large viscosity change brought about by the detergent itself, together with the constancy of viscosity on reduction suggests that these bonds contribute little to the cross-linking within the molecule. The absence of any large change in the optical rotation indicates that the two halves of each cystine residue are so located relative to each other that their disulfide bond does not prevent the assumption of secondary structure in its neighborhood.

Finally, we note the difference in the optical rotatory properties between fully reduced bovine γ-globulin and fully reduced human serum albumin, both in 0.1 *M* decyl sulfate. The albumin molecule, as we have already pointed out, seems under these conditions to acquire additional secondary structure relative to its native form, as revealed by the decrease of levorotation. The reduced γ-globulin molecule, on the other hand, appears to be less well organized in its secondary structure relative to the native protein. The disorganization of the unreduced molecule which combination with the detergent has caused persists in the fully reduced molecule. Since we have found that the elevated levorotation is retained after removal of the detergent, we may conclude that in this protein the stability of the secondary structure is dependent on the integrity of the tertiary organization.

PHILADELPHIA, PA.

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, DUKE UNIVERSITY SCHOOL OF MEDICINE]

Recovery of Native Bovine Serum Albumin after Precipitation with Trichloroacetic Acid and Solution in Organic Solvents[1]

BY GEORGE W. SCHWERT

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Bovine serum albumin, after precipitation with trichloroacetic acid, solution of the precipitate in alcohol or acetone, and removal of the solvent and trichloroacetic acid by dialysis, has been shown to be identical in sedimentation and electrophoretic behavior, in solubility, and crystallizability with untreated crystalline bovine serum albumin.

Recently, Levine[2] and Delaville, *et al.*,[3] have reported that human and bovine serum albumin, after precipitation with trichloroacetic acid (TCA), are soluble in organic solvents. Levine[2] found the TCA precipitate of bovine serum albumin (BSA) to be soluble only in polar solvents, such as methanol, ethanol and acetone, and also found that BSA which had been precipitated with perchloric acid or benzenesulfonic acid did not dissolve in organic solvents. Further, heat-denatured BSA was not soluble in organic solvents containing TCA and no precipitate appeared when alcoholic solutions of TCA-precipitated BSA were heated at 70° for 1 hour. Delaville, *et al.*,[3] in devising a method for the estimation of the albumin–globulin ratio of serum, made use of the fact that the TCA precipitate of serum globulins, unlike that of serum albumin, is insoluble in alcohol.

Although TCA is a powerful denaturing agent for most proteins, and although aqueous solutions of alcohol denature BSA unless the temperature is low,[4] the observations of Levine[2] raise the possibility that BSA is not denatured by treatment with TCA and solution of the precipitate in organic solvents.[5] The following experiments were carried out to test this hypothesis.

Experimental

Preparation of Protein.—Portions of two lots of Armour crystallized BSA were used for these experiments. One of these (Lot 20) had been stored at 5° for several years. The other (Lot P67704) was recently purchased. In a typical preparation 2 g. of BSA was dissolved in 50 ml. of water and precipitated by the addition of an equal volume of 10% TCA. The precipitate was collected by centrifugation at 1400 × *g* for 10 min., was suspended in 20 ml. of water and dissolved in 80 ml. of acetone or ethanol.[6] These steps were carried out at room temperature. The organic solvent solution was dialyzed for about 24 hours at 5° against several changes of distilled water,[7] the solution was pervaporated to a volume of about 50 ml. and again dialyzed for about 24 hours against several changes of distilled water at 5°. At this stage the protein solution is perfectly clear, but, as judged by a striking silkiness when the solution is stirred, contains a trace of suspended crystalline material.[8] The precipitate was removed by filtration and the protein was dried by lyophilization. BSA treated in this way is designated TCA–BSA.

Sedimentation Measurements.—Determinations of the sedimentation rates of BSA (Lot 20) and of TCA–BSA (Lot 20) were made in the Spinco Model E ultracentrifuge by techniques which have been described previously.[9] Measurements were made in *p*H 5.00 acetate buffer, Γ/2 0.2. Rotor temperature was assumed to increase linearly

(1) Supported by grant G1161 from the National Science Foundation.

(2) S. Levine, *Arch. Biochem. Biophys.*, **50**, 515 (1954).

(3) M. Delaville, G. Delaville and J. Delaville, *Ann. pharm. franc.*, **12**, 109 (1954).

