terial of refractive index characteristic of lipid-free protein with mixed plasma lipids in volume fraction corresponding to the analytical figure for the lipoproteins.

Specific refractive increments in terms of nitrogen range from 1.17×10^{-3} (g. N/liter)$^{-1}$, characteristic of lipid- and carbohydrate-poor proteins, to 4.05×10^{-3} (g. N/liter)$^{-1}$ for β_1-lipoprotein. The variation between proteins is roughly inversely proportional to the nitrogen factors.

Corresponding nitrogen factors are given together with empirical rules relating the weight content cholesterol of normal human plasma lipoprotein fractions to weight content nitrogen and refractive index increment in terms of weight of dried protein.

The conditions for dry weight measurements on plasma lipoproteins are re-examined experimentally.

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

Preparation and Properties of Serum and Plasma Proteins. XIII. Crystallization of Serum Albumins from Ethanol–Water Mixtures[1a,b]

BY E. J. COHN, W. L. HUGHES, JR., AND J. H. WEARE

I

Roughly half of the proteins of plasma are albumins. The albumins all appear to be of closely the same molecular weight and of closely the same isoelectric point. They are not only among the most stable, but also among the most soluble of the plasma proteins. In the classical procedure for the separation of proteins by "salting-out" the albumins remain soluble in neutral, two molal, that is to say in half-saturated, ammonium sulfate solution.[2] In the methods which have been developed recently for the separation of proteins in ethanol–water mixtures of controlled *p*H, ionic strength, and temperature,[3,4] the albumins remained in solution when the γ-globulins and certain of the β-globulins were precipitated from an ethanol–water mixture of mole fraction 0.091 at *p*H 6.8 and $-5°$ and the remaining β-globulins and α-globulins were precipitated by increasing the ethanol to mole fraction 0.163 at *p*H 5.8 and $-5°$. The supernatant solution, in the case of the fractionated human plasma proteins, contained 92% of the serum albumins.[4] The albumins were readily precipitated by adjusting the *p*H to 4.8. The fraction separated by this change in *p*H in a 0.163 mole fraction ethanol–water mixture at $-5°$ has been called Fraction V in our system of plasma fractionation. It has proved a convenient starting material (1) for purification of the albumins, by precipitating most of the remaining globulins (in Fraction V-1) from a more concentrated solution of the crude fraction at lower ethanol and salt concentrations, and (2) for crystallization of the albumins.

The albumins of different species vary greatly in the ease with which they may be crystallized. Horse serum albumin crystallizes readily from half-saturated ammonium sulfate solution upon acidification[5,6,7]; that is to say, by the method for egg albumin used by Hopkins and Pinkus[8] and later studied in such great detail by Sørensen and Høyrup.[9] Crystallization of human serum albumins from concentrated ammonium sulfate solutions has been carried out by Adair and Taylor[10] and special conditions developed, so that the procedure might consistently yield crystalline preparations, by Kendall.[11] The albumins from bovine plasma have, however, resisted crystallization from ammonium sulfate solution.

The difference in the ease of crystallization of these different species may be related to differences in the polypeptide structure of the albumins, or in the nature and amounts of the carbohydrate or lipid present. Hewitt and Kekwick demonstrated that horse serum albumin could be fractionated into a carbohydrate-free and a carbohydrate-rich fraction,[6,12] and McMeekin succeeded in crystallizing an "albumin" fraction with a carbohydrate content as high as 5.5%.[13] The nitrogen content of his carbohydrate-rich fraction

(1a) This work has been supported by grants from the Rockefeller Foundation and from funds of Harvard University. It was aided early in 1941 by grants from the Committee on Medicine of the National Research Council, which included a grant from the American College of Physicians. From August, 1941, to July, 1946, it was carried out under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

(1b) This paper is Number 60 in the series "Studies on Plasma Proteins" from the Department of Physical Chemistry, Harvard Medical School, Boston, Massachusetts, on products developed by the Department of Physical Chemistry from blood collected by the American Red Cross.

(2) The earlier literature with respect to the "salting-out" of the plasma proteins is referred to in the first paper in this series: E. J. Cohn, T. L. McMeekin, J. L. Oncley, J. M. Newell and W. L. Hughes, Jr., THIS JOURNAL, **62**, 3386 (1940).

(3) E. J. Cohn, J. A. Luetscher, Jr., J. L. Oncley, S. H. Armstrong Jr., and B. D. Davis, *ibid.*, **62**, 3396 (1940).

(4) E. J. Cohn, L. E. Strong, W. L. Hughes, Jr., D. J. Mulford, J. N. Ashworth, M. Melin and H. L. Taylor, *ibid.*, **68**, 459 (1946).

(5) S. P. L. Sørensen, *Compt. rend. trav. lab. Carlsberg*, **18**, No. 5, 1 (1930).

(6) L. F. Hewitt, *Biochem. J.*, **30**, 2229 (1936).

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

(8) F. G. Hopkins and S. N. Pinkus, *J. Physiol.*, **23**, 130 (1898).

(9) S. P. L. Sørensen and M. Høyrup, *Compt. rend. trav. lab. Carlsberg*, **12**, 164 (1917).

(10) M. E. Adair and G. L. Taylor, *Nature*, **135**, 307 (1935).

(11) F. E. Kendall, *J. Biol. Chem.*, **138**, 97 (1941).

(12) R. A. Kekwick, *Biochem. J.*, **32**, 552 (1938).

(13) T. L. McMeekin, THIS JOURNAL, **62**, 3393 (1940).

was 15.1%, and of his carbohydrate-free fraction was 16.1%.

Kendall's preparations of crystallized human serum albumins were free from carbohydrate when analyzed by the orcinol method of Sørensen and Haugaard.[14] After four crystallizations, however, one-half per cent. lipid was readily removed with dry ether in a Soxhlet extractor. The extracted protein still contained large quantities of lipid and a yellow pigment. As much as 1.8 to 2.9% lipid was extracted with hot alcohol if the albumin had previously been denatured by heating in aqueous solution. Completely soluble in acetone and free from cholesterol, "the lipid could be extracted from chloroform or ether with NaOH and reëxtracted from the aqueous solution with ether after acidification. Both the color and the odor associated with human serum followed the lipid through these extractions. It would thus appear that crystalline human serum albumin is associated with a free fatty acid which cannot be extracted without first denaturing the albumin."[11] The amount of lipid varied in Kendall's different preparations. By fractional crystallization, albumin was obtained with a lipid content as low as 0.4%, the fraction of low lipid content being the more soluble. The less soluble fraction separated from it also had a reduced lipid content of 1.3%, however. Presumably, therefore, repeated recrystallization did not yield one fraction richer and one fraction poorer in lipid than the starting material, but two fractions both of which were lower in lipid content than the starting material.

All of the evidence deduced from the diverse investigations upon the serum albumins thus suggests that this large and important class of proteins may, *in vivo*, be combined with other substances, carbohydrate or lipid, and that the complexes so formed are still capable of crystallization.

