Preparation of Bovine Mercaptalbumin and an Investigation of Its Homogeneity*

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ABSTRACT: The microheterogeneity of plasma albumin, originally proposed as a means of explaining the electrophoretic separation of rapidly interconverting conformers, has been supported by a variety of evidence. Plasma albumin is known to consist primarily of mercaptalbumin, with significant amounts of two nonmercaptalbumin components. It was therefore of interest to prepare the mercaptalbumin component in the purest form possible and ascertain whether or not it is heterogeneous. A new method for the preparation of defatted mercaptalbumin by ion-exchange chromatography is described.

Evidence is presented that this mercaptalbumin preparation, made from either crystallized or noncrystallized bovine plasma albumin, is virtually free of lipid-bound albumin, dimer, or oligomers of albumin, and of any forms with abnormal disulfide pairing such as are believed to arise on aging albumin in solution. The mercaptalbumin is more homogeneous than the parent plasma albumin as judged by the solubility–pH profile method and, in fact, exhibited the solubility behavior in concentrated ammonium sulfate solutions expected for a homogeneous protein on the basis of the Gibbs phase rule. Also, in contrast to the parent albumin, moving boundary electrophoresis in the pH range of the N–F isomerization did not yield separate N and F boundaries. However, separate boundaries were obtained corresponding to two detergent complexes which exist in a cooperative equilibrium having a very high order dependence on detergent ion concentration. This result suggests that mercaptalbumin retains a microheterogeneity with respect to detergent binding affinity. Reversible boundary spreading confirmed that while the mercaptalbumin is more homogeneous than the parent plasma albumin, there remains an electrical heterogeneity. Analysis of the data indicated that the distribution of time-averaged net charge in the population at the mean isoelectric pH is gaussian, with a standard deviation of approximately 0.6 electronic charge unit.

Sogami and Foster (1963) concluded that plasma albumin is microheterogeneous, *i.e.*, it consists of a large number of closely related species. They based their conclusion principally on two observations: N and F forms, although rapidly interconverting, were electrophoretically resolvable, and the apparent order in H^+ ions of the N–F transition was lower than expected on the basis of titration data. Other evidence in support of albumin heterogeneity has been the fractionation of albumin by partial precipitation in concentrated KCl solution (Petersen and Foster, 1965a; Habeeb, 1968; McMenamy and Lee, 1967) and by partial denaturation (Štokrová and Šponar, 1963) into fractions differing in titration behavior, disulfide reducibility, and stability toward denaturation.

The most widely used criterion of albumin heterogeneity has been its solubility–pH profile in 3.0 M KCl, a method developed by Petersen and Foster (1965a,b), and later modified by Sogami and Foster (1968). It has been shown that more heterogeneous albumin preparations have broader solubility–pH profiles, *i.e.*, larger values of ΔpH_{10}^{90} as defined by Sogami *et al.* (1969).

Recent investigations of microheterogeneity have placed considerable emphasis on purity of the albumin preparation, specifically on removal of nonmercaptalbumins and bound lipid impurities. Sogami and Foster (1967) and Foster (1967) pointed out the large decrease in ΔpH_{10}^{90} observed upon defatting albumin. McMenamy and Lee (1967) confirmed this result, and also reported that a preparation defatted by the procedure of Goodman (1957) could not be fractionated in 3.0 M KCl. They concluded that their preparation was most likely not microheterogeneous, but Wong and Foster (1969b) showed this preparation also to be heterogeneous by solubility in ammonium sulfate.

Andersson (1969) prepared defatted mercaptalbumin and investigated its microheterogeneity, also by the solubility–pH method. He reported two results that indicated his preparation was highly purified: a low value of ΔpH_{10}^{90} and identical solubility–pH profiles of fractions prepared by partial precipitation. He concluded that "Defatted mercaptalbumin appears to be completely homogeneous. . ."

It was the purpose of the research presented in this paper to undertake the preparation of defatted mercaptalbumin, and to investigate critically the question of its microheterogeneity, using techniques not employed by Andersson (1969).

Experimental Section

Materials. Bovine plasma albumin preparations were purchased from Armour Pharmaceutical Co. Crystallized preparations contained 0.67 ± 0.02 mole of SH/mole of albumin (lots D71002, D71309). Uncrystallized preparations (fraction V) contained 0.59 ± 0.02 mole of SH/mole of albumin (lots D27309, F32101, F33106). In all cases, the monomer content was 90–95% as estimated from disc gel electrophoresis or Sephadex G-150 chromatography.

Dialysis membranes and bags were made from cellulose dialysis tubing (Union Carbide Corp). The tubing was cut into convenient lengths and then repeatedly rinsed in boiling water for 10- to 20-min durations until A_{220} (absorbance at 220 nm)

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of the rinse water was less than 0.02. Care was taken not to contaminate the dialysis bags with the hands. The clean bags were stored under water at 2° until used.

Sephadex G-150 and Sulfoethyl Sephadex were purchased from Pharmacia Fine Chemicals, Inc. The anion exchanger AG1-X8 (Bio-Rad Laboratories) was prepared for use by rinsing with hot water, then cycling between 1.0 M HCl and 0.8 M NaOH.

All inorganic chemicals employed were reagent grade. Organic chemicals were the best grade commercially available, except for decylbenzenesulfonate, which was synthesized by oleum sulfonation of decylbenzene according to the method of Gray *et al.* (1955).

Detergent Binding. Equilibrium dialysis was conducted in plastic (Plexiglas) dialysis cells, agitated by rotation at 5 rpm. They were agitated with repeated changes of buffer solution before use in order to leach out impurities from the Plexiglas and the cellophane membranes. Each membrane was tested for leakage before use by dialyzing a solution of charcoal-defatted albumin against solvent. Experiments showed that equilibrium was achieved after 22-hr dialysis. The absorbance of the dialysate at 223 nm was measured, and the equilibrium decylbenzenesulfonate concentration was calculated from a measured extinction coefficient of 1.31×10^4 M^{-1}. The average moles of detergent bound per mole of albumin (ν) was calculated assuming a molecular weight of 66,000 for albumin.

