17] and for our repeatedly recrystallized carbohydrate-free serum albumin even after exhaustive fractionation with ammonium sulfate, as is demonstrated by the measurements in Table I of solubility in 1.8 *M* ammonium sulfate at *p*H 4.9. The solubility measurements were made by the method previously described.[18] Not only was the solubility of the albumin greater the greater the amount of saturating body in the system but it diminished at each equilibration of approximately twenty-four hours with fresh aliquots of solvent.

TABLE I

SOLUBILITY OF CARBOHYDRATE-FREE MIXED SERUM ALBUMINS IN 1.8 *M* AMMONIUM SULFATE AT *p*H 4.9 AND 25°

No. of equilibrations of one day each	Vol. of solvent, cc.	Soly., g./liter Solute A 0.37 g. protein	Soly., g./liter Solute B 1.85 g. protein	Solubility A / Solubility B
2	40	0.287	0.802	2.8
3	40	.132	.335	2.5
4	40	.072	.195	2.7

Further Fractionation of Carbohydrate-free Crystalline Serum Albumin.—The procedure of J. D. Ferry for "crystallization of a fraction of horse serum albumin from salt-free solution"[10] was followed next. A salt-free solution of purified albumin containing 26 g. of protein in 260 cc. was brought cautiously to *p*H 4.0 by adding 0.1 normal sulfuric acid (approximately 0.00025 mole of acid being required for each gram of protein) and then concentrated in a negative pressure dialyzing apparatus[19] at 4°. When the protein concentration reached about 25% the solution was brought to room temperature. Crystallization began within an hour and was allowed to continue for four hours. The crystals were removed by centrifugation and washed three times, each time with a volume of water equal to the volume of the precipitate. The supernatant liquor was again concentrated at 4° and a second and third crop of crystals removed. Crystallization was continued until no further separation occurred. The first crop of crystals generally amounted to 10 g. of protein, the second to 3 g. and the third to 0.3 g., leaving 12.5 g. of protein dissolved in solution under these conditions. Thus, about one-half of the original serum albumin was converted into water insoluble crystalline material.

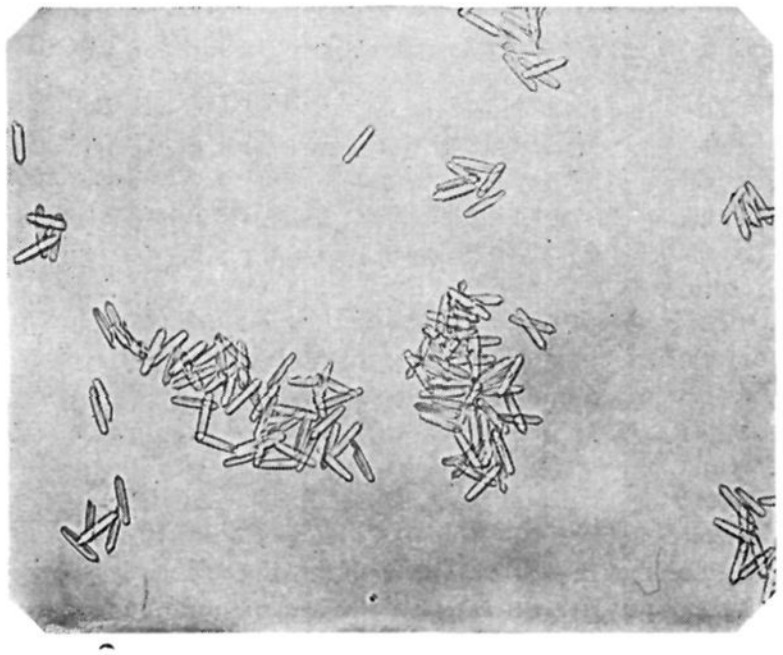

Fig. 2.—Microphotograph of serum albumin sulfate crystallized from water.

Properties of Serum Albumin Crystallized from Water at *p*H 4.0.—The serum albumin sulfate had the same crystal form of hexagonal prisms with pointed ends as the serum albumin from which it was derived. A solution obtained by repeated extraction of the crystals always had a *p*H of 4.0 ± 0.05 and gave a test for sulfate ions indicating that the substance was an albumin sulfate. For complete removal of sulfate, precipitation with barium or electrodialysis was required. After the removal of sulfate by electrodialysis, the solution had a *p*H close to that of the solution from which the albumin sulfate was prepared, indicating that the isolectric points of the several proteins were not sensibly different and close to *p*H 4.8.

The nitrogen factor of the albumin crystallized as sulfate was appreciably higher than that of the carbohydrate-free crystalline serum albumin from which it was derived. The latter carbohydrate-free unfractionated serum albumin was estimated to contain 16.1% nitrogen by macro-Kjeldahl and 16.2% nitrogen by Nesslerization. These results are in good agreement with the older values, but are higher than some recent reports. Abderhalden[20] found the nitrogen content of serum albumin to be 15.89, while Hewitt[4] reports from 14.1 to 14.4%, and Adair and Robinson[21] 15.6% nitrogen.

The isoelectric albumin prepared from the crystalline sulfate was found to contain 16.8% nitrogen by the Kjeldahl method. Protein concentration was determined by evaporation to constant weight of the salt-free solution at the isoelectric point in an oven at 105°, and also by weighing the dried coagulated protein. The results from the two methods were in good agreement.