In the course of the development of fractionation procedures in ethanol–water mixtures at low temperatures, methods of protein crystallization in these systems have been developed which have proved readily adaptable to large scale production, making possible the preparation of albumins of a very high degree of purity. It has proved possible to remove serologically identifiable globulin impurities to any desired extent. The success of these methods has depended, in part, upon the discovery of the importance of the higher aliphatic alcohols and other substances as aids in crystallization.

II. MATERIALS

Albumins.—The starting material for all of the following procedures has been the albumin fraction of plasma prepared by methods already described.[4] Fraction V so prepared (by Methods 2, 5 or 6) has been brought to the desired crystallizing condition, or the supernatant from Fraction IV, concentrated in a vacuum still (by Methods 3 or 4), has been crystallized by dialyzing to the appropriate conditions.

Long Chain Alcohols.—The use of long chain aliphatic alcohols and certain other substances has made possible the reproducible crystallization of serum albumins from ethanol–water mixtures. These substances form slightly dissociated complexes with the serum albumins, being carried with them through repeated recrystallizations in the concentrated solutions employed.

The effective concentration of these crystallization aids has varied from 0.01 to 1%. Concentration has affected the rate of crystallization and secondarily the yield. All of the aliphatic alcohols containing five or more carbon atoms which have been tested—*n*-pentanol, *n*-hexanol, *n*-heptanol, *n*-octanol, *n*-nonanol, *n*-decanol—have proved effective, as has a corresponding halide, *n*-amyl chloride.

Benzene and toluene have also aided the crystallization of serum albumins, as have chloroform and ethylene dichloride when present in high concentrations of roughly one per cent.

The effectiveness of the higher aliphatic alcohols increased with increase in the length of the carbon chain, at least up to decanol. The very long chain alcohols, such as stearyl or cetyl alcohol, however, showed considerably less effect, perhaps because they are so insoluble in the ethanol–water–protein systems. Ethyl ether, while ineffective alone, augmented the action of effective reagents. In the crystallization of human serum albumin, 0.3% ethyl ether in combination with 0.03% of *n*-decanol was as effective as 0.1 to 0.2% of decanol alone. Nevertheless, the alcohol has generally been used without ethyl ether, because of the high volatility and inflammability of ether.

III. METHODS

Since most of the apparatus and techniques employed have been fully described in paper IV of this series,[4] only those additional techniques or variations essential to crystallization will be described here.

In the interests of the stability of the final product, all operations have been carried out close to the freezing point of the system. Since crystallization proceeds slowly, it has proved necessary to crystallize from concentrated protein solutions in order to obtain good yields within a practical period of time.

The Sharples super-centrifuge has proved particularly useful in separating crystalline precipitates. Since the resulting pastes often contain more than 40% solids, efficient purification has been possible even from concentrated protein solutions. When thorough washing of the crystals was required, they were separated by filtration to produce a loose filter cake.

Exhaustive clarification of all solutions by filtration through diatomaceous filter cakes or asbestos filter pads has been used for the removal

(14) M. Sørensen and C. Haugaard, *Compt. rend. trav. lab. Carlsberg*, **19**, No. 12, 1 (1933); *Biochem. Z.*, **260**, 247 (1933).

of insoluble impurities. By taking advantage of the time lag in crystallization, it was sometimes possible to filter off a more rapidly forming amorphous globulin precipitate before crystallization of the albumin from the solution was appreciable.

After seeding to initiate crystallization, agitation of the solution was avoided, in order that the crystals formed might be as large as possible. If the crystals tended to settle, the solution was occasionally stirred gently to resuspend them.

In the definition of crystallizing conditions, constant use has been made (1) of the point of incipient amorphous precipitation, since conditions far removed from this point have not yielded crystals, and (2) of the considerable difference in solubility of crystalline and amorphous forms. Since it has been found impractical to readjust the solvent composition while crystallization was progressing, the solution to be crystallized was adjusted until it was on the verge of precipitating amorphously, then seeded and allowed to crystallize undisturbed. This procedure permitted the largest possible yield at constant conditions without the concomitant precipitation of impurities.

Tests of Physical Chemical Homogeneity

Repeated recrystallization has proved necessary to remove the last traces of impurities. Following each operation, tests were carried out to demonstrate that the albumin had not been measurably altered. For this purpose, analyses were carried out with the ultracentrifuge, viscosimeter, and nephelometer. The last proved most useful and informative when the observations were carried out in the following manner.

Thermal Stability.—The thermal stability of albumin solutions has been found to increase with recrystallization of the albumin. This effect may be due to the removal of either globulin impurities or partially denatured albumin. The method adopted for estimating the stability of the standard human serum albumin prepared for clinical use[15] has been modified for these measurements. The albumin solutions were prepared in a medium of constant composition so that the intrinsic stability of the albumin molecules could be measured independently of environmental variables. The conditions that were chosen were 1% protein dissolved in an acetate buffer of *p*H 4.9, and an ionic strength of 0.2. The protein solutions, after filtration through asbestos pads,[16] were diluted with an appropriate acetate buffer to give these final conditions.

When such solutions were heated at 57°, the nephelometric readings remained low and constant for several hours, then increased rapidly, accompanied by first, visible opalescence, then flocculation. This test has proved particularly useful in determining that a crystallization process has removed impurities without damaging the albumins (see Table I).

Tests of Chemical Purity

Impurities have included small amounts of the various plasma protein components, salts, fatty acids, and pigment. Sodium acetate and sodium chloride have been present during crystallization and therefore have been present in small but calculable amounts in all crystalline products.

Euglobulin Precipitation.—A solution of Fraction V, dialyzed against cold distilled water, gave a slight precipitate. Upon repeated crystallization, the amount of this precipitate decreased until it could no longer be observed.

Precipitin Test for Globulins.—The most sensitive tests employed (not only for eu- but also for pseudoglobulins) depended upon immunological methods. The test solutions were prepared by sensitizing rabbits to bovine or human serum. The anti-albumin components were then removed by precipitation with limited amounts of purified albumin. Such absorbed serum precipitated strongly when mixed with crude serum albumin solutions.

The serum was titrated with serial dilutions of the albumin and the critical dilution, at which precipitation first occurred, determined. The corresponding dilution was determined for a solution containing a known concentration of the globulins occurring in Fraction V, and from the ratio of these critical dilutions, the concentration of globulins in the sample was calculated. This test has detected less than 0.01% of globulins in albumin preparations.[17]

Carbohydrate.—The glycoprotein impurities have been followed by means of the orcinol reaction.[14] Since the crude albumin (Fraction V) contained 0.5% carbohydrate or less and the sensitivity of the test in our hands has been about 0.1% carbohydrate, this test has proved useful only in the first stages of purification.

Pigment.—The absorption spectrum of serum albumins has been measured for 25 to 30% aqueous solutions *versus* a water blank in the Beckmann quartz spectrophotometer (Model DU). As shown in Fig. 5, serum albumins show progressively increasing absorption with decreasing wave length. However, the amount of absorption at all wave lengths from 300 to 500 mμ decreases upon recrystallization of the albumin (compare curves B and C, Fig. 5).

