

Bovine Mercaptalbumin and Non-mercaptalbumin Monomers

INTERCONVERSIONS AND STRUCTURAL DIFFERENCES*

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SUMMARY

The monomer macroheterogeneity of commercial bovine serum albumin preparations is entirely due to oxidation of the mercaptalbumin sulfhydryl group. When the sulfhydryl group is oxidized to oxidation states higher than disulfide, circular dichroism and fluorescence data show that a tryptophanyl and a cystinyl residue are perturbed. Measurements conducted through the pH range of the N-F transition (pH 5 to 3.5) place these residues in the crevice which is opened up in the course of the transition. Studies with undefatted and defatted monomers indicate that one of the primary fatty acid binding sites is also located in this part of the crevice and that oxidation of the cysteinyl sulfhydryl group alters the binding of fatty acid at this site.

The macroheterogeneity of bovine serum albumin with respect to sulfhydryl content has been common knowledge for many years. The presence of non-mercaptalbumin monomers in albumin has been attributed by King (1) and by Andersson (2) to the formation of mixed disulfides between the mercaptalbumin and the plasma cysteine and glutathione. Andersson has demonstrated the presence of such mixed disulfides in some non-mercaptalbumin fractions of BSA.¹ Fractionation of BSA on DEAE-Sephadex A-50 has shown the presence of three non-mercaptalbumin monomers (3). Two of these monomers were also obtained following fractionation of an aged mercaptalbumin preparation (0.8 mole of sulfhydryl per mole of albumin) which had not been in contact with small molecule thiols (3). It therefore appeared that oxidative pathways other than mixed disulfide formation were operative in the formation of non-mercaptalbumin monomer.

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¹ The abbreviations used are: BSA, bovine serum albumin; CD, circular dichroism; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); HSA, human serum albumin.

In the present studies, three general approaches have been taken to further investigate the four BSA monomers which have been isolated by chromatography on DEAE-Sephadex and to examine the factors involved in the formation of non-mercaptalbumin. Since it appeared that non-mercaptalbumin monomers could be generated from mercaptalbumin, it was assumed that the observed heterogeneity was not due to variations in primary structure.

The first series of experiments were concerned with fatty acid heterogeneity. Experimental evidence has indicated the importance of free fatty acids both *in vitro* (4) and *in vivo* (5, 6) in the formation of the tertiary structure of albumin. In addition, broadening of the albumin pH solubility profiles has been observed in the presence of fatty acid (7-9). The albumin fractions isolated by DEAE-Sephadex chromatography differed in their fatty acid content (3) and Peterson and Sober (10) have observed that the addition of oleic acid to purified mercaptalbumin resulted in the formation of a number of overlapping chromatographic components having a higher affinity than the original mercaptalbumin for the adsorbent. Thus, on a superficial level, it was necessary to determine whether much of the observed heterogeneity could be due to fatty acid heterogeneity.

In the second aspect of this investigation, the conversion of mercaptalbumin to non-mercaptalbumin monomer was studied under controlled conditions in order to obtain a more precise delineation of the nature of the non-mercaptalbumin monomers.

The third approach to the problem was concerned with the possible existence of secondary or tertiary structural differences between the monomers. It was thought that any change such as the suggested albumin disulfide interchange (2) might be detectable by suitable measurements. The two types of measurement which were employed were fluorescence and circular dichroism spectral determinations. BSA undergoes a reversible isomerization, the so-called N-F transition (11), between pH 4.5 and 3.5. The N or high pH form of the molecule is characterized by the presence of many masked carboxylate ions, presumably in some crevice (or crevices) of the molecule. In the opened up, low pH, or F form of the molecule, all the carboxylate groups titrate normally. Since many of the physical properties of the BSA change markedly in the course of this transition it was thought that measurements conducted over the pH range of the transition might prove a sensitive index of any small structural differences that could exist between the monomers.