(15) Steinhardt, *J. Biol. Chem.*, **129**, 135 (1939).
(16) Mellanby, *J. Physiol.*, **33**, 338 (1905–1906).
(17) S. P. L. Sörensen, and M. Sörensen, *Compt. rend. trav. Lab. Carlsberg*, **19**, No. 11, 1 (1933).
(18) Cohn, McMeekin, Edsall and Weare, THIS JOURNAL, **56**, 2270 (1934).
(19) Sörensen, *Compt. rend. trav. Lab. Carlsberg*, **12**, 26 (1917).
(20) Abderhalden, *Z. physiol. Chem.*, **37**, 495 (1903).
(21) Adair and Robinson, *Biochem J.*, **24**, 993 (1930).

Tryptophan has been present in the purest preparations of serum albumin but to a very small extent and it therefore seemed important to determine the amount of this amino acid in our preparations both before and after crystallization of the albumin sulfate. For this purpose Lugg's modification[22] of the Folin–Ciocalteu–Marenzi[23] method was used and a tryptophan content of 0.53% estimated for the albumin both before and after crystallization as a sulfate. This value is in good agreement with that of Folin and Marenzi[23] and is approximately double the value reported by Hewitt.[4,24]

The albumin sulfate was electrophoretically homogeneous at *p*H 7.4, but still showed a small quantity of a second component at *p*H 4.0. Its solubility in water at 4° was very high but at room temperature only moderate. The data in Table II illustrate its solubility in water at 25°. The *p*H was 4.0 ± 0.02 and the solvent was saturated with toluene.

TABLE II

SOLUBILITY OF CRYSTALLINE SERUM ALBUMIN SULFATE AT *p*H 4.0 AND 25°

No. of equilibrations of one day each	Vol. of solvent, cc.	Soly., g./liter: Solute A 0.6 g. protein	Soly., g./liter: Solute B 3.0 g. protein
1	5	31.7	49.3
2	5	29.1	46.4
3	5	34.4	47.5
4	5	14.2[a]	45.0
5	5		43.9
6	5		41.4
			26.0[a]

[a] The solute protein was largely denatured at this stage as was shown by absence of crystalline form and almost complete insolubility in water.

Analysis for the sulfate ion in these crystals yielded the result that each gram of the protein was combined with 0.00035 equivalent of sulfate, a value considerably in excess of the amount of sulfate, 0.00026 equivalent per gram, necessary to titrate the albumin from the isoelectric point to a *p*H 3.95.

The sulfate ion was removed from albumin sulfate by dialysis and electrodialysis and the protein again crystallized from ammonium sulfate. The solubility of these crystals at 25° was determined in a 1.47 *M* ammonium sulfate solution, and the results are given in Table III. Although

TABLE III

SOLUBILITY IN 1.47 *M* AMMONIUM SULFATE OF ALBUMIN ISOLATED BY CRYSTALLIZATION AS SULFATE AT *p*H 4.0 AND 25°

No. of equilibrations of one day each	Vol. of solvent, cc.	Soly., g./liter[a]: Solute A 0.2 g. protein	Soly., g./liter[a]: Solute B 1.0 g. protein
2	40	0.62	(1.03)
3	40	.54	0.50
4	40	.80	.54
5	40	(1.07)	.57
6	40		.49

[a] The *p*H of the filtrate was determined each day. For the more dilute series it always remained 4.8 ± 0.04 and for the more concentrated series 4.63 ± 0.03. In subsequent experiments it should be possible to control the *p*H in these unbuffered solutions even more carefully.

there is some drift from day to day in these unbuffered solutions, there is no evidence of increase in solubility with increase in saturating body or of decrease in solubility with increase in the number of days during which the saturating body was equilibrated with fresh aliquots of solvent. The extent of the purification achieved by crystallization as the albumin sulfate is perhaps best demonstrated by a comparison of Table III with Table I. The results reported in Tables II and III indicate that at least one component of the serum albumin system has been separated as a chemical individual. Further studies both of its composition and properties are being undertaken and will be reported subsequently.

I am indebted to Professor Edwin J. Cohn for his suggestions and advice throughout this investigation.

Summary

1. Carbohydrate-free crystalline serum albumin has been separated into two fractions by crystallizing the sulfates from water.

2. The solubility in salt solution of the protein regenerated from the insoluble albumin sulfate was found to be essentially independent of the amount of protein in the system, and of the volume of solvent with which it was equilibrated, suggesting that this albumin component had been separated as a chemical individual.

3. The percentage of nitrogen in this serum albumin was found to be higher, tyrosine lower, and tryptophan the same as in unfractionated carbohydrate-free crystalline serum albumin.

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(22) Lugg, *Biochem. J.*, **31**, 1422 (1937).

(23) Folin and Ciocalteu, *J. Biol. Chem.*, **73**, 627 (1927); Folin and Marenzi, *J. Biol. Chem.*, **83**, 89 (1929).

(24) Preliminary results on the tyrosine content of the albumin sulfate suggest that it is appreciably lower than that of the material from which it was derived but not by a sufficient amount to account for the differences in nitrogen percentage.