Fatty acids have been extracted from albumin and estimated by the following procedures: (1) by extraction with methanol after denaturing the albumins in boiling water (Kendall's method[11]), (2) by pouring a 5% aqueous solution of albumin into 4 volumes of methanol, or (3) by repeated extractions of the albumins dried from the frozen state with 10 parts of anhydrous methanol at −5°. The method which has proved most satisfactory for the routine quantitative estimation of fatty acids follows[19]:

One gram of the albumin to be tested was dissolved in 20 cc. of an ammonium acetate–acetic acid buffer of *p*H 4.7 and ionic strength 0.05.[20] This solution was then pipetted dropwise into 80 cc. of absolute methanol in a centrifuge tube. The precipitated albumin was centrifuged and washed once with 100 cc. of methanol.

The combined methanol extracts were evaporated just to dryness on a steam-bath. The residue was extracted three times with 15-cc. portions of ether. The combined ether extracts were filtered and evaporated. Finally, the residue was heated on a steam-bath for one hour to remove volatile acids such as acetic acid. The oil was then dissolved in 3 cc. of boiling 75% methanol and titrated while boiling with standard sodium hydroxide in 75% methanol using phenolphthalein as indicator.

This method, when tested with stearic acid, gave the theoretical neutralization equivalent. It has been possible to detect approximately 0.05 mole of fatty acid per mole of albumin. The albumin was not grossly denatured by this procedure, being readily soluble in water after re-

(15) G. Scatchard, S. T. Gibson, L. M. Woodruff, A. C. Batchelder and A. Brown, *J. Clin. Invest.*, **23**, 445 (1944).

(16) Pads prepared by Republic Filter Paper Co., and designated Seitz "Serum #3" have been found satisfactory.

(17) This test was first carried out by C. A. Janeway on samples of our crystallized bovine serum albumins. More recently we have found it equally applicable for testing the purification of human serum albumins. F. E. Kendall has also analyzed certain of our crystallized bovine serum albumins, using Heidelberger's quantitative precipitin techniques, and obtained similar results.[18]

(18) F. E. Kendall, personal communication.

(19) We are indebted to Mrs. B. J. Livingstone for carrying out these analyses.

(20) Ammonium acetate was used because it is readily volatile on a steam-bath.

moval of the methanol. If such extracted albumin was completely denatured by heating, less than 0.05 equivalent of fatty acid per mole of albumin could be extracted by a second methanol treatment. If the initial albumin precipitate in 80% methanol was heated to denature it, the extraction was less efficient presumably because the precipitate changed from a flocculent to a gummy consistency, preventing efficient washing.

The ether-soluble oil, which partially crystallized on standing, was completely soluble in aqueous alkali. It had a neutralization equivalent of 350 (titrated in boiling 75% methanol with phenolphthalein as indicator) and contained less than 0.5% nitrogen. The crystalline fraction, after repeated crystallization from ethanol–water mixtures, had a melting point of 67–67.5° (stearic acid, m. p. 69.5°; mixed m. p. 68.5–69°).

IV. CRYSTALLIZATION OF BOVINE SERUM ALBUMINS

The first crystals of bovine serum albumin were observed while dialyzing an albumin preparation against 0.05 mole fraction ethanol at 0°. The yields by this method were poor, and crystallization commenced very slowly even following seeding. However, recrystallization was accomplished without difficulty.

It has subsequently been found that the ease of crystallization by this method and the yield obtained were markedly influenced by the previous treatment of the albumins. Thus, after the addition of aliphatic alcohols such as decanol, or after a heat treatment in a stabilized aqueous system which removes globulins (see below), or after a previous crystallization at high ethanol concentration, albumins were readily crystallized from solutions low in ethanol and electrolyte.

Crystallization at High Ethanol Concentration.—Bovine serum albumins crystallized readily from 0.163 mole fraction ethanol at a sufficiently low temperature. Since the serum albumins are very soluble, the *p*H was maintained close to the isoelectric point if satisfactory yields were to be obtained. The range of solvent compositions for the preparation of this type of crystal have been

Ethanol	vol. % at 25°	33	to	40
	mole fraction	0.12	to	0.163
Ionic strength $\Gamma/2$		0.35	to	0.65
*p*H		5.3	to	5.7
Temperature, °C.		+5	to	−10°
Protein concentration, %		10	to	20

Within the above range the best defined crystals as illustrated in Fig. 3 have been obtained at ionic strengths above 0.4. At lower ionic strengths, the crystal form was globular. The high temperature coefficient of solubility for albumin has been used advantageously for the preparation of large crystals by slowly cooling a solution of the proper composition over the course of ten to twenty hours.

The details of one of a series of crystallization procedures modified from that described previously[21] follows.

(21) Memorandum of April 11, 1942, to the National Research Council and the Committee on Medical Research of the Office of Scientific Research and Development.

Method IV.[22] Crystallization of Bovine Serum Albumins at 35% Ethanol; $\Gamma/2 = 0.35$; *p*H 5.6; $t = -5°$.—To 1 kilogram of Fraction V paste (wet weight)[23] at −5°, add 300 ml. of 0.4 *M* *p*H 5.5 acetate buffer in 35% ethanol precooled to −10° and 430 ml. of 1.0 *M* sodium acetate in 25% ethanol likewise at −10°. Good results in dissolving the Fraction V are obtained by adding all of the buffer and about half of the sodium acetate and stirring with a slow stirrer for an hour. The rest of the sodium acetate is then added when all of the lumps have disintegrated. Allow to stand several days at −5° to crystallize. Add 1 liter of 35% ethanol to dilute the crystal suspension and lower the ionic strength just before centrifuging; centrifuge at −5°: yield 80% of Fraction V.

The crystals are dissolved in 1.5 volumes of 15% ethanol—0.02 *M* sodium chloride at −5° and clarified by filtration through washed, calcined diatomaceous earth. This solution may be recrystallized by readjusting to the conditions for the first crystallization or it may be recrystallized at 22% ethanol with the aid of a very small amount of decanol (enough to give a final concentration of 0.02%) by the method described below.

Crystallization at Low Ethanol Concentrations.—Since crystallization from lower ethanol concentration produced greater purification as judged by the removal of carbohydrate and of globulin, as well as a more stable product, it was adopted as a standard method of crystallization as soon as a reproducible method of obtaining good yields had been developed.

Crystals as shown in Fig. 4 have been obtained within the following ranges provided that the combination of variables was so chosen that the albumin concentration exceeded the solubility of crystalline serum albumins

Ethanol	vol. % at 25°	0	to	25.
	mole fraction	0	to	0.09
Decanol,[24] moles/liter		0	to	0.02
Ionic strength $\Gamma/2$		0.0	to	0.05
*p*H		5.0	to	6.0
Temperature, °C.		+25	to	−5°
Protein concn.	%	5	to	20
	moles/liter	0.001	to	0.003

In the interests of stability, conditions close to the freezing point of the solvent have always been chosen.

The best procedure thus far developed follows.