Moving-boundary electrophoresis analysis of the albumin–detergent solutions was performed with the Beckman Model H electrophoresis apparatus, according to the procedure of Decker and Foster (1966).

Comparative Solubilities. Whenever the solubilities of two or more albumin samples were compared at the same pH and salt concentration, they were dialyzed against the same outside dialysate, after the example of Wong and Foster (1969a). The albumin samples (in approximately 25% ammonium sulfate) were dispensed into dialysis bags, about 2 ml per bag. It was found important for good agitation with minimum surface denaturation that the bags be tied with a bubble of air inside so that the bags were filled and rounded.

The bags were placed in a vial with 20–100 ml of ammonium sulfate solution and agitated for 20–30 hr. Agitation was accomplished by shaking at 30 cpm or by rotating at 1.5 rpm. It was found that rotation was necessary when more than two dialysis bags were equilibrated against the same outside solution.

After equilibration, and before the precipitate had settled, an aliquot of the slurry was withdrawn into a syringe fitted with a relatively large diameter plastic needle (1-mm inside diameter). The aliquots were diluted to volume with water and total albumin concentration determined. The precipitate was then removed by centrifugation for a few minutes at approximately 15,000 rpm. An aliquot of the supernatant was diluted to volume to determine its albumin concentration. Temperature was 25 ± 1° during agitation, transferring of solutions, and centrifugation.

Approach to equilibrium of precipitation by dialysis was studied by experiments similar to the test for reversibility of precipitation by Wong and Foster (1969a); *i.e.*, equilibrium was approached from two directions: all protein precipitated and all dissolved. Salt concentrations and pH were chosen so that at equilibrium about half the albumin was in solution. Within experimental error, it was observed that equilibration of salt concentration across the dialysis membrane occurs in 3 hr, and equilibration of pH in 5 hr. Supernatant albumin concentration reached its equilibrium value in 15 hr. For all results reported, dialysis lasted 20–30 hr. Error in determination of solubility is estimated to be about ±5%.

Reversible Boundary Spreading. The experimental technique used was that of Alberty *et al.* (1948), except that the long diffusion time recommended by Bhargava and Foster (1970) was used. The data treatment was similar to that of Bhargava and Foster, one notable exception being that σ (the standard deviation of the gradient of refractive index) was calculated by a least-squares treatment of the data. Average values of D^* (apparent diffusion coefficient) for right and left boundaries were used to calculate h (standard deviation of the mobility distribution). All reported experiments were performed at pH near the isoelectric pH.

Ion-Exchange Chromatography. For analytical ion-exchange chromatography a salt gradient was used. The ionic strength was varied by linearly increasing the NaCl concentration, keeping pH and sodium acetate concentration constant. The column (empty) was 40 × 2.5 cm. Flow rate, maintained by a peristaltic pump, was about 35 ml/hr while pouring the column and about 30 ml/hr while eluting. The sample was prepared for application by first dialyzing it against buffer for at least 3 hr.

A somewhat different technique was used for the routine preparation of bovine mercaptalbumin. A water-jacketed column (2.5 × 90 cm) was fitted at the bottom with a Sephadex flow adapter. Sulfoethyl Sephadex C-50 was equilibrated with "pouring buffer" (0.02 M Na acetate–0.18 M NaCl, pH 4.30 ± 0.02) and the column poured at a flow rate of about 35 ml/hr to a bed height of 75 ± 5 cm at 3 ± 3°. Meanwhile 5 g of Armour fraction V was charcoal defatted according to the Sogami and Foster (1968) modification of the method of Chen (1967). The pH of the charcoal-defatted albumin was adjusted to 4.3 and then the albumin was dialyzed against approximately 500 ml of the pouring buffer at 3 ± 3° for at least 3 hr. Then the sample was layered onto the resin and eluted at about 30 ml/hr with about one column volume (400 ml) of column pouring buffer at 3 ± 3°.

At this point the more positive protein impurities had been eluted through the column, and the albumin was still in the top few centimeters of resin. The albumin was then eluted by switching to the "eluting buffer" (0.02 M Na acetate–0.27 M NaCl, pH 4.30 ± 0.02), and then eluting at 20–30 ml/hr. The temperature fluctuations during elution with the eluting buffer were controlled to ±0.02°, the temperature being about 4°. The tubes were analyzed for albumin and the position of the peak maximum was carefully determined. Approximately 1 g of mercaptalbumin, with 0.95 ± 0.02 mole of SH/mole of albumin, was obtained by pooling the tubes from 130 ml before the peak maximum to 60 ml beyond it.

The preparation of the sample by dialysis, the initial elution with pouring buffer, and the close temperature control during elution were found necessary to eliminate artificial peaks in the elution pattern otherwise obtained.

Miscellaneous Methods. Analysis for reactive SH was according to the method of Ellman (1959) except that Tris-HCl buffer was used to adjust the pH to 8.05 ± 0.10, and at least a tenfold molar ratio of 5-5′-dithiobis-(2-nitrobenzoic acid) to albumin was used. A dependence of SH analysis on albumin concentration was noted by Janatová *et al.* (1968b), and as a consequence the analyses were routinely done at 0.2% bovine plasma albumin. Solutions of 5,5′-dithiobis-(2-nitrobenzoic acid) were made up fresh daily. Accuracy is estimated to be ±0.03 mole of SH/mole of albumin.

Disc gel electrophoresis (Davis, 1964) was done in standard 7% gels, approximately 5 cm in length. The stacking gel was

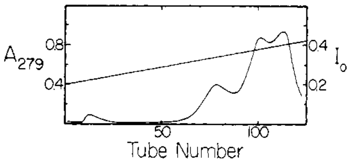

FIGURE 1: Sulfoethyl Sephadex chromatography of fraction V. Conditions were 0.02 M Na acetate, 33 × 2.5 cm resin bed, pH 4.30, 7.0 ml/tube, 27-ml/hr flow rate, 25°, 0.44-g sample. For a given tube number the value of A_{279} is a measure of the albumin in that tube, and I_0 is the calculated ionic strength of the eluent that was entering the top of the column as the reference tube was being collected. The buffer was made up to ionic strength by adding NaCl.