TABLE I
Characterization of albumin preparations

Armour BSA (lot)	Dimer content	Sulfhydryl content	Fatty acid content
	%	mole/mole	
D71209	5-6	0.64	0.60
E71503	5-6	0.65	0.66
F71601	5-6	0.61	0.62

Since the albumin should not have been exposed to pH values greater than 7.4 during its preparation and since the pH of the albumin solutions was never allowed to rise above 7 during these investigations, it is unlikely that the observed macroheterogeneity is directly related to the alkali-induced interconversions recently reported by Nikkel and Foster (12).

EXPERIMENTAL PROCEDURE

Materials—Crystalline bovine serum albumin, Lots D71209, E71503, and F71601, was obtained from the Armour Pharmaceutical Co. A comparison of the sulfhydryl content, dimer content (as determined from sedimentation velocity analysis), and fatty acid content of these preparations is given in Table I.

5,5'-Dithiobis(2-nitrobenzoic acid) was obtained from Aldrich Chemical Co. Iodoacetamide was prepared according to the method of Anson (13) and was recrystallized. Thioglycolate was obtained from Matheson, Coleman and Bell and was redistilled *in vacuo* prior to use. Oleic acid, Lot 75L750, was obtained from Fisher Scientific Co. and linoleic acid was obtained from Hormel. Boron trifluoride in methanol (14% w/v) and sil-3-trimethylchlorosilane, Lot 040766, were obtained from Applied Science Laboratories, Inc., State College, Pa. Hexamethyldisilazane, Lot 16, was obtained from Dow Corning Corp. Pyridine, reagent grade, was obtained from Merck. Dialysis tubing was obtained from Union Carbide Corp. and was washed well with distilled water prior to use. DEAE-Sephadex A-50, Sephadex G-150, and Sephadex G-200 were obtained from Pharmacia. Celite 535 was obtained from Johns Manville. The starch-hydrolyzed, Lot 253-1, used in the starch gel electrophoresis experiments was obtained from the Connaught Medical Research Laboratories, University of Toronto, Canada. Darco-activated charcoal was obtained from Atlas Chemical Industries. *N,N*-Methylenebisacrylamide, acrylamide, 2-amino-2-hydroxymethyl-1,3-propanediol and *N,N,N',N'*-tetramethylethylenediamine were obtained from Canaco. Riboflavin was obtained from Nutritional Biochemicals Corp. All other chemicals used were reagent grade.

Protein solutions of large volume were concentrated by use of a Diaflo UM-1 membrane ultrafilter (Amicon Corp.) under nitrogen pressure; in some instances a PM-10 filter was used. Small volumes were concentrated by use of a Sartorius membrane filter (Collodion bag, porosity 5 nm, size 8 ml) from Carl Schleicher and Schuell, Keene, N. H., or by use of a 10-ml Diaflo ultrafilter with PM-10 membrane. Protein solutions were clarified by passage through Millipore filters of various sizes (0.22, 0.45, and 1.2 μ).

The molecular weight of albumin was taken as 67,000. An extinction coefficient ($E_{1\%}^{1\text{cm}}$ (279 nm)) of 6.67 had been determined for the BSA preparation (3).

Absorbance Measurements—These were made on a Zeiss PMQII Spectrophotometer with Beckman quartz cells of 1.0-cm path length.

Sedimentation Velocity Analyses—Sedimentation velocity anal-

yses were performed by Mr. John Trojanowski on a Spinco model E Ultracentrifuge with the schlieren optical system. Ultracentrifuge plates were read on a modified Nikon Shadow Graph, model 3A (Nippon Kogaku K. K., Tokyo, Japan), equipped with a Mann two-dimensional Comparator, type 829 (David W. Mann, Inc., Lincoln, Mass.).

Conductivity Measurements—These were performed on a Conductivity Bridge, type 3216B, with LKB 3225J-2 cells (cell constants, 4.60 and 4.625 cm^{-1}), from L. K. B. Instruments, Inc.