Method VII. 1. Crystallization at 22% Ethanol; 0.1% Decanol; $\Gamma/2 = 0.05$; *p*H 5.3; $t = -5°$.—To 1 kilogram of Fraction V[23] (wet weight) at −10 to −15° add 500 ml. of 15% ethanol at −5° and stir slowly until a uniform suspension is obtained. Then add 500 ml. of 15% ethanol at −5°. Add sufficient 0.2 *M* sodium bicarbonate solution, precooled to 0°, to bring the *p*H (1:10 dilution) to 5.3. This should require 125–150 ml. of 0.2 *M* sodium bicarbonate. Some temperature rise

(22) In the development and application of these methods to the large scale production of crystallized bovine serum albumin, we have enjoyed the close collaboration of J. D. Porsche and J. B. Lesh of Armour and Company.

(23) This paste, obtained by removing Fraction V[4] in the Sharples super-centrifuge, contained approximately 25% protein by weight. When dissolved in 25 parts of water it had a *p*H o f5.0, having been precipitated from a solution of *p*H 5.3. This was done purposely in bovine plasma fractionation since the convenience of obtaining albumin paste with the lowest possible salt content more than offset the slightly greater loss of albumin into the supernatant from Fraction V.

(24) Lower alcohols such as *n*-pentanol or *n*-hexanol were also effective, particularly below 0.05 mole fraction ethanol. However, a higher concentration of these alcohols was necessary.

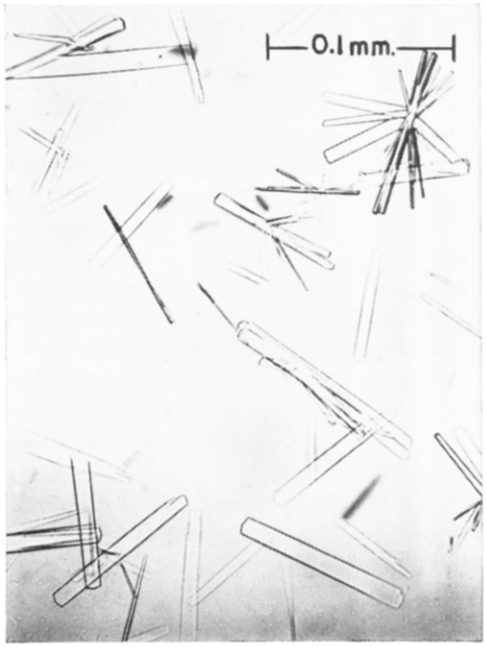

Fig. 1.—Human serum albumins crystallized from 0.09 mole fraction ethanol; 0.1% decanol; $\Gamma/2 = 0.1$; *p*H 5.5; −5°.

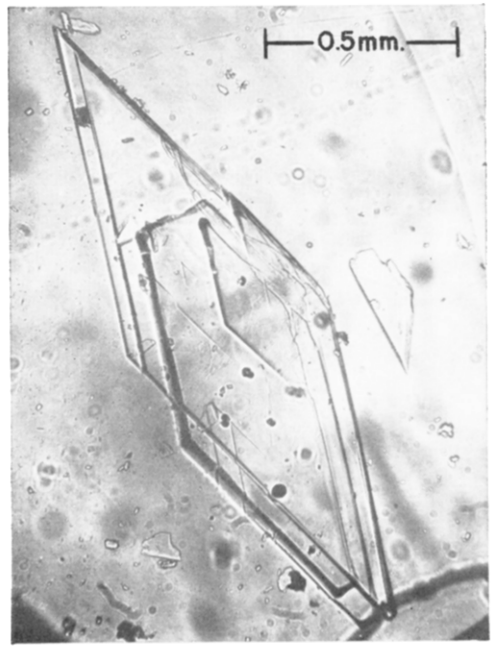

Fig. 2.—Human serum albumins crystallized from water saturated with decanol; $\Gamma/2 < 0.001$; *p*H 4.9; 0°.

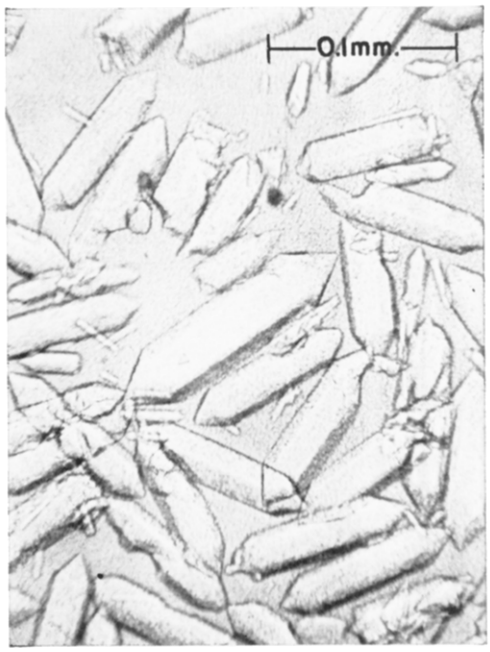

Fig. 3.—Bovine serum albumins crystallized from 0.14 mole fraction ethanol; $\Gamma/2 = 0.4$; *p*H 5.5; −5°.

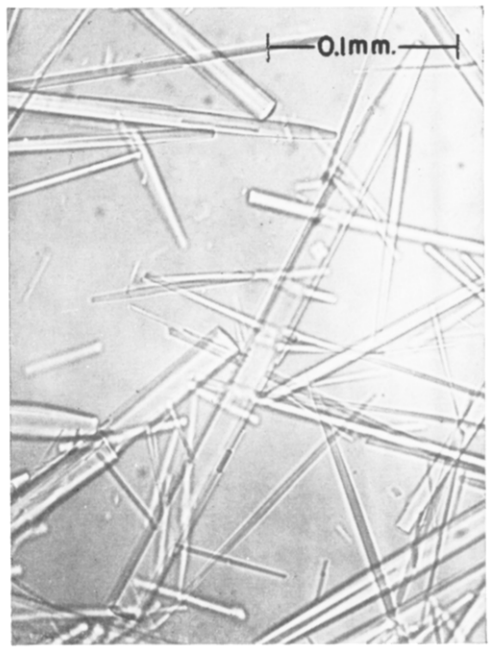

Fig. 4.—Bovine serum albumins crystallized from 0.05 mole fraction ethanol; $\Gamma/2 = 0.02$; *p*H 5.1; −5°.

TABLE I

CRYSTALLIZATION OF BOVINE SERUM ALBUMIN BY METHOD VII

	Conditions for crystallization				Properties of crystals			
	Ethanol mole fract.	Γ/2	Temp., °C.	pH	Protein, %	Yield from Fract. V, %	Globulin (precipitin), %	Carbohydrate, %	Stability[a] hours
Fraction V (amorphous ppt.)	0.163	0.08	−5	5.0	0.7	(100)	1.2	0.5	
First crystn.	.082[b]	.05	−5	5.3	12	(65)[c]	0.4	.2	21
Second crystn.	.058	.02	−5	5.1	7	45[d]	.1	< .1	20
Third crystn.	.040	.005	−4	5.1	9	29[d]	.06	< .1	25
Fourth crystn.	.051	.02	−5	5.1	9	25[d]	.03	< .1	26

[a] Hours for a 1% solution of the albumin in a *p*H 4.9, ionic strength 0.2 acetate buffer to increase 20 nephelometric units at 57°. [b] 0.1% decanol was also present. [c] Albumin may be crystallized from the mother liquor from the first crystallization following heat denaturation of globulin, by adjusting to conditions for second crystallization. [d] The mother liquors from the second, third, and fourth crystallizations may be combined, concentrated, and further amounts of serum albumin crystallized under conditions for the second crystallization.

occurs during these additions, and care must be taken that the maximum temperature does not exceed −5°.