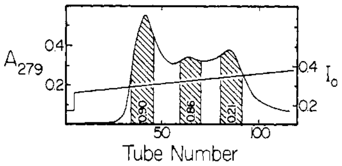

FIGURE 2: Sulfoethyl Sephadex chromatography of crystallized plasma albumin. Conditions were 0.02 M Na acetate, 37 × 2.5 cm resin bed, pH 4.30, 10 ml/tube, 33-ml/hr flow rate, 25°, 0.49-g sample. The numbers under the peaks indicate the moles of SH/mole of albumin for the pooled tubes. Note that the first major component is larger here than in Figure 1.

replaced by Sephadex G-150 that had been equilibrated against the Tris-glycine buffer of pH 8.5.

Values of pH were determined with a Beckman Research pH meter, always at room temperature. It is estimated that reported values are accurate to ±0.03 pH unit. Sargent buffers were used for standardization. Routine spectrophotometric measurements were made with a Hitachi Perkin-Elmer spectrophotometer, using 0.1-mm slit width. Their accuracy is estimated to be ±2%. Optical rotation measurements were made with a Cary Model 60 spectropolarimeter.

Charcoal defatting was conducted according to the procedure of Chen (1967) as modified by Sogami and Foster (1968). Solubility–pH profiles in 3.0 M KCl were carried out by the method described by Sogami and Foster (1968).

Albumin concentrations were determined spectrophotometrically, using $\epsilon_{279}^{1\%} = 6.67$. Concentrated solutions were either diluted before measuring A_{279}, or the absorbance was kept in the accurate range of the spectrophotometer by varying cell path length from 1 to 10 mm.

Results

Ion-Exchange Chromatography. The monomer of Armour fraction V was prepared by Sephadex G-150 exclusion chromatography. It was free of dimer and other proteins as judged by disc gel electrophoresis. However, analysis by salt gradient elution on Sulfoethyl Sephadex gave evidence that this protein was quite heterogeneous. At least three major components were present, as indicated by Figure 1. The eluting buffer was 0.02 M Na acetate, made up to desired ionic strength with NaCl. It should be emphasized that experiments with higher concentrations of acetate yielded much poorer resolution of the peaks. As a consequence of this observation, all Sulfoethyl ion-exchange chromatography was performed at 0.02 M Na acetate concentration.

Similar analysis of unpurified, crystallized bovine plasma albumin was performed, and the results are shown in Figure 2. The results of SH analysis, also reported in Figure 2, show that the first two major components are mostly mercaptalbumin, and the third mostly nonmercaptalbumin. The existence of two mercaptalbumin components was unexpected. Separation on DEAE-Sephadex A-50 of mercaptalbumin from nonmercaptalbumin by Janatová *et al.* (1968a,b) showed no evidence of two major mercaptalbumin components.

The elution pattern of charcoal-defatted crystallized bovine plasma albumin is shown in Figure 3. Of the 3 major components observed in Figures 1 and 2, the first eluted has been almost completely eliminated by charcoal defatting. Also, SH analysis indicates that the leading peak in Figure 3 is virtually pure mercaptalbumin. Our interpretation of these results is that the bound lipid impurities caused the mercaptalbumin and the nonmercaptalbumin each to separate into two components in Figures 1 and 2: a component that elutes early with bound fatty acid, and one that elutes later without fatty acid. Charcoal defatting caused each to elute as only one component, hence the simpler elution pattern of Figure 3. In harmony with this interpretation is the finding of Chen (1967) that crystallized plasma albumin has approximately 2.4 times as much bound fatty acid as fraction V (both Armour preparations), because an examination of Figures 1 and 2 indicates that the first major peak is larger for crystallized albumin than for fraction V.

The analytical Sulfoethyl Sephadex chromatography experiments described provided a basis for the development of a routine procedure, described under Experimental Section for the preparation of bovine mercaptalbumin from fraction V or from crystallized albumin. The analysis of column fractions during a routine preparation of bovine mercaptalbumin is shown in Figure 4. In addition to the results shown there disc gel electrophoresis indicated that only monomer was present in tubes 91–160. A trailing band on disc gel electrophoresis was observed in tubes 20 and 60, and in trace amounts in tube 75.

Solubility–pH Profiles in 3.0 M KCl. The results of solubility pH profiles, presented in Figure 5, indicate that mercaptalbumin prepared from fraction V is identical with that prepared from crystallized plasma albumin. The data points scatter within experimental error around the curved line. The value of ΔpH_{10}^{90} for mercaptalbumin, 0.11 ± 0.02 pH unit, is equal to the lowest reported value, that of Andersson (1969), who found that at higher albumin concentration (0.4 g/100 ml), ΔpH_{10}^{90} was 0.11 for defatted mercaptalbumin.

Data of Sogami and Foster (1968) gave a ΔpH_{10}^{90} value of 0.17 for plasma albumin. Andersson (1969) reported values of 0.18 and 0.22 for plasma albumin. Therefore ΔpH_{10}^{90} is reduced by 35–50% by removal of nonmercaptalbumin, which is

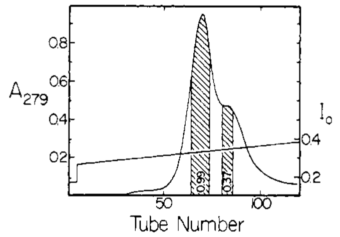

FIGURE 3: Sulfoethyl Sephadex chromatography of charcoal-defatted, crystallized plasma albumin. The conditions are as for Figure 2, except that the resin bed was 4 cm shorter. Note that there are apparently only two peaks, as opposed to three in Figures 1 and 2.