Absorption Spectra—Absorption spectra were measured with a Cary model 15 recording spectrophotometer. Scans in the ultraviolet were made while purging the instrument with high purity nitrogen. Beckman quartz cells of 1.0-cm path length were used.

Fluorescence Measurements—Fluorescence measurements were performed on an Aminco-Bowman spectrofluorimeter which had been adjusted as recommended by Chen (14). A constant temperature of 25° was maintained by the use of a Heto circulating water bath. Tryptophan spectra ($\lambda_{\text{excitation}}$, 280 or 295 nm) were almost identical with the corrected tryptophan spectrum of Teale and Weber (15). The collisional quenching constant (K_Q) of tryptophan in the presence of various iodide to chloride concentration ratios (total ionic strength = 0.4) was 12.1 M^{-1} , in good agreement with the values reported by Lehrer (16). The absorbance of protein solutions at the exciting wave length was never greater than 0.1 and the concentration dependence of the emitted fluorescence intensity was linear over a concentration range greater than that employed in the measurements. The fluorescence intensity of a standard mercaptalbumin-NaCl solution at 343 nm was measured before and after all spectral and single wave length measurements and the data were corrected when necessary to the same standard value at 343 nm.

Fluorescence spectra were obtained for all samples in which pH was the variable. Since the HCl-titrated samples varied slightly in concentration, the reported spectra are adjusted to a standard concentration of 0.005 g per dl. In all experiments involving pH as a variable, an exciting wave length of 280 nm was used.

In the initial iodide-quenching experiments complete spectra were obtained. Since the shape of the fluorescence spectra in the presence and absence of iodide were the same and since the band half-widths were essentially the same as those of tryptophan, it was assumed that the quantum yield was proportional to the peak fluorescence and that measurement of fluorescence intensities at λ_{max} could be substituted for whole spectra measurements. The wave length of λ_{max} was 343 nm for all BSA preparations and did not change significantly (< 2 nm) in the presence of even the highest iodide concentration. Most peak fluorescence data were obtained with an exciting wave length of 295 nm. In a few experiments exciting wave lengths of 280 and 305 nm were used.

When a quencher such as iodide is added to a solution containing a fluorescent molecule, the ratio of the fluorescence quantum yields without and with quencher is given by the relationship:

$$F_0/F = (1 + K \cdot X)(1 + \kappa \tau \cdot X) \quad (1)$$

where F_0 and F are the fluorescence quantum yields without and with the quencher, K is the binding constant of the quencher to the fluorescent molecule, κ is the rate of encounter by diffusion between the fluorophore and the quencher, τ is the mean lifetime of the excited fluorophore, and X is the molar concentration of the quencher. The product, $\kappa \tau$, is the well known Stern-Volmer collisional quenching constant, K_Q . The first term in Equation 1 thus represents "static quenching" and the second "collisional

quenching." In the absence of static quenching, the equation reduces to the well established Stern-Volmer relationship (17).

$$F_0/F = 1 + K_Q X \quad (2)$$

In the case of a protein molecule containing 2 nonidentical tryptophanyl residues, the collisional quenching is described by the following relationship.

$$F = \frac{F'_0}{1 + K'_Q X} + \frac{F''_0}{1 + K''_Q X} \quad (3)$$

where F'_0 and F''_0 are the fluorescence quantum yields of each tryptophanyl residue in the absence of quencher, K'_Q and K''_Q are the respective quenching constants, and F is the observed fluorescence.

If one of the tryptophanyl residues is unavailable to the quencher, the equation reduces to the form:

$$F = \frac{F'_0}{1 + K'_Q X} + F''_0 = \frac{F'_0}{1 + K'_Q X} + F'_0 - F'_0 \quad (4)$$

where F'_0 is the total fluorescence quantum yield in the absence of quencher. A plot of F'_0/F versus X would not be linear. However, a plot of $F'_0/\Delta F$ versus $(1/X)$ should be linear where $\Delta F = F'_0 - F$. The reciprocal of the ordinate value at $(1/X) = 0$ represents the fraction of F'_0 due to the quenchable tryptophanyl residue and the ratio of intercept to slope gives K'_Q . F'_0 , F''_0 , and K'_Q can also be determined directly from Equation 4.