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

(5) This possibility is supported by unpublished observations, made by Dr. R. G. Parrish in this Laboratory in 1952, that alcohol solutions of TCA precipitated BSA remain clear and fluid at room temperature for a period of several days and set to a clear gel only after a period of weeks.

(6) If organic solvents are added to the packed precipitate, it is difficult to suspend the precipitate fast enough to prevent the formation of particles of gel which dissolve very slowly. If, however, the precipitate is first suspended in a small volume of water, it dissolves readily when the organic solvent is added.

(7) If alcohol is used as the solvent for TCA–BSA, the protein precipitates during the dialysis and later redissolves. Dr. Joseph C. Rupp has pointed out that this precipitation can be avoided by using acetone as the solvent.

(8) These particles are barely resolved by the light microscope. The author is indebted to Dr. D. Gordon Sharp for electron micrographs which reveal the particles to be highly asymmetric parallelograms in plain view. The crystals are insoluble in water but readily soluble in ethanol. The ultraviolet absorption spectrum of a dilute solution in ethanol is characterized by a sharp maximum at 218 mμ. The nature of this material is being investigated.

(9) G. W. Schwert, *J. Biol. Chem.*, **179**, 655 (1949).

with time and the mean temperature during the period when boundary positions were being recorded was used to correct the sedimentation rate to 20°.[10] One degree was subtracted from the temperature measurements made on the stationary rotor to account for the adiabatic cooling of the rotor during acceleration to 59,780 r.p.m.[11,12] Since, as shown in Fig. 1,

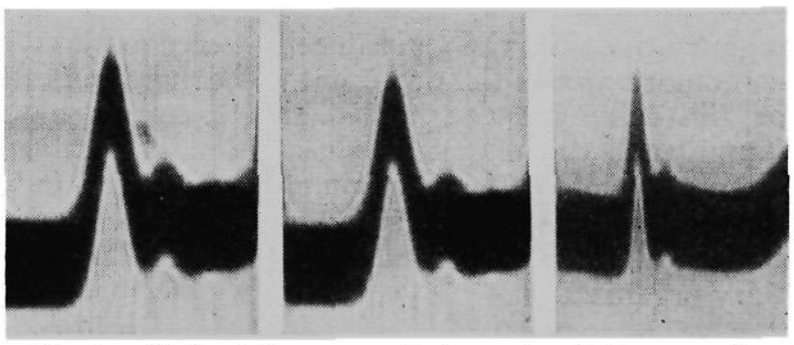

Fig. 1.—Philpot–Svensson photographs of ultracentrifuge boundaries: left, BSA (lot 20) in *p*H 5.00, Γ/2 0.2, acetate, 16.9 mg./ml., bar angle 45°; middle, TCA–BSA (lot 20) in *p*H 5.00, Γ/2 0.2 acetate, 18.0 mg./ml., bar angle 50°; right, approximately 1% solution of TCA precipitate of BSA (lot 20) in absolute alcohol, bar angle 50°. Photographs were made approximately 88 min. after reaching full rotor speed. Meniscus is at left edge of each photograph.

the original BSA preparation and TCA–BSA in acetate buffer, and TCA-precipitated BSA in alcohol solution, exhibit two centrifugal components,[13] 5 photographs of boundary positions were made at 8 minute intervals after sedimentation had been carried on long enough to resolve the two components. The results of these measurements are shown in Fig. 2.

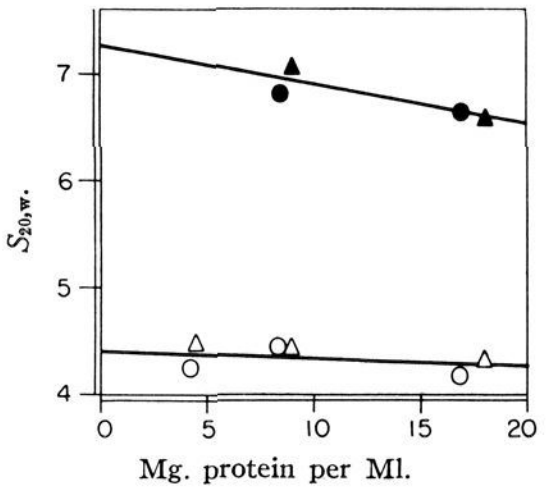

Fig. 2.—Variation of sedimentation constant of BSA and of TCA–BSA with concentration in *p*H 5.00, Γ/2 0.2 acetate buffer. Filled symbols are for the heavy component and open symbols for the light component. O, BSA (lot 20); Δ TCA–BSA (lot 20). At the lowest concentration of protein used for these measurements, the concentration of heavy component was too small to permit of accurate measurement of boundary displacement.