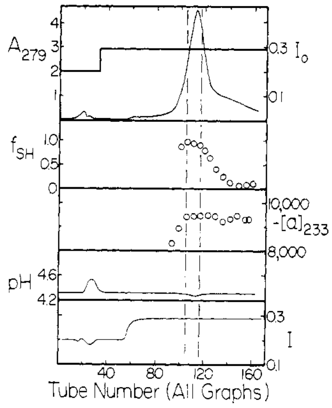

FIGURE 4: Analysis of effluent fractions in the chromatographic preparation of bovine mercaptalbumin. The eluting buffer was 0.02 M Na acetate plus NaCl to give an ionic strength of 0.29 (I_0, top panel). The ionic strength of the eluted fractions is given as I (bottom panel). Also shown are: A_{279}, pH, f_{SH} (moles of SH/mole of albumin), and $[\alpha]_{233}$ (specific rotation measured at 233 mμ and pH 5.4). The volume/tube was 15.4 ml.

added testimony to the sensitivity of solubility–pH profiles to albumin heterogeneity.

Fractionation by partial precipitation in 3.0 M KCl was not employed as a method of investigating heterogeneity of mercaptalbumin, because of possible complications arising from recent observations that a partial loss of solubility accompanies precipitation of albumin in KCl solutions[1] (McMenamy and Lee, 1967).

Solubility in Ammonium Sulfate Solutions. Attempts were made to fractionate bovine mercaptalbumin in ammonium sulfate solutions. The advantage over low pH precipitation in KCl is the absence of hysteresis effects, as reported by Wong and Foster (1969a).

A sample of mercaptalbumin was dialyzed against concentrated ammonium sulfate solution in an attempt to fractionate by partial precipitation. Equilibrium of precipitation was assumed after 20 hr of dialysis. Analysis of dialysate indicated that pH was 5.32; ammonium sulfate concentration 28.6%. The supernatant concentration was 0.63 g/100 ml, and total protein concentration 1.52 g/100 ml. The solubilities of the supernate and precipitated fractions were compared at 0.6 g/100 ml of total albumin concentration. The results, presented in Table I, show that the solubilities of the fractions are identical and therefore no true fractionation has been achieved. The results in Table I are in agreement with the report by Wong and Foster that no hysteresis effect accompanies precipitation of albumin in ammonium sulfate at pH near 5. However, the same results contrast with a report by the same authors that plasma albumin was fractionated by partial precipitation in ammonium sulfate solutions into fractions of significantly differing solubilities.

The Gibbs phase rule predicts that a homogeneous protein should show no dependence of solubility on the total amount of protein added per unit volume. Taylor (1953) called this constant solubility test "the most exacting test of homogeneity yet applied to proteins."

The experimental procedure for the constant solubility test was the comparative solubility method, modeled after the work of Wong and Foster (1969a). The method was adapted for this purpose by the simple expedient of using samples of differing total protein concentrations. The advantage of the method is the precise matching of pH and salt concentration achieved. The importance of control of salt concentration is apparent from the results of Wong and Foster, and by an examination of the results in Table I. The importance of control of pH is evident from the pH dependence of albumin solubility, shown in Figure 6.

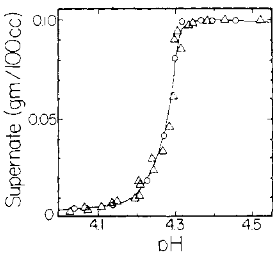

FIGURE 5: Solubility–pH profile of bovine mercaptalbumin in 3.0 M KCl: (△) prepared from crystallized plasma albumin; (○) prepared from fraction V. Total albumin concentration was 0.10 g/100 ml.

TABLE I: Comparative Solubilities of Mercaptalbumin Fractions.

Dialysate Ammonium Sulfate Concentration (%)	Dialysate pH	Solubility of Precipitate Fraction (S_p) (%)	Solubility of Supernatant Fraction (S_s) (%)	S_p/S_s
28.5	5.22	0.416	0.438	0.95
28.8	5.25	0.339	0.335	1.01
29.1	5.24	0.227	0.225	1.01
29.7	5.25	0.122	0.114	1.07
				1.01 av

Experiments demonstrated that equilibration of salt concentrations was achieved within 3 hr and of pH within 5 hr. For constant solubility tests, dialysis was continued for about 25 hr.

The results of one constant solubility test are shown in Figure 7. The observed solubility behavior is that predicted by Gibbs phase rule for a homogeneous protein. In contrast, concentration dependence of solubility[2] has been reported for plasma albumin preparations (Foster *et al.*, 1965; Wong and Foster, 1969a). In an attempt to understand the differing solubility behavior of mercaptalbumin and plasma albumin, the solubilities of mercaptalbumin and nonmercaptalbumin were

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[1] Similar observations have been made in our laboratory by Dr. L. J. Kaplan.

[2] It is clear, in retrospect, that Wong and Foster (1969a) did not recognize the critical pH dependence of the solubility of plasma albumin in concentrated ammonium sulfate, and the pH control exercised in many of their experiments was not adequate to justify comparisons of the solubility behavior of various preparations and subfractions. Those experiments conducted by the comparison method which they proposed, *i.e.*, by dialysis of various preparations or fractions against a common solution, did not suffer from this objection. Indeed, this comparison method has been extremely useful in the present work and in particular is the basis of the constant solubility test we have employed.

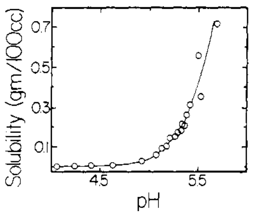

FIGURE 6: Dependence on pH of the solubility of bovine mercaptalbumin in 29.6% ammonium sulfate.

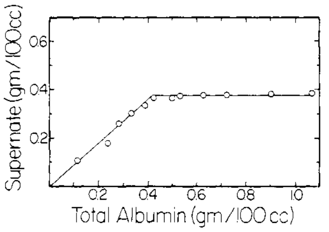

FIGURE 7: Constant solubility test for bovine mercaptalbumin in 29.1% ammonium sulfate solution at pH 5.28. A two-phase system, supernatant and precipitate, was observed for the data points on the line running parallel to the abscissa, indicating that solubility is concentration independent, a property predictable for a homogenous preparation by the Gibbs phase rule.