The albumin may still be only partially dissolved. To complete the solution, small amounts of water, 100 cc. at a time, precooled to 0°, are added. Allow fifteen minutes between successive additions to permit equilibrium to be established. Undissolved albumin can be readily distinguished from small amounts of undissolved globulin, for as the last albumin dissolves, the appearance of the solution changes sharply from a milky white to a hazy gray-green color.

After standing twelve hours at −5° the solution is filtered. This is best accomplished by suspending in it 15 g. of washed, fine, calcined diatomaceous earth, and then filtering this mixture on Buchner funnels which have been precoated with a coarser diatomaceous earth. A filtrate may require two or more complete passages through filter cakes to remove haze and attain any desired clarity.

To crystallize the filtrate, add through a capillary pipet, with careful stirring, $^1/_{100}$ of the volume of a solution containing 10% decanol and 60% ethanol, precooled to −10°, and seed the resulting solution with the needle type of crystal.

After allowing two to three days for crystallization to become complete, the crystals are centrifuged. Suspend the centrifuged crystals, with gentle mechanical stirring, in one-third their weight of 0.005 *M* sodium chloride, precooled to 0°. When suspension is complete, with careful stirring add slowly water precooled to 0°, in amount equal to 1.7 times the weight of the crystals. There is approximately 7% ethanol at this stage of the process, and the temperature cannot, therefore, be lower than −2.5° and must not exceed −1°. The resulting solution is clarified by filtration through diatomaceous earth.

2. **Recrystallization.**—The maximum amount of 53% ethanol which can be added to the above filtrate without precipitating the albumin at the freezing point of the resulting solution is now determined on an aliquot. This amount, precooled to −30°, is added through a capillary to the filtrate in about thirty minutes, with careful stirring so that there is no local excess. In order to maintain the solution at all times within two degrees or less of its freezing point, some additional cooling is required at this point. The cooling may be accomplished by precooling the filtrate before the addition of ethanol. The solution is then seeded and allowed to stand at not more than one degree above the freezing point. The crystals are separated in the Sharples centrifuge at a temperature just above the freezing point of the mother liquor. The crystals are suspended with gentle stirring in one-third their weight of 0.005 *M* sodium chloride, precooled to 0°. When suspension is complete, water at 0°, equivalent to 1.7 times the weight of crystals, is added, the temperature being maintained below −1°. The solution is clarified by filtration through diatomaceous earth at a temperature of not more than one degree above its freezing point.

3. **Recrystallization.**—The procedure given in step 2 above is repeated.

4. **Recrystallization.**—To the filtrate from step 3 add one-fourth volume of 53% ethanol at −30° containing 6.0 g. sodium chloride per liter.[25] Determine the freezing point and amorphous precipitation point and adjust both to −5 to −6° by addition of small amounts of 53% ethanol. Seed and allow to stand at −5° for at least twenty-four hours. Separate the crystals in the Sharples centrifuge at −5° and suspend them in one-half their weight of 0.005 *M* sodium chloride preparatory to drying from the frozen state.

In method VII the albumin was repeatedly recrystallized to obtain the purest albumin possible as judged by loss of globulin by the precipitin test. Consequently, the yield was never more than 20 to 25% of the starting material (Fraction V). However, the protein was recovered from the mother liquors of the second, third and fourth crystallizations by amorphous precipitation (see Paper IV) and then readjusted to crystallizing conditions, thereby obtaining a further 15% of crystallized albumin indistinguishable from the first fraction.

The mother liquors from the first crystallization, while still rich in albumin, crystallized with difficulty, presumably because of their high globulin content. Following partial removal of globulin by differential heat denaturation,[26] approximately 40% of the protein was crystallized from this fraction.

One method employing sodium mandelate to stabilize the albumin[27] selectively follows.

The mother liquor from the first crystals was frozen and dried under vacuum. The resulting powder was dissolved in 3.2 parts by weight of an aqueous solution containing 0.13 *M* sodium mandelate and 0.01 *M* sodium bicarbonate. The resulting 25% protein solution contained 0.1 *M* sodium mandelate and had a *p*H of 6.0.[28] This solu-

(25) After three crystallizations at successively lower ionic strengths, the ionic strength was raised in the last crystallization to permit a possible fractionation from impurities with a solubility like albumin at 0 ionic strength.

(26) E. J. Cohn, *Chem. Rev.*, **28**, 395 (1941).

(27) The stabilizing action of the salts of fatty acids has been extensively investigated and reported by Luck, *et al.*: G. A. Ballou, P. D. Boyer, J. M. Luck and F. G. Lum, *J. Clin. Invest.*, **23**, 454 (1944); G. A. Ballou, P. D. Boyer, J. M. Luck and F. G. Lum, *J. Biol. Chem.*, **153**, 589 (1944); G. A. Ballou, P. D. Boyer and J. M. Luck, *ibid.*, **159**, 111 (1945); P. D. Boyer, F. G. Lum, G. A. Ballou, J. M. Luck and R. G. Rice, *ibid.*, **162**, 181 (1946); P. D. Boyer, G. A. Ballou and J. M. Luck, *ibid.*, **162**, 199 (1946).

(28) A *p*H slightly acid to that of optimum albumin stability aided subsequent removal of the coagulated protein.

tion was heated in a water-bath at 68.0 ± 0.2° for ten hours. During this heating the solution first became turbid, then a heavy coagulum separated, leaving a clear supernatant,[29] which was free of globulin at the end of the heating period when tested by the precipitin test described above.

The heated solution was cooled, diluted with 7 volumes of 23% alcohol at −5° and centrifuged. The supernatant was completely clarified by filtration through asbestos filter pads. It was then brought to suitable conditions for the precipitation of bovine serum albumin (35% ethanol, *p*H 5.2, 3% protein) and the precipitate was centrifuged. The precipitate was dissolved in an equal volume of water and the *p*H readjusted to 5.3 with sodium bicarbonate. Upon seeding, it crystallized readily in the needle form of crystal. Yield was 40% of the protein in the mother liquor from the first crystals.

V. CRYSTALLIZATION OF HUMAN SERUM ALBUMINS

The crystallization of human serum albumins from ethanol–water mixtures reveals striking similarities and differences to that of bovine serum albumins. Both show two separate sets of crystallizing conditions: one at high ethanol concentrations and one at low ethanol concentrations, and both show the same physical chemical response to their environment. However, the crystallization of bovine serum albumin appears to be unaffected by decanol and related compounds at high ethanol concentrations; and even at low ethanol concentrations, crystallization may proceed without these reagents. The crystallization of human serum albumin has thus far only been accomplished with the aid of some such substance as decanol.[30]

The various conditions under which human serum albumin has been crystallized are summarized in Table II.