Electrophoresis.—Electrophoretic measurements were made at 1.00° in *p*H 8.60 veronal buffer, Γ/2 0.1, with the Tiselius apparatus supplied by Frank Pearson Associates. Electrophoresis patterns, recorded by the scanning technique of Longsworth,[14] are shown in Fig. 3. The mobility of BSA (lot 20), calculated to the ordinate dividing the descending boundary into equal areas, was found to be

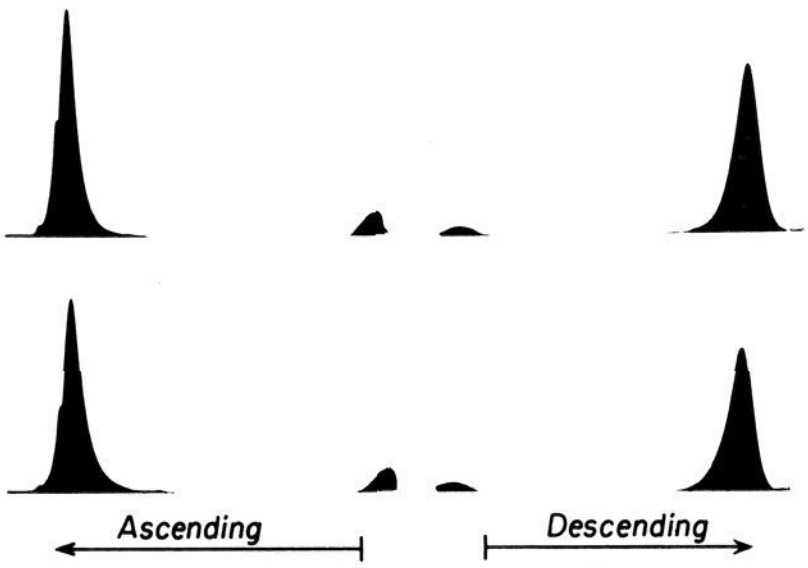

Fig. 3.—Longsworth photographs of electrophoresis boundaries of BSA (lot 20) (upper photographs) and of TCA–BSA (lot 20) (lower photographs). Photographs were made after 9960 sec. at a field strength of 8.67 v./cm.

6.4×10^{-5} cm.²/volt-sec. and that of TCA–BSA was 6.2×10^{-5} cm.²/volt-sec.

Salting-out Curves.—Nine-ml. portions of ammonium sulfate solutions of various concentrations were added to 1-ml. portions of approximately 1% solutions of BSA and of TCA–BSA in *p*H 5.00 acetate buffer, Γ/0.2. The solutions were mixed and allowed to stand for approximately 45 hours at 23° with occasional mixing. At the end of this period, the precipitates were collected by centrifugation at 11,000 × *g* for 10 min. The protein concentration in the supernatant solutions was determined from absorbancy measurements at 280 mμ in the Beckman model DU spectrophotometer. The value of the specific absorbancy was taken as 6.6.[15] The results of these measurements are shown in Fig. 4.

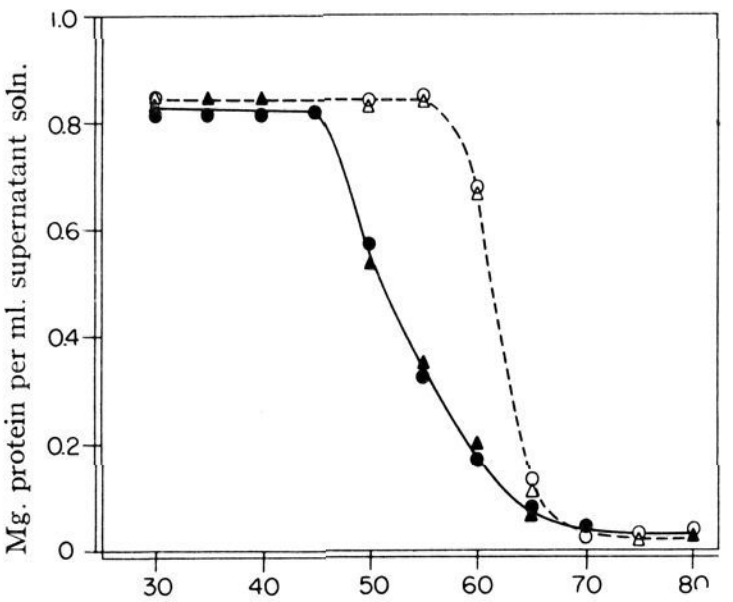

Fig. 4.—Salting-out curves for BSA and for TCA–BSA: O, BSA; Δ, TCA–BSA. Open symbols are for lot P67704 and filled symbols for lot 20.