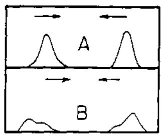

FIGURE 8: Schlieren electrophoresis patterns in the pH range of the N–F transition for (A) mercaptalbumin, and (B) unpurified, crystallized albumin: (A) pH 3.95, 2.04 × 10^4 sec in 0.99-V/cm gradient; (B) pH 3.98, 2.28 × 10^4 sec in 1.15-V/cm gradient. Both were at 22°, 0.02 M Cl^-. The boundaries moved in the direction of the arrows.

TABLE II: Comparative Solubilities of Mercaptalbumin and Nonmercaptalbumin.

Dialysate Ammonium Sulfate Concentration (%)	Dialysate pH	Solubility of Mercaptalbumin (S_{MA}) (%)	Solubility of Nonmercaptalbumin (S_{NMA}) (%)	S_{NMA}/S_{MA}
28.6	5.24	0.372	0.465	1.25
28.7	5.23	0.336	0.417	1.24
29.2	5.24	0.225	0.273	1.21
29.5	5.24	0.162	0.186	1.16
29.9	5.30	0.147	0.168	1.14
				1.20 av

compared. Both samples were collected from a routine Sulfoethyl Sephadex separation of mercaptalbumin and nonmercaptalbumin. The mercaptalbumin contained 0.95 mole of SH/mole of albumin, and the nonmercaptalbumin 0.10 mole/mole. The solubilities were compared at the same total albumin concentration (0.5%). The data in Table II indicate that nonmercaptalbumin is about 20% more soluble than mercaptalbumin under the conditions of the experiment.

N–F Isomerization. The pH range of the N–F isomerization was located, for mercaptalbumin in 0.02 M Cl^-, by measuring the pH dependence of $[\alpha]_{313}^{22}$ with a Cary Model 60 recording spectrophotometer. The abrupt transition in $[\alpha]_{313}^{22}$ centered about pH 3.95 was assumed, after Sogami and Foster (1968), to indicate the occurrence of the N–F isomerization.

Unsuccessful attempts were made to separate N and F isomers of mercaptalbumin by moving-boundary electrophoresis. One such attempt is represented in Figure 8, together with a control experiment with unpurified, crystallized plasma albumin. The control sample shows separation of N and F isomers, while no separation is observed with mercaptalbumin.

It is interesting to note that the more homogeneous preparation (mercaptalbumin) shows no separation, whereas the more heterogeneous preparation (unpurified plasma albumin) does. It had been previously argued by Foster *et al.* (1965) that separation of the N–F isomers was evidence for heterogeneity. The results just described support that line of reasoning.

Separation of Detergent Complexes. The separation of detergent complexes of mercaptalbumin–decylbenzenesulfonate was observed by moving-boundary electrophoresis at 22°, pH 6.44, ionic strength 0.15, in a solvent 0.05 M in LiCl, 0.021 M in Li_2HPO_4, and 0.037 M in LiH_2PO_4. The schlieren patterns, shown in Figure 9, are reasonably enantiographic. The separation of the two peaks seen in Figure 9 was observed to be reversible, *i.e.*, when the potential gradient was reversed the two peaks recombined. Binding data were measured by equilibrium dialysis, accompanying the electrophoretic experiments. Two complexes were observed in the range $24 < \bar{\nu} < 40$. Their stoichiometry was determined by the graphical technique of Decker and Foster (1966), as seen in Figure 10. The results indicate that both mercaptalbumin and plasma albumin preparations have the same stoichiometry

$$\mathrm{A(DecBS)_{16}} + 31\ \mathrm{DecBS} = \mathrm{A(DecBS)_{47}} \qquad (1)$$

where A represents albumin and DecBS represents decylbenzenesulfonate. The albumin with less bound detergent is the slow-moving complex, and the other is the fast complex.

At the same pH and temperature, but in 0.10 M LiCl solvent, Decker and Foster (1966) found the stoichiometry

$$\mathrm{A(DBS)_{11}} + 27\ \mathrm{DBS} = \mathrm{A(DBS)_{38}} \qquad (2)$$

where DBS represents dodecylbenzenesulfonate.

The association of albumin with decylbenzenesulfonate, at $\bar{\nu} = 23$, was concluded to be complete within 45 sec, as a consequence of the observed time independence of the complex ultraviolet perturbation difference spectrum of albumin and decylbenzenesulfonate. The minimum time in which the protein and detergent could be mixed, the sample cell filled, and the scan started was 45 sec. The solvent was the same as that for binding studies and electrophoresis separations.

The dissociation of decylbenzenesulfonate from plasma albumin was concluded to be almost complete within 30 sec, as determined by rapidly passing mixtures of albumin and detergent through a small column containing Bio-Rad AG 1-X8 anion exchanger. The albumin entered the column with 37–57 moles of detergent bound per mole of albumin and was eluted 30 sec later with only 2 ± 1 moles of detergent

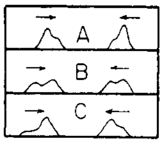

FIGURE 9: Schlieren electrophoresis patterns showing separation of albumin–detergent complexes: (A) $\bar{\nu}$ = 24.4, 0.95 × 10^4 sec of electrophoresis; (B) $\bar{\nu}$ = 31.8, 1.44 × 10^4 sec; (C) $\bar{\nu}$ = 39.4, 1.44 × 10^4 sec. E was 0.97 V/cm in all cases.

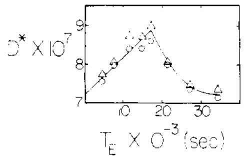

FIGURE 11: Reversible boundary spreading of bovine mercaptalbumin: (△) left boundary; (○) right boundary. Conditions were 22°, 0.01 M Na acetate, 0.97 V/cm. The line is calculated for h = 0.45 × 10^{-5} cm²/V sec.

remaining per mole of albumin. Measurement of detergent remaining was made from a calibration curve of moles of detergent per mole of albumin *vs.* A_{256}/A_{279}. These wavelengths were chosen because A_{256} is sensitive to detergent concentration and A_{279} to albumin concentration.