Circular Dichroism Measurements—These measurements were performed on a Durrum-Jasco UV5 scanning spectropolarimeter equipped to make circular dichroism measurements; the sensitivity of the machine was increased by a Sproul Scientific SS-20 CD modification. Circular dichroism measurements were performed at 25° on 0.025 to 0.15% protein solutions in 0.1 M KCl. An Opticell quartz cell of 1.000-cm optical path length and a scale factor of 3.03×10^{-5} or 6.06×10^{-5} dichroic absorption difference per cm on the recorder chart were used for measurements in the near-ultraviolet (360 to 250 nm). A scale factor of 30.3×10^{-5} dichroic absorption difference per cm on the recorder chart was used for measurements in the far-ultraviolet. All reported CD data were corrected for solvent base-line nonideality and the base-line and protein dichroisms were matched at 450 nm (*i.e.* in a spectral region where no absorption occurs). No corrections for refractive index dispersion were made. All spectra were measured in triplicate and the average values have been reported; the variance among the triplicates for a given sample was within 0.3 cm on the recorder chart. The more detailed study of defatted, iodoacetamide-treated BSA-A and defatted BSA-B1 and BSA-B2 was conducted at constant wave lengths by reading samples and reagent blanks at 350 nm (where no protein absorption occurs) and at 262 and 269 nm or 209 and 220 nm. The circular dichroism was calibrated with (dry) *d*-camphorsulfonic acid, assuming a circular dichroic absorption coefficient of 2.2 at 290 nm.

The modified CD instrument measures the observed difference in absorption between left- and right-circularly polarized light, $k_L - k_R$. The circular dichroic absorption coefficient, $\Delta\epsilon$, is then calculated according to the relationship

$$\Delta\epsilon = (\epsilon_L - \epsilon_R) = \frac{k_L - k_R}{c \times l}$$

where c is the molar concentration and l is the optical path length in centimeters. All the results have been expressed in terms of

the molar ellipticity, $[\theta]$, using the relationship

$$[\theta] = 2.303 \frac{4500}{\pi} (\epsilon_L - \epsilon_R)$$

where $[\theta]$ has the units of degree cm² per decimole.

To minimize any degradation of the albumin, whole spectra samples were used within 48 hours of their preparation. In the more detailed constant wave length measurements, samples were used within 6 hours of their preparation.

pH Measurements—pH measurements were performed on a Radiometer pH Meter 4, type PHM 4c, with a Radiometer combination glass electrode, type GK 2021 B. A Heto constant temperature circulating bath, model 623 (Heto, Birkerød, Denmark), circulated water ($25 \pm 0.1^\circ$) through two identical Pyrex-jacketed vessels (inside diameter, 2 cm; height, 8 cm). A 5-ml Pyrex burette, graduated to 0.01 ml and equipped with a platinum tip, was used to deliver acid; drawn polyethylene tubing was attached to the platinum tip in order to titrate directly into the solution. Samples were stirred with Teflon-coated magnets driven by a Toya magnetic stirrer (Toya Kagakusangyo Co., Ltd., Japan).

The pH meter was standardized with standard buffer solutions of pH 4.00 (Wills) and 6.86 (Harleco). pH 4.00 buffer, 5 ml, was added to one titration vessel and 5 ml of pH 6.86 buffer were added to the second titration vessel. The buffer adjustment dial and temperature control dial of the pH meter were adjusted until both buffers gave the correct pH reading on the meter.

To prepare albumin solutions of known pH for the CD and fluorescence studies, 5 ml of the protein in 0.1 M KCl were introduced into the titration vessel with a volumetric pipette (Pyrex, Class A, 5 ± 0.01 ml). Using the burette, 0.1 or 0.01 N HCl was added to the albumin solution until the desired pH was attained; the volume of acid added was recorded. The solution was stirred for 10 min, the stirrer was turned off, and after 2 min the pH was read and recorded. The concentration of the titrated solution was calculated by multiplying the concentration of the albumin solution before titrating (determined accurately by the microbiuret method) by the appropriate dilution factor.