TABLE II

CONDITIONS FOR THE CRYSTALLIZATION OF HUMAN SERUM ALBUMIN

Ethanol mole fraction	Additional reagent	Γ/2	*p*H	Temp., °C.
0	Decanol	< 0.001	4.9	0
0.02 to 0.06	$CHCl_3$	0 to 0.05	4.9	+10 to − 5
0.09 to 0.163	Decanol, $CHCl_3$, benzene, etc.	0.05 to 0.3	5.3	+10 to −10

The rhombic plates (Fig. 2) obtained by dialysis of Fraction V, or a more purified albumin, *vs.* distilled water have been obtained only in the presence of relatively large amounts of decanol and then only in poor yield. However, the crystals once obtained were readily recrystallized from water in good yield. It seems probable that these crystals represent a true fractionation of the serum albumins.[31]

(29) The partial purification of human serum albumin by a similar heat treatment in mandelate or caprylate solution has been accomplished by Luck, *et al.* (personal communication).

(30) Historically, the first crystals of human serum albumin from ethanol–water mixtures, were observed while dialyzing the salts from a vacuum still concentrate of serum albumin. Decanol had been added during distillation to prevent foaming.

(31) A fraction of the human serum albumins has recently been crystallized as a mercury salt; Hughes, THIS JOURNAL, **69**, 1836 (1947).

The crystallization of human serum albumins under the other two sets of conditions has been carried out with yields of 90% or more so that these conditions would appear better for separating the albumins from other proteins than for separating the different albumins from each other.

Crystallization with the aid of chloroform from 0–0.06 mole fraction ethanol permitted recrystallization at successively lower ethanol concentration: a method similar to the very successful method VII for bovine serum albumins. However, the process was handicapped by the high concentration (over 1%) of chloroform required.[32] Not only did this make it difficult to dissolve the chloroform in the albumin solution, but the high chloroform concentration seemed to cause the concomitant precipitation of some of a yellow pigment impurity so that it was not separated from the albumin. If the chloroform was added directly to the protein solution, it could be dissolved only by vigorous stirring; if it was added dissolved in ethanol, it caused severe local precipitation.

The most successful method for the crystallization of human serum albumins thus far developed is in the region of high ethanol concentration (see Table II and Fig. 1) using decanol or a mixture of decanol and ethyl ether as the crystallizing aid. Fraction V paste obtained from normal human plasma[4] may be readily crystallized under these conditions by the following procedure.

Crystallization at 25% Ethanol; 0.2% Decanol; Γ/2 = 0.15; *p*H 5.3; $t = -5$°C.—To 1 kilogram of Fraction V paste[4] (25–30% protein in 40% ethanol) is added 1 liter of water, and the mixture is gently stirred. To prevent excessive temperature increase during this operation due to the heat of dilution of the ethanol, it is desirable either to precool the Fraction V to −10 to −15° or to freeze the water partially. In this way, it is possible to obtain a solution at a temperature of −5°. During the dissolution of the protein, sufficient sodium bicarbonate is added to raise the *p*H to 5.2. At this point, the solution should be filtered free of insoluble material by passage through asbestos filter pads. (If an appreciable amount of insoluble material is present, the addition of small amounts of calcined diatomaceous earth to the solution may markedly accelerate this filtration.)

The clarified solution is now brought to ionic strength 0.15 by the addition of the calculated amount of chilled 2 *M* sodium acetate solution—allowance is made for the contribution of the paste and the sodium bicarbonate. The solution, which should now have a *p*H of 5.3 ± 0.1 and an ethanol concentration of approximately 16% is ready to adjust to crystallizing conditions (25 to 30% ethanol and 0.05 to 0.3% decanol) by the addition of the appropriate amounts of these reagents. Since these may vary from preparation to preparation, they are best determined on each lot by aliquot experiments as follows:

First, the maximum amount of 75% ethanol which can be added without causing protein precipitation above −7° is determined, then to a series of samples are added increasing amounts of decanol dissolved in this amount of 75% ethanol and the samples are allowed to stand at −5° to crystallize—they should be seeded, if possible.

After allowing one to two days for crystallization,[33] the

(32) The protein solution was nearly saturated with chloroform, when crystallization took place.

(33) Much longer times are necessary if no seed crystals are available.

aliquots are examined to determine the minimum amount of decanol giving a maximum yield of crystals. Excess decanol is to be avoided not only because it is difficult to remove from the final product but also because excess decanol may produce such small crystals that their technical manipulation is difficult.

The correct ethanol–decanol–water mixture is now added through a capillary with efficient stirring and cooling, and the solution, after seeding, is allowed to stand at −5° for several days undisturbed except for occasional resuspension of the crystals. The progress of crystallization may be readily followed by refractive index measurements on the supernatant fluid. If sufficient decanol has been used, the refractive index will decrease to a constant value within two to three days.

The crystals, because of their laminar shape, may be efficiently separated from the mother liquor by a Sharples super-centrifuge. The resulting pastes contain more than 40% protein; yield was up to 90% of the protein in Fraction V.

Recrystallization may be readily accomplished following resolution in water, by readjustment to the conditions of protein concentration, *p*H, ionic strength, and ethanol concentration used for the first crystallization. It is not necessary to add more decanol as sufficient is carried along with the crystals. In adjusting the ionic strength, it is preferable to add sodium acetate rather than sodium chloride in order to keep the solution well buffered, so as to minimize changes in *p*H during crystallization.

VI. PROPERTIES OF THE CRYSTALLIZED SERUM ALBUMINS

Many of the properties of the highly purified serum albumins, that have been made available in large amounts by these methods, have received extensive study. Some of these studies, as indicated below, are already published. Others continue.

Composition.—Except for small amounts of electrolytes, which may be removed by electrodialysis, and of fatty acids, which may be removed by alcohol extraction, these crystallized serum albumins are believed to be composed exclusively of amino acid residues. Brand[34] has reported practically complete amino acid analyses of these proteins.[35] The orcinol test for carbohydrate[14] has been uniformly negative.

Affinity for Small Molecules.—The extraordinary capacity of serum albumins to combine with a wide variety of smaller molecules and ions has recently been stressed.[36] The ability of serum albumins to bind large anions, as reported by Grollman,[37] Bennhold,[38,39] Fairley,[40] and Kendall,[11] has been confirmed for these crystallized fractions.[27,41,42,43,44,45,46]

The experiments here reported indicate that even in the crystalline state, not only do serum albumins bind anions, but also certain neutral molecules such as long chain alcohols. Furthermore, the presence of such molecules strongly influences the crystallizability of the protein.