These results are consistent with the recent conclusion of Johnson and Muller (1970), based on nuclear magnetic resonance measurements, that the residence time of a fluorinated detergent on albumin is no longer than 10^{-2} sec.

Reversible Boundary Spreading. Reversible boundary spreading experiments were performed on bovine mercaptalbumin, and Figure 11 shows the results of a typical experiment. The line drawn thru the points is the expected dependence of D^* on t_E for h = 0.45 × 10^{-5} cm/V sec, calculated for a gaussian distribution of mobilities according to Alberty *et al.* (1948). Here D^* is the apparent diffusion coefficient, t_E is time of electrophoresis, and h is the standard deviation of the gaussian distribution. The close fit of the line with the points indicates that mercaptalbumin is electrophoretically heterogeneous, and is an indication that the mobility distribution is gaussian (Brown and Cann, 1950).

Another test of a gaussian distribution of mobilities is that the gradient maintain its gaussian shape after application of a potential gradient. This requires that X_j graph as a straight line *vs.* z, with a slope of σ, where X_j is the coordinate in the electrophoresis cell, and z is the limit of integration of the gaussian distribution, both corresponding to a given Rayleigh fringe, j (Bhargava and Foster, 1970). Figure 12 indicates that an approximately linear dependence of X_j on z is maintained during electrophoresis and therefore it is concluded that the mobility distribution is very nearly gaussian.

Reversible boundary spreading experiments were done with different preparations and under different conditions, and the results are given in Table III. The mobilities were calculated as averages of left and right boundaries during forward and reverse electrophoresis.

The experimental value of h for mercaptalbumin was lower than literature values for other plasma-albumin preparations. At 22° in 0.01 M Na acetate the experimental value for mercaptalbumin (0.47 × 10^{-5} cm²/V sec) is only 80% as large as Bhargava and Foster's value of h for charcoal-defatted crystallized bovine plasma albumin under the same conditions. At 3° in 0.10 M Na acetate the experimental value for the mercaptalbumin is only 70% as large as the value of Baldwin *et al.* (1951) for crystallized plasma albumin (their value at 0°).

Microheterogeneity and Aging. When albumin is subjected to high pH and low salt concentration for long periods of time it is said to be "aged." Sogami *et al.* (1969) first noted an increase of ΔpH_{10}^{90} of the pH–solubility profile in 3.0 M KCl on aging albumin solutions and concluded it results from permutation in disulfide pairing. Bhargava and Foster (1970) showed that aging causes a large increase in the electrophoretic boundary spreading of albumin.

Experiments were conducted in which albumin was aged and then eluted thru a Sulfoethyl Sephadex column. In one such experiment charcoal-defatted fraction V was deionized by dialysis against water at pH 5.1, then the pH raised to 9.0, and the albumin stored for 1 day at 2°. The aged albumin was used as the starting material in a routine preparation of mercaptalbumin. Reversible boundary spreading experiments were performed on the charcoal-defatted fraction V, aged charcoal-defatted fraction V, and on the mercaptalbumin prepared from aged charcoal-defatted fraction V.

As seen in Table III, the aged fraction V showed a separate component by moving-boundary electrophoresis—a component with mobility approximately 2.0 × 10^{-5} cm²/V sec more positive than the unaged fraction V, and consisting

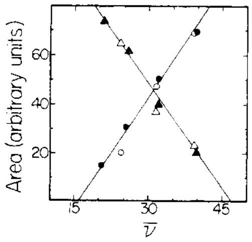

FIGURE 10: Determination of stoichiometry of detergent complexes: (○) fast mercaptalbumin peak, (●) fast plasma albumin peak; (△) slow mercaptalbumin peak, (▲) slow plasma albumin peak. The areas under the individual peaks of the electrophoresis patterns (see Figure 10) are plotted *vs.* $\bar{\nu}$. The plot is after Decker and Foster (1966), and the interception of the lines with the abscissa indicates the stoichiometry. The indicated stoichiometry is AD_{16} for the slow peak, and AD_{47} for the fast peak.

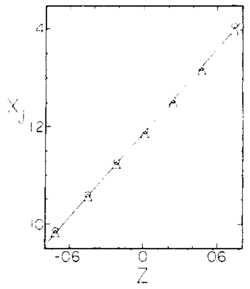

FIGURE 12: Dependence of X_j on z in a reversible boundary spreading experiment: (○) right boundary; (△) left. Data were taken after 4.5 hr of electrophoresis, 2.4-V/cm gradient, 3°, 0.03 M Na acetate. The fit of the points to a straight line is evidence that the schlieren pattern has remained gaussian, which in turn means that the mobility distribution is gaussian (Brown and Cann, 1950).

TABLE III: Reversible Boundary Spreading Results.

Preparation	Temp (°C)	I[a]	E(V/cm)	pH	($h \times 10^5$)[b]	($\mu \times 10^5$)[b]
Mercaptalbumin[c]	22	0.01	0.99	4.90	0.50	−0.05
Mercaptalbumin[c]	22	0.01	0.99	4.86	0.45	+0.20
Mercaptalbumin[d]	22	0.01	0.97	4.91	0.45	−0.12
Mercaptalbumin[d]	22	0.01	0.99	4.86	0.49	+0.20
Iodoacetamide-blocked mercaptalbumin[e]	22	0.01	0.99	4.90	0.44	+0.05
Mercaptalbumin[d]	3	0.01	2.61	4.92	0.25	+0.23
Mercaptalbumin[d]	3	0.01	4.20	4.90	0.26	+0.23
Mercaptalbumin[d]	3	0.03	2.39	4.82	0.17	+0.13
Mercaptalbumin[d]	3	0.03	2.38	4.89	0.18	−0.25
Mercaptalbumin[d]	3	0.10	1.61	4.62	0.10	+0.25
Charcoal-defatted fraction V	3	0.03	2.37	4.84	0.44	+0.06
Aged, charcoal-defatted fraction V	3	0.03	2.37	4.85	[f]	−0.19 +1.81
Mercaptalbumin from aged albumin[g]	3	0.03	2.37	4.86	0.18	−0.12