When less accurate pH measurements were needed (*e.g.* in the preparation of buffers), a Radiometer titrator, type TTT 1c, attached to the Radiometer combination glass electrode, was used.

Protein Concentration—Protein concentration was routinely determined by a modification of the biuret reaction described by Janatova *et al.* (3). When desired, estimates of the protein concentration were obtained by measuring the absorbance of the solution at 279 nm.

Sulfhydryl Content—Sulfhydryl content was determined by a spectrophotometric titration with 5,5'-dithiobis(2-nitrobenzoic acid) by the procedure of Ellman (18) as modified by Janatova *et al.* (3).

Fatty Acid Content—Fatty acid content was assayed by a modification of the method of Dole (19) described by Scheider and Fuller (20).

Starch Gel Electrophoresis—Starch gel electrophoresis was performed according to the method of Smithies (21). Starch-hydrolyzed, 33 g, was suspended in 300 ml of buffer (0.03 M H₂BO₃-0.003 M NaOH, pH 8.60) and the suspension was stirred well and heated with a bunsen burner until it became gelatinous. Heating was continued for about 45 s until the gel became transparent. Electrophoresis was conducted at 2° with the gel in a vertical position at a potential gradient of approximately 3 volts per cm for 15 to 17 hours. The proteins were stained with a saturated

solution of Amido black 10B in methanol-acetic acid-water (5:1:5) and the gels were destained with methanol-acetic acid-water (5:1:5).

Disc Gel Electrophoresis—Disc gel electrophoresis was performed as described by Davis (22), with 5 to 8% gels. From 5 to 30 μ l of an approximately 0.1% protein solution were added to 20 μ l of a 20% sucrose solution and this was layered onto the spacer gel. Gels were stained with a saturated solution of Amido black 10B in methanol-acetic acid-water (5:1:5) and were destained in methanol-acetic acid-water (5:1:5). Gels were stored in 7% formic acid.

Gas-Liquid Chromatography—Gas-liquid chromatography was performed in order to determine the fatty acid distributions in albumin samples. Fatty acids were isolated from albumin by suspending the protein (0.1 to 0.2 g) in 7 ml of chloroform-methanol, 1:1. The mixture was vigorously shaken, allowed to stand for 10 min, and was centrifuged to remove the protein. The supernatant was transferred to a clean test tube and 1 ml of distilled water, 1.6 ml of 1% formic acid, and 3 ml of chloroform were added. The tube was vigorously shaken, centrifuged, and the chloroform layer was drawn off. Fatty acid analysis was also performed on the heptane extract (as in the Dole method) and on unextracted protein.

To determine the total fatty acid content (nonesterified plus esterified fatty acids), fatty acid methyl esters were made by drying the chloroform extract and adding 1.0 ml of BF_3 in methanol and heating in a sand bath at 50–80° for 15 min. Then 0.5 ml of saturated NaCl was added and the methyl esters were extracted with three 3-ml portions of petroleum ether. The extract was dried and redissolved in carbon disulfide and the methyl esters were chromatographed at 197° on an ethylene glycol succinate stationary phase, with nitrogen as the carrier gas. A Barber-Coleman gas chromatograph, model 10, was used.

For detection of only the esterified fatty acids present in a sample, methyl esters were made by transesterification of the esterified fatty acids. The chloroform extract was dried, 1.0 ml of 0.5 N sodium methoxide was added, and the reaction was allowed to stand at room temperature for 5 min. The methyl esters were then extracted with three 3-ml portions of petroleum ether, dried, redissolved in carbon disulfide, and chromatographed.