Crystallization.—BSA (lot P67704) was recrystallized from aqueous ethanol according to the directions of Cohn, Hughes and Weare[15] for the second crystallization of BSA. TCA–BSA was prepared by the method described with the exceptions that: (a) between the two dialysis steps the *p*H of the solution was adjusted to 5.1 with 0.2 *M* $NaHCO_3$, and (b) the final solution was not lyophilized but was concentrated by pervaporation to a protein concentration of 20%. These modifications were made to bring the solution to the concentration, *p*H and ionic strength required for crystallization.[15] A heavy crop of very long needles, similar

(10) G. W. Schwert, *Arch. Biochem. Biophys.*, **48**, 310 (1954).

(11) D. F. Waugh and D. A. Yphantis, *Rev. Sci. Inst.*, **23**, 609 (1952).

(12) A. Biancheria and G. Kegeles, THIS JOURNAL, **76**, 3737 (1954).

(13) W. L. Hughes in H. Neurath and K. Bailey, "The Proteins," Academic Press, Vol. II, New York, N. Y., 1954., pp. 663–754, states that the amount of fast-sedimenting impurity, which is an albumin dimer, varies with the age and history of the preparation.

(14) L. G. Longsworth, THIS JOURNAL, **61**, 529 (1939).

(15) E. J. Cohn, W. L. Hughes, Jr., and J. H. Weare, *ibid.*, **69**, 1753 (1947).

to those shown by Cohn, Hughes and Weare in Fig. 4 of their publication,[15] was obtained.

Thermal Stability.—When an approximately 1% solution of recrystallized BSA in *p*H 5.00 acetate buffer, Γ/2 0.2, is heated at the rate of 1° per min., incipient turbidity appears at 64°. A similar solution of crystallized TCA–BSA shows incipient turbidity at 65°. This slight elevation of the turbidity point probably results from the stabilizing influence of traces of TCA in the crystallized TCA–BSA since, if TCA is added to 1% solutions of recrystallized BSA in *p*H 5.00 acetate buffer to give concentrations of 6×10^{-5} *M*, 6×10^{-4} *M* and 6×10^{-3} *M* TCA, the turbidity points are elevated to 64.5, 65 and 67°, respectively.

Reaction with *p*-Chloromercuribenzoate.—The extent of mercaptide formation when BSA (lot P67704) or crystallized TCA–BSA was treated with *p*-chloromercuribenzoate (PCMB) was determined by measuring the increase in absorbancy at 255 mμ.[16] The final concentration of PCMB was 4.96×10^{-5} *M* and the final concentration of BSA or of TCA–BSA was 2.35×10^{-5} *M*. Using Boyer's value for the increase in molar absorbancy accompanying mercaptide formation at *p*H 4.6, 6.2×10^3, duplicate determinations with each protein preparation gave good agreement in indicating 0.69 mole of mercaptide formed per mole (69,000 g.) of TCA–BSA and 0.85 mole of mercaptide formed per mole of BSA.

Other Observations.—Levine[2] found that casein and conalbumin, after precipitation with TCA, do not dissolve in organic solvents and that *n*-propyl, isopropyl and *n*-butyl alcohols do not dissolve TCA-precipitated BSA. It has been found in this Laboratory that crystalline chymotrypsinogen, chymotrypsin, trypsin and ribonuclease do not exhibit this solubility effect in ethanol but that crystalline zinc insulin does exhibit this property. Also, the TCA precipitate of BSA is found to dissolve in benzyl alcohol.

Dr. Walter L. Hughes[17] has found that BSA is insoluble in glacial acetic acid but does dissolve if a small amount of water is added to the solvent. The question whether this behavior arises from a requirement for mixed solvents *per se*, for solubility or for a solvent of appropriate dielectric constant appears to be resolved in favor of the latter hypothesis by the observation that the solubility of recrystallized BSA is greater than 2 g. per 100 ml. of 10% TCA in absolute methanol, is approximately 0.2 g. per 100 ml. of 10% TCA in absolute ethanol, and is very small in 10% TCA in acetone. It thus appears that the water contained in centrifugally packed TCA precipitates of BSA contributes markedly to the solubility of these precipitates in ethanol or acetone.