[a] Ionic strength (molarity of sodium acetate). [b] In units of $cm^2\ V^{-1}\ sec^{-1}$. [c] Prepared by routine procedure from crystallized plasma albumin. [d] Prepared by routine procedure from fraction V. [e] Mercaptalbumin prepared by routine procedure from fraction V, then SH groups blocked with iodoacetamide. [f] Two peaks visible, therefore not gaussian and h not calculated. The smaller peak (18% of area) was more positive. [g] Prepared by routine procedure from aged, fraction V.

of 18% of the albumin. This component appears not to have been noticed by Bhargava and Foster (1970). Due to this separate component, an h value could not be calculated for the aged fraction V. The schlieren pattern for the charcoal-defatted fraction V showed almost complete absence of aged protein and had an h value 2.5 times as large as mercaptalbumin. The mercaptalbumin prepared from aged fraction V was indistinguishable from the usual mercaptalbumin preparations in both h value and isoelectric pH.

Discussion

The classical method for the preparation of mercaptalbumin from plasma albumins (Hughes and Dintzis, 1964) is based on crystallization of the mercury dimer. While this method is elegant in principle, being based on the reaction of Hg^{2+} with the free SH group of the mercaptalbumin, it possesses several practical drawbacks. In particular, the procedure is laborious and time consuming. Janatová *et al.* (1968a,b) have developed a chromatographic method based on DEAE-Sephadex. Our initial studies with Sulfoethyl Sephadex were motivated by a desire to use conditions of lower pH to minimize the possibility of disulfide interchange occurring during the chromatographic separation. It quickly became apparent that this system, particularly at low acetate concentration, provided a more sensitive separation method. In particular, the separation of the ligand-bound components from both mercaptalbumin and nonmercaptalbumin in nondefatted samples indicates this method to be particularly valuable for preparation of mercaptalbumin which is free of bound fatty acids. The separation achieved between mercaptalbumin and nonmercaptalbumin components appears to be at least as good and perhaps better than that achieved by Janatová *et al.* The method provides mercaptalbumin containing at least 0.95 mole/mole of free SH in yields of 40–50% based on the total mercaptalbumin in the starting material.

The basis for the ion-exchange separation is of some interest. Estimation of the differences in net charge of albumin components produced by bound fatty acids or by the SH blocking groups (cysteine and glutathione) indicate that net charge alone cannot account for the observed separations. Whatever the basis for the separations, Sulfoethyl Sephadex chromatography appears to be remarkably sensitive to subtle differences in the albumin components.

Comparison of the elution patterns of undefatted (Figure 2) and charcoal-defatted (Figure 3) albumin suggests several tentative conclusions. (1) The bound lipids (presumably fatty acids) must be associated with only part of the protein molecules, the balance being lipid free. This implies that the impurities are inertly bound, *i.e.*, interchange slowly. This is not surprising in view of the known difficulty with which these impurities are removed. (2) Charcoal defatting is very effective in that the component corresponding to lipid-bound protein is almost completely eliminated in Figure 3. (3) Charcoal defatting does not produce any irreversible alterations of the protein, since no new components are seen in the elution patterns following defatting.

The results of electrophoresis of aged mercaptalbumin, given in Table III, indicate that aging produces a new component which has markedly altered electrophoretic properties. This new component can be removed by Sulfoethyl Sephadex chromatography. The balance of the mercaptalbumin appears to be unchanged on the basis of h value. The difference in mobility between native and aged components ($2.0 \times 10^{-5}\ cm^2/V$) corresponds to a charge difference of approximately 6 e based on μ/Q of 0.31 for the conditions of the experiment (see Table IV). To account for the observed mobility difference between native and altered albumin, other molecular differences must have accompanied the

TABLE IV: Calculation of σ_Q.

Ionic Strength	Temp (°C)	($\mu/Q \times 10^5$ (cm^2 V^{-1} sec^{-1})	$h \times 10^5$ (cm^2 V^{-1} sec^{-1})	$\sigma_Q = h/(\mu/Q)$
0.01	3	0.42	0.25	0.60
0.03	3	0.31	0.18	0.58
0.10	3	0.20	0.10	0.50
0.01	22	0.71	0.47	0.66

rearrangement of disulfide pairing. It is therefore concluded that the aging of albumin is a cooperative phenomenon.

Bovine mercaptalbumin, as prepared by the ion-exchange method described, was free of dimer and other proteins as judged by disc gel electrophoresis. Fatty acid impurities were removed, both by charcoal defatting and by Sulfoethyl chromatography. The mercaptalbumin was almost free of nonmercaptalbumin, as judged by SH analysis (routinely 0.95 mole of SH/mole of albumin). Experiments included in Table III indicated that it was also free of aged albumin. A further indication of its homogeneity is its low value of ΔpH_{10}^{90}, equal to the lowest value reported, which was also for defatted mercaptalbumin (Andersson, 1969). In addition, isoelectric focusing of this mercaptalbumin preparation by Kaplan (1970) indicated that it was more homogeneous than the monomer of charcoal-defatted plasma albumin.

The mercaptalbumin preparation could be separated electrophoretically into two complexes in the presence of detergent. The evidence presented that association and dissociation of detergent with albumin is rapid is interpreted to mean that the interconversion of albumin molecules between the complexes is rapid. If this is so, separation of complexes suggests microheterogeneity, according to the argument of Sogami and Foster (1963) and Foster *et al.* (1965).

On the other hand, separation of N and F forms could not be achieved with highly purified mercaptalbumin. It was concluded by Foster and Aoki (1957) that the N–F isomerization is approximately 12th order in protons under conditions corresponding to the electrophoretic experiments. The complex formation of mercaptalbumin with decylbenzenesulfonate was observed to be about 31st order in detergent ions (Figure 10 and eq 1). Foster *et al.* (1965) pointed out that the more highly cooperative an isomerization, the more favorable are the chances of detecting microheterogeneity. Evidently the mercaptalbumin preparation has a microheterogeneity that can only be detected by the more highly cooperative of the two isomerization reactions of albumin.