To check for the possible presence of peroxidized or branched chain fatty acids the samples were hydrogenated prior to esterification. The chloroform extract was dried, dissolved in 1.75 ml of methanol, and hydrogenated under a stream of hydrogen gas, with a few milligrams of PtO_2 as a catalyst. The resultant fatty acids were then acidified with 0.2 ml of glacial acetic acid and esterified with BF_3 in methanol, as described previously. The methyl esters were then chromatographed. As a result of hydrogenation, all fatty acids except those which are peroxidized or branched chain will be reduced to the saturated acids. Upon subsequent esterification and chromatography, no peaks other than those for the straight chain, saturated acids are normally observed, for the hydroxy esters which result from reduction of the peroxy acids are very polar, resulting in chromatographic bands which are greatly broadened, decreased in amplitude and eluted at very long retention times. To reduce this problem, the silyl derivatives of the hydroxy esters were made in some cases. To the dried petroleum ether extract were added, in order, 50 μ l of pyridine, 30 μ l of hexamethyldisilazane, and 15 μ l of sil-3-trimethylchlorosilane. The reaction mixture was allowed to stand at room temperature for 15 min and then 2 ml of petroleum ether were added and the sample was immediately placed

in an ice bath. Just before chromatography, the sample was dried and redissolved in carbon disulfide.

Interpretation of Chromatograms—Observed retention times were compared with those obtained from a standard mixture of fatty acids (16:0, 18:0, 20:0, and 22:0) and identification of the fatty acids was made by comparison of equivalent chain lengths with the table of equivalent chain lengths published by Hofstetter *et al.* (23). An internal standard (15:0) was routinely added to the fatty acid unknowns and fatty acids were quantitatively estimated by measuring the peak at its maximum and multiplying by the retention time.

Iodoacetamide Treatment—Iodoacetamide treatment of albumin was performed in order to mask the reactive sulfhydryl group. The pH of a 4 to 6% albumin solution was raised to 8 with 0.1 N NaOH. The reaction vessel was covered with aluminum foil and then approximately 2 moles of iodoacetamide per mole of albumin sulfhydryl were added to the albumin solution. The reaction was allowed to continue at room temperature for 1 hour, during which time the solution was stirred with a magnetic stirring bar and the pH was maintained at 8. The albumin solution was then dialyzed exhaustively against distilled water at 2°, the dialysis vessel was wrapped in aluminum foil. Following dialysis the sulfhydryl content of the albumin solution was determined by spectrophotometric titration with DTNB; no reactive sulfhydryl was detected.

Reduction of BSA Fraction C Monomer with Thioglycolate—This reduction was performed according to the method of Katchalski *et al.* (24), in a manner identical with the reduction of the BSA Fraction B1 and B2 monomers (3).

Removal of Fatty Acids from Albumin—Removal of fatty acids was effected by the charcoal treatment of Chen (9).

Addition of Oleic Acid to Defatted BSA Fraction A—This was performed according to a modification of the method of Spector and Hoak (25) for the addition of long chain fatty acid to protein solutions. Oleic acid-coated Celite particles were prepared as follows. Celite 535, 1.0 g, was placed in a beaker to a depth of less than 5 mm. Oleic acid, 0.0285 g, was dissolved in 25 ml of hexane and was added to the Celite, giving a suspension containing 1 mmole of oleic acid per 10 g of Celite. The hexane was evaporated under a stream of nitrogen and the dry particles were transferred to a screw capped vial and were well mixed.

Iodoacetamide-treated, charcoal-defatted mercaptalbumin, 0.4 g, was dissolved in 20 ml of 0.1 N KCl and a few drops of NaOH were added to adjust the pH to 7.4. To 19.5 ml of the albumin solution were added 300 mg of the oleic acid-coated Celite. Nitrogen was blown over the solution and then the flask was stoppered. The suspension was stirred with a magnet stirrer at room temperature for 1½ hours. At the end of the incubation period the albumin-fatty acid-Celite mixture was decanted into a plastic tube and centrifuged at 2° for 15 min at 15,000 $\times g$. The supernatant was recentrifuged at 2° for 15 min at 15,000 $\times g$. The final supernatant was then passed through a 1.2 μ Millipore filter and the pH of the solution readjusted to 7.4 with NaOH. The fatty acid content of the resulting albumin solution was determined by the method of Dole (19) and the protein content was determined by the microbiuret method.