Serum albumins crystallized by our methods have contained fatty acids, but in smaller amounts than found by Kendall.[11] The crystallized albumins usually contained larger amounts of fatty acid than the Fraction V from which they were prepared, but never more than 1 mole of acid per mole of albumin. After their removal at low temperature by methanol extraction of the crystallized albumin in the form of a dry powder, the albumin crystallized poorly when brought to the usual crystallization conditions. The crystallizability was improved by the addition of 1 mole of sodium oleate or stearate per mole of albumin.[47] Such extracted albumin, although readily soluble, when analyzed in the ultracentrifuge showed about 30% of a faster component moving ahead of the main albumin component.

Optical Properties.—The optical rotation and refractive index increment are given in Table III. The purest crystallized serum albumins were almost colorless except in concentrated solution when they had a light, straw-yellow color. Typical graphs of the optical density *versus* wave length are plotted in Fig. 5. There was appreciable variation from preparation to preparation. Results obtained with the preparation showing least absorption have been plotted. Since the plot of optical density *versus* $1/\mu^4$ was never found to be linear, only a portion of the absorption in even the best preparation can be due to Tyndall scattering, and there must be present an impurity absorbing between 300 and 400 mμ. Further studies on the absorption and its relation to Tyndall scattering are in progress.

Another pigment with an absorption maximum at 405 mμ, characteristic of albumin-bound hematin,[45] has occasionally been found in crystallized serum albumin (curve D, Fig. 5) when the starting plasma had been contaminated with hemoglobin.

(34) E. Brand, *Ann. N. Y. Acad. Sci.*, **47**, 187 (1946). Brand's data are given by J. T. Edsall in "Advances in Protein Chemistry," Vol. 3, 1947, p. 383.

(35) It should be emphasized that since these preparations are not homogeneous as judged by solubility measurements, they may represent a mixture of proteins whose composition with respect to amino acids, fatty acids or other small molecules may vary from preparation to preparation.

(36) J. T. Edsall, "Advances in Protein Chemistry," Vol. 3, 1947, p. 383.

(37) A. Grollman, *J. Biol. Chem.*, **64**, 141 (1925).

(38) H. Bennhold, *Ergeb. inn. Med. u. Kinderheilk.*, **42**, 273 (1932).

(39) H. Bennhold, E. Kylin and S. Rusznyak (editors), "Die Eiweisskörper des Blutplasmas," Theodore Steinkopf, 1938, 470 pp.

(40) N. Hamilton Fairley, *Quart. J. Med.*, **10**, 115 (1941).

(41) G. Scatchard, L. E. Strong, W. L. Hughes, Jr., J. N. Ashworth and A. H. Sparrow, *J. Clin. Invest.*, **24**, 671 (1945).

(42) B. D. Davis and R. J. Dubos, *Archiv. Biochem.*, **11**, 201 (1946).

(43) L. Fieser and H. Heymann, in preparation (combination of antimalarial naphthoquinone with albumin).

(44) I. M. Klotz, J. M. Longfellow and O. H. Johnson, *Science*, **104**, 264 (1946); I. M. Klotz, F. M. Walker and R. B. Pivan, THIS JOURNAL, **68**, 1486 (1946); I. M. Klotz, *ibid.*, **68**, 2299 (1946).

(45) M. Rosenfeld, personal communication (combination of hemin with albumin).

(46) N. Martin, in preparation (Preparation and Properties of Serum and Plasma Proteins. XV. Interactions with Bilirubin).

(47) The exact role of fatty acid in albumin crystallization will be further studied when milder methods for their removal have been found. It seems possible that they may also affect classical salting-out crystallization procedures and that lipases (42) may also play an important role.

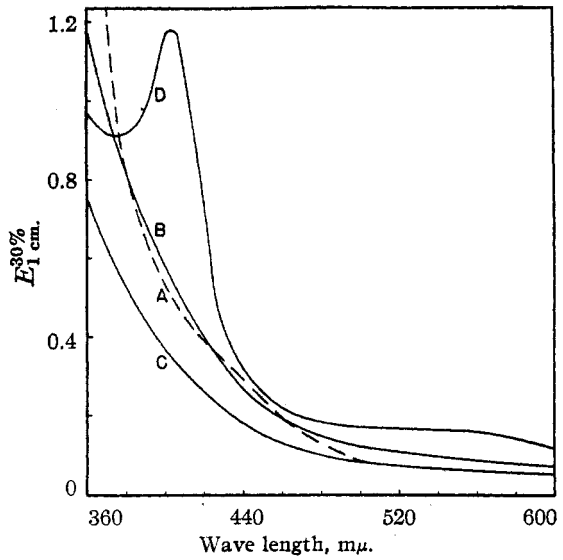

Fig. 5.—Absorption spectra of 30% aqueous solutions of serum albumins: A, Prep. 10 of human serum albumins crystallized with decanol and twice recrystallized; B, Prep. 26 of bovine serum albumins crystallized once with decanol by method VII; C, albumins in curve B after 3 recrystallizations; D, Prep. 51 of bovine serum albumins which contained hemin after 3 recrystallizations.

It has been demonstrated that human serum albumin crystallized by these methods is capable of combining with as much as 3 moles of bilirubin per mole of albumin.[46] Recrystallized albumin, however, is essentially free of this pigment unless the slight inflection at 450 mμ in curve A, Fig. 5, represents a trace of this substance.[48]

Physical Chemical Properties.—The isoelectric point of crystallized serum albumins determined as the *p*H of a 1% electrodialyzed albumin varied from 4.8 to 5.1. This variation may possibly be related to the fatty acid content, since after its removal by methanol extraction, the isoelectric point of both human and bovine serum albumins was raised to *p*H 5.3.

The thermal stability of carefully prepared crystallized serum albumins is considerably better than that of crude Fraction V or of any other fraction of plasma which we have thus far investigated. The relation of the solvent to the stability has been extensively investigated, particularly with regard to *p*H, protein concentration, ionic strength, and specific stabilizing anions.[15,27]

References to the measurement of sedimentation, diffusion, osmotic pressure, viscosity, dielectric constant, and electrophoretic mobility are given in Table III. With respect to electrophoretic analysis, these preparations, while homogeneous at *p*H 7.4 or 8.6, still showed the two components found by Luetscher[49] in serum albumins at *p*H 4.0.

Measurements in a viscous solution (*i.e.* 90% glycerol) have revealed double refraction of flow and made possible estimation of particle lengths in accord with those calculated from the sedimentation and diffusion constants.[50]

(48) The bilirubin in normal plasma, while precipitating in Fraction V, appears to be associated with an α_1-globulin component which is readily separated from the albumin by precipitation in Fraction V-1—the precipitate formed when Fraction V is brought to 3% protein at *p*H 4.5 in 10% ethanol and 0.01 *M* salt at −3°. (These are the conditions for the purification of the albumin in Fraction V, as already described.[4]) Further purification and characterization of this pigment protein will be reported later by G. Derouaux and others in this Laboratory.