A stronger case for microheterogeneity of mercaptalbumin is seen in the reversible boundary spreading results. Figures 11 and 12 indicate a gaussian distribution of mobilities at the mean isoelectric pH. Presumably the time-average net molecular charge distribution is also gaussian. The standard deviation of the charge distribution σ_Q can be calculated from the relation

$$\sigma_Q = \frac{h}{\mu/Q} \tag{3}$$

where Q is the net time-averaged molecular charge in units of e, h is the standard deviation of the mobility distribution, and μ is electrophoretic mobility. In a homogeneous protein it would be expected that the net time-averaged charge would be the same for all molecules and therefore both h and σ_Q would be zero.

To calculate σ_Q, μ/Q was estimated employing the Henry equation, with an effective radius of albumin of 49 Å (Aoki and Foster, 1957). The results of this calculation, presented in Table IV, show that h varies approximately as expected for a σ_Q value of 0.6 ± 0.1 e. If the reversible spreading were due to some artifact such as thermal convection, electroosmosis or pH gradients it would be most surprising that the resulting h values would vary with ionic strength and temperature as predicted by the Henry equation. We conclude that reversible boundary spreading is a true measure of electrical heterogeneity and that the mercaptalbumin preparation is indeed heterogeneous.

The results presented in this paper indicate that bovine mercaptalbumin is heterogeneous. However the cause of the observed heterogeneity remains unknown, and therefore it is impossible to attribute it unambiguously to either macroheterogeneity or microheterogeneity. Nevertheless, the preparation was shown to be one component by the best available separation techniques, and, therefore, is assumed to be free of macroheterogeneous impurities. Consequently, the preparation is empirically described as microheterogeneous.

It is surprising that a protein preparation could meet the constant solubility test in high salt solutions, and yet exhibit a distribution of mobilities, at the mean isoelectric pH, corresponding to the rather large standard deviation of 0.6 electronic charge unit. Perhaps these charge differences are masked in concentrated ammonium sulfate solutions. Clearly there is much yet to be learned about the solubility of this protein in concentrated salt solutions and the significance of the phase-rule behavior.

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Proteolytic Fragmentation of Elasmobranch Immunoglobulins*

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ABSTRACT: Lemon shark 7S IgM, when digested by papain or trypsin, yielded a 6S (mol wt ~112,000) product plus small peptides. Upon reduction, the 6S fragment was cleaved into two 4.5S fragments (mol wt ~60,000). Analysis of heavy and light chains of the proteolytic products (either 6S or 4.5S) showed the light chains to be intact (mol wt ~22,000), but the heavy chain reduced in size from 70,000 to 43,000. Antigenic analysis of this heavy-chain fragment showed it to be missing some of the determinants of the intact heavy chain and hexose determinations showed it to have lost ~20% of its carbohydrate. It was concluded from these experiments that lemon shark 7S IgM can be cleaved by proteolysis to yield a 6S F(abμ')$_2$, which upon reduction yields two 4.5S fragments. Reduction and subsequent proteolysis of lemon shark 7S IgM, or proteolysis of 7S IgM subunits produced by reduction of lemon shark 19S IgM, yielded 3.5S (Fabμ) material closely resembling the 4.5S fragment. This was considered to be the "core" of these immunoglobulin molecules. In these fragments light chains remained intact and the heavy chain was reduced to approximately light-chain size. Antigenic analysis showed the 3.5S fragment to be deficient in heavy-chain determinants to the 4.5S molecule and carbohydrate analysis showed 80% reduction in hexose content.

The surge of interest, during recent years, in the phylogenetic development of immunological competency has resulted in the demonstration that representatives of all major classes of vertebrates are capable of producing humoral antibodies in response to antigenic stimulation (reviewed, Clem and Leslie, 1969). However, in order to understand the mechanisms operative in the evolution of antibody structure and function, it is necessary to thoroughly characterize the immunoglobulins of various lower vertebrates and to relate these proteins to the immunoglobulins of mammals. Initial efforts in this direction from several laboratories have focused on the elasmobranchs and have resulted in the demonstration that dogfish (Marchalonis and Edelman, 1965), lemon (Clem and Small, 1967), nurse (Clem *et al.*, 1967), and leopard (Suran *et al.*, 1967) sharks each have 19S and 7S immunoglobulins. The shark 19S immunoglobulin is a pentamer of disulfide-linked subunits, each composed of two heavy (mol wt ~70,000) and two light (mol wt ~22,000) polypeptide chains, containing a relatively high carbohydrate content. These structural studies suggest that the shark 19S immunoglobulin belongs to the IgM class as defined for higher animals (World Health Organization, 1964). Based upon immuno- and physicochemical studies, the shark 7S immunoglobulin appears to belong to the same immunoglobulin class as the shark 19S molecule and therefore can be referred to as 7S IgM. The other immunoglobulin classes commonly found in mammals appear to be absent in sharks.

With the realization that enzymatic digestion of macromolecules may elucidate gross structural details, *i.e.*, differentiate between loosely structured regions (exceedingly enzyme susceptible) and highly structured regions such as helical areas (enzyme resistant) (Mihalya and Harrington, 1959), several investigators have studied the products of proteolysis of mammalian IgM (Miller and Metzger, 1966; Onoue *et al.*, 1966; Onoue *et al.*, 1968a; Mihaesco and Seligmann, 1967; Inman and Hazen, 1968; Goodman and Inman, 1969; Chen *et al.*, 1969; Beale, 1969; Suzuki, 1969). IgM fragments corresponding to IgG fragments [Fcμ, F(abμ')$_2$, and Fabμ] have been demonstrated to occur, and the polypeptide chain arrangement of IgG and IgM is thus probably quite similar.

With this high degree of structural similarity between different mammalian immunoglobulin classes and the gross similarity of lower vertebrate immunoglobulins to one of the

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