Fractionation of Albumin on DEAE-Sephadex A-50 and Sephadex G-150 or G-200—Fractionation of albumin was performed according to the method of Janatova *et al.* (3). In some cases it was desired to fractionate smaller samples of albumin than those used in the procedure cited. The same procedure was used, with all volumes and flow rates scaled down appropriately from the conditions of the larger fractionation schemes.

TABLE II
Nonesterified fatty acid compositions^a of Armour BSA

Nonesterified fatty acid	Armour BSA			
	Lot E71503	Lot K450-11	Lot F71601	Lot F71601 after passage down DEAE-Sephadex
14:0	0.026	0.024	0.018	0.018
16:0	0.234	0.223	0.198	0.196
16:1	0.045	0.038	0.034	0.036
18:0	0.212	0.208	0.228	0.221
18:1	0.354	0.365	0.331	0.338
18:2	0.089	0.102	0.142	0.150
18:3	0.014	0.017	0.010	0.011
20:0	0.004	0.003	0.008	0.004
20:4	0.012	0.011	0.015	0.017

^a Moles per mole of total fatty acid.

RESULTS

Fatty Acid Heterogeneity

Nature of Fatty Acids Bound to Albumin—The fatty acid compositions of several preparations of BSA were determined by gas-liquid chromatographic analysis. Chloroform-methanol extracts, heptane extracts (as in the Dole assay) and whole protein gave identical fatty acid distributions. The results of the analyses performed on the total fatty acids extracted from BSA with chloroform-methanol 1:1 are summarized in Table II; these results for Armour BSA are in good agreement with the fatty acid distributions reported for Poviet BSA and Behringwerke BSA (26). Only very slight variations were noticed among the fatty acid distributions of the three different Armour preparations, which perhaps reflects the large plasma pool represented in the commercial preparations.² Eight fatty acids constituted the bulk of the fatty acids associated with BSA: oleic, palmitic, stearic, linoleic, palmitoleic, myristic, linolenic, and arachidonic acids.

Further gas-liquid chromatographic analysis indicated that the fatty acids bound to BSA may be considered as essentially nonesterified. Esterified fatty acids comprised less than 1% of the total detected fatty acids extracted from BSA by chloroform-methanol, 1:1; the peaks observed in the test for esterified fatty acids were tentatively designated as 20:1 and 20:3. Chromatograms of samples which had been hydrogenated prior to esterification showed no new peaks in comparison with the chromatograms of samples which had been esterified without hydrogenation, and it was therefore assumed that no peroxidized or branched chain fatty acids were present in the samples in significant amounts. The amounts of 14-, 16-, 18-, and 20-carbon acids present after chromatography of the samples which had been hydrogenated prior to esterification were the same as those obtained with samples which had been esterified without hydrogenation; the agreement between the two data sets affords an indication of validity in the identification of the fatty acid components.

Albumin fractions which had been obtained by DEAE-Sephadex A-50 chromatography of BSA had differing fatty acid contents (Table III); the fatty acid content of Fraction A (mercaptalbumin) was lower than those of the later eluted fractions. The fractions (see Fig. 1) have been characterized previously (3): Fraction A is mercaptalbumin (*i.e.* albumin monomer with

² R. Seidel, personal communication.

TABLE III
Fatty acid and sulphydryl content of albumin fractions

BSA fraction	Fatty acid content		Sulphydryl content	
	Native	Charcoal-defatted	Native ^a	Charcoal-defatted
moles/mole albumin monomer				
A	0.22	0.05	0.98	0.92
B1	0.97	0.13	0.16	0.12
B2	0.99	0.13	0.06	0.02
C	1.04	0.17	0.36	0.44
E	0.57	0.18	0.14	0.18
Calculated average (mole/mole whole albumin)	0.48		0.65	0.45

^a Previously published data (3).