Discussion

The sedimentation diagrams shown in Fig. 1 indicate that the state of dispersion of BSA is not changed by treatment with TCA and organic solvents. Although the salting-out curves shown in Fig. 4 indicate that BSA from lot 20 is not wholly native BSA, this old preparation exhibits fine detail in sedimentation and electrophoresis experiments which would not be found with a newer preparation. The reproduction of this detail after the TCA-solvent treatment seems to be a convincing demonstration that none of the various molecular species present is altered by this treatment. The values found for sedimentation constant and electrophoretic mobility appear to be in satisfactory agreement with those given by Kegeles and Gutter[18] and by Armstrong, Budka and Morrison,[19] respectively. The close agreement of the salting-out curves for the two lots of BSA before and after TCA-solvent treatment, and the fact that BSA can be crystallized by an established procedure after this treatment seem to establish the unchanged character of the protein. The fact that the thermal stability of TCA–BSA is within the range shown by native BSA when stabilized by traces of TCA, is in agreement with this conclusion, as is the observation that the content of reactive sulfhydryl groups is essentially unchanged after TCA-organic solvent treatment. The slight reduction in the quantity of reactive sulfhydryl groups which accompanies the formation of TCA–BSA from BSA is consistent with Boyer's observation[16] that the amount of reactive sulfhydryl groups is reduced by one precipitation of BSA by ammonium sulfate. Hughes[13] reports $^2/_3$ of the molecules of serum albumin to contain a thiol group.

Levine[2] has reasoned that since BSA in which the amino groups were covered by benzoylation can still be precipitated by TCA and since this precipitate is soluble in organic media, salt formation between the charged amino groups on the protein and trichloroacetate ion does not make a significant contribution to the solubility effect. In view of the fact that benzoylated BSA can also be precipitated by acidification with HCl, that this precipitate is likewise soluble in alcohol,[20] and that serum albumin is known to bind trichloroacetate ion very strongly,[21] it appears that the explanation of this phenomenon lies in the repression of ionization of carboxyl groups on the protein by the low *p*H of the TCA solution and the masking of cationic charges by ion pair formation with trichloroacetate. The observations[20] that tyrosine and histidine are not soluble in 10% TCA in 95% ethanol while arginine and lysine are soluble in this solvent, lend support to this view.

The solubility of BSA in organic solvents containing TCA appears to increase with the dielectric constant of the organic solvent, and, for solvents of low dielectric constant, to be increased by the addition of water. Since, however, it is a familiar observation that TCA-precipitated BSA can be dissolved in water only after excess TCA has been removed by washing the precipitate, it appears that the dependence of solubility on dielectric constant is reversed at high dielectric constants. This prolamine-like solubility effect appears to merit further investigation.

A point which remains to be explained is the fact that, aside from insulin, which is resistant to denaturation, only serum albumin, which is very sensitive to many forms of denaturation, shows this behavior. It seems possible that TCA in high concentrations exhibits the property of per-fluorooctanoic acid of stabilizing the specific internal hydrogen bonded structure of the protein.[22,23]

The possibility that this technique may be of use in the preparation of human serum albumin is being investigated by Dr. Joseph C. Rupp of the Department of Biochemistry of the University of Virginia.

DURHAM, NORTH CAROLINA

(16) P. D. Boyer, THIS JOURNAL, **76**, 4331 (1954).

(17) Personal communication.

(18) G. Kegeles and F. J. Gutter, THIS JOURNAL, **73**, 3770 (1951).

(19) S. H. Armstrong, Jr., M. J. E. Budka and K. C. Morrison, *ibid.*, **69**, 416 (1947).

(20) G. W. Schwert, unpublished observation.

(21) G. Scatchard and E. S. Black, *J. Phys. Colloid Chem.*, **53**, 88 (1949).

(22) H. B. Klevens, Abstracts of 121st National Meeting of the American Association for the Advancement of Science, Berkeley, December 26–31, 1954.

(23) E. Ellenbogen and P. H. Maurer, Abstracts of 128th Meeting of the American Chemical Society, Minneapolis, September 11–16, 1955.