TABLE III

CONSTANTS FOR CRYSTALLIZED SERUM ALBUMINS[a]

Per cent. nitrogen		16.0[b]
Optical rotation $[\alpha]^{25°}_{5461}$		−78 ± 2°
$[E]^{1\%}_{1\text{ cm.}}$ at 280 mμ	human	5.3
	bovine	6.6
Refractive index increment, $\Delta n/\Delta W$(g. prot./liter)		1.86×10^{-4}[c]
Partial specific volume		0.733[d]
Isoelectric point	human	4.9
	bovine	5.1
Electrophoretic mobility	*p*H 7.7, Γ/2 0.2 phosphate	− 5.2[e]
	*p*H 8.6, Γ/2 0.1 barbiturate	− 6.0[e]
Sedimentation constant $s_{20,w}$		4.6[d,f]
Diffusion constant $D_{20,w}$		6.1[d,g]
Intrinsic viscosity		0.042[d]
Molecular weight		69,000[h]
Dimensions	length, Å.	150[d]
	diameter, Å.	38[d]

[a] Values given are for human serum albumins. With the exception of the isoelectric point and extinction coefficient identical values within the experimental error have been obtained for bovine serum albumins. This is substantiated in the succeeding footnotes. [b] Brand[34] reported 15.95% for human serum albumin and 16.07% for bovine serum albumins. These values should vary from preparation to preparation depending on the amount of decanol or other crystallizing aid present. [c] S. H. Armstrong, Jr., M. J. E. Budka, K. C. Morrison and M. Hasson, in press. [d] J. L. Oncley, G. Scatchard and A. Brown, *J. Phys. Colloid Chem.*, **51**, 184 (1947). [e] S. H. Armstrong, Jr., M. J. E. Budka and K. C. Morrison, THIS JOURNAL, **69**, 416 (1947). [f] J. L. Oncley has obtained a value of 4.5 (the same within the experimental error) for bovine serum albumin, personal communication. [g] The same value (within the experimental error) of 6.4 has been reported for bovine serum albumin, E. J. Cohn, *Trans. Coll. Physicians Phila.*, [4] **10**, 149 (1942). [h] This value has been obtained for osmotic pressure measurements of both human and bovine serum albumins. G. Scatchard, A. Batchelder and A. Brown, *J. Clin. Invest.*, **23**, 458 (1944). G. Scatchard and A. Brown, THIS JOURNAL, **68**, 2320 (1946). The same value has been calculated from sedimentation and diffusion measurements.[d]

Summary

1. Methods are described for the crystallization of human and of bovine serum albumins from ethanol–water mixtures of controlled *p*H and ionic strength at low temperature.

2. Certain auxiliary substances, such as decanol, are demonstrated to aid crystallization.

3. Criteria of physical homogeneity and lack of chemical alteration of the serum al-

(49) J. A. Luetscher, THIS JOURNAL, **61**, 2888 (1939).

(50) J. T. Edsall and J. Foster, in preparation.

bumins, purified by these methods, are given.

4. Criteria of the purification achieved are presented.

5. Constants defining the properties of these purified serum albumins are tabulated.

BOSTON, MASS. RECEIVED MARCH 21, 1947

[CONTRIBUTION FROM THE NOYES CHEMICAL LABORATORY, UNIVERSITY OF ILLINOIS]

Properties of Polymers as Functions of Conversion. III. Molecular Weights of Bottle Polymerized GR-S[1]

BY F. T. WALL AND L. F. BESTE[2]

Numerous investigations have been carried out on various properties of polymers, such as the intrinsic viscosity, the molecular weight and the composition of copolymers. Much of this work, however, has been directed toward understanding the nature of the final product rather than the nature of the products obtained at different stages of conversion. It is the purpose of this present report to indicate and to interpret the molecular weight changes which occur with increasing conversion for the GR-S system.

The first paper[3] of this series dealt with the theoretical equations by which conversion data might be analyzed. In the second paper[4] the concept of "partial conversion properties" was defined and detailed intrinsic viscosity data were used for illustrative purposes. Although intrinsic viscosity gives some indication about the molecular weight it is felt that the viscosity does not provide a truly reliable index of that quantity. In particular, if one is interested in counting molecules it becomes necessary to determine a number average molecular weight which is usually quite different from that obtained by a viscosity measurement.[5] To obtain a number average molecular weight it is necessary to use some method involving a colligative property of the polymer solution such as its osmotic pressure. In this paper there will be reported the results of three separate studies of osmotic molecular weights of bottle-polymerized GR-S covering wide ranges of conversion.

Partial Conversion Properties

As indicated earlier,[4] if the molecules formed up to a certain stage of a polymerization reaction remain unchanged throughout the remainder of the polymerization, then any subsequent changes in the average or cumulative properties of the polymer can be attributed to the new molecules formed. Actually, since polymer molecules once formed can undergo further reactions, the apparent increment properties often reflect only the net result of a number of simultaneous processes. For this reason we shall employ the term "partial conversion property" to denote an *apparent* property of a polymer increment. "Partial conversion properties" will be denoted by double bars placed over the appropriate symbols. Thus, the partial conversion molecular weight will be indicated by and defined as

$$\overline{\overline{M}} = dW/dN \qquad (1)$$

where W is the weight of polymer and N the corresponding number of moles. The average or cumulative molecular weight will of course be given by $\overline{M} = W/N$.

The partial conversion molecular weight is easily computed by the following method. Since $N = W/\overline{M}$, it follows that

$$\frac{dN}{dW} = \frac{\overline{M} - W(d\overline{M}/dW)}{\overline{M}^2} \qquad (2)$$

From this we see that the partial conversion molecular weight is given by

$$\overline{\overline{M}} = \frac{\overline{M}^2}{\overline{M} - W(d\overline{M}/dW)} \qquad (3)$$

The partial conversion molecular weight is important in the study of polymerization kinetics since it is precisely the reciprocal of the net rate of formation of new molecules with respect to conversion.

Experimental

Three series of bottle-polymerized GR-S were investigated. The first two series were polymerized in 4-oz. bottles in triplicate and the last series was polymerized in a 32-oz. bottle from which samples were removed at various times by means of a hypodermic syringe.[6] For all three sets, the polymerization bottles were tumbled end-over-end at 21 revolutions per minute in a bath whose temperature was controlled at 50 ± 0.05° by means of an appropriate thermostat. The charge for the first series was made up according to the following recipe

Material	Parts by weight
Proctor and Gamble SF Flakes	5.25
Potassium persulfate	0.30
Pure dodecyl mercaptan	0.269
Water	180.0
Styrene	27.0
Butadiene	73.0

(1) This investigation was carried out under the sponsorship of the Office of Rubber Reserve, Reconstruction Finance Corporation, in connection with the Government Synthetic Rubber Program (first reported October 1, 1945).

(2) Present address: Rayon Department, Technical Division, E. I. du Pont de Nemours and Co., Buffalo 7, New York.

(3) F. T. Wall, THIS JOURNAL, **67**, 1929 (1945).

(4) F. T. Wall, R. W. Powers, G. D. Sands and G. S. Stent, *ibid.*, **69**, 904 (1947).

(5) P. J. Flory, *ibid.*, **65**, 372 (1943).

(6) R. L. Frank, C. E. Adams, J. R. Blegen, R. Deanin and P. V. Smith, *Ind. Eng. Chem.*, in press.