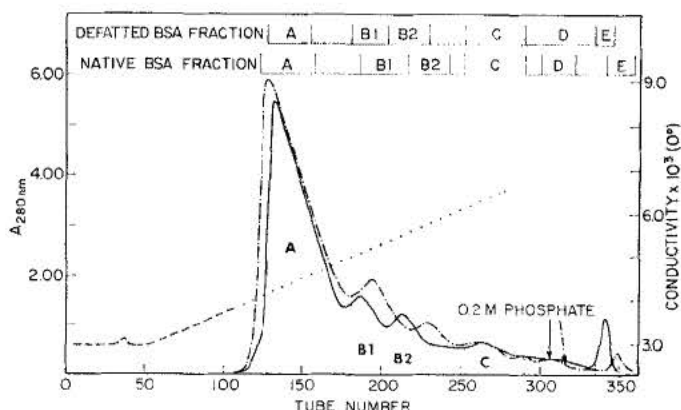


Fig. 1. The elution profiles of native and charcoal-defatted BSA after fractionation on DEAE-Sephadex A-50. —, absorbance of eluate of native BSA at 280 nm; —, absorbance of eluate of charcoal-defatted BSA at 280 nm; ---, conductivity of eluate.

1.0 sulphydryl group per molecule); Fraction B1 is non-mercaptalbumin monomer which is readily convertible to mercaptalbumin on addition of small molecule thiols, with the liberation of 1 mole of cysteine per mole of albumin³; Fraction B2 is a non-mercaptalbumin monomer which is not readily reducible to mercaptalbumin on addition of small molecule thiols; Fraction C contains a nonmercaptalbumin monomer and a sulphydryl-containing dimer; and Fraction E consists of sulphydryl-free dimers and small amounts of higher polymers.

It can be seen that the fatty acid content of the albumin prior to fractionation (Table I) was greater than that calculated from summation of the fatty acid contents of the fractions (Table III). To determine whether certain fatty acids were being selectively adsorbed to the ion exchange resin, the fatty acid composition of a BSA preparation (Armour, Lot F71601) was determined both before and after passage down a DEAE-Sephadex A-50 column. The fatty acid content of the albumin was 0.62 mole per mole of albumin before passage over the Sephadex and 0.48 mole per mole of pooled eluate albumin after passage over the resin. The fatty acid distributions, as determined by gas-liquid chromatography, are included in Table II. The distributions were in good agreement, indicating that passage over the ion exchange resin had resulted in a general decrease in fatty acid content, without selective removal of any particular fatty acid. The similarity of the binding constants of the long chain fatty

³ L. Edwards, unpublished data.

acids to BSA (27) would argue against any pronounced selective removal of fatty acid.

Three BSA fractions were examined by gas-liquid chromatography: Fraction A, Fraction B1, and Fraction B2 to C. The fatty acid distributions are presented in Table IV. The fatty acid distribution in Fraction A (mercaptalbumin) was distinctly different from the fatty acid distributions of the later eluted fractions, the difference in the amount of oleic acid (18:1), the major fatty acid component, being most pronounced.

Removal of Fatty Acids from Albumin—The charcoal treatment was effective in reducing the fatty acid content to less than 0.1 mole of fatty acid per mole of albumin. Ultracentrifugal analysis indicated that the dimer content of the defatted albumin (5 to 6%) was unchanged from that of the native protein and this conclusion was supported by starch gel electrophoresis of the native and defatted albumin preparations. The sulfhydryl content of the defatted albumin (0.42 mole per mole of albumin) was, however, lower than that of the native protein (0.64 mole per mole of albumin).

Fractionation of Defatted BSA on DEAE-Sephadex A-50—The defatted BSA preparation described above was fractionated on DEAE-Sephadex A-50 by exactly the same procedure as that followed for the previously reported fractionation of native albumin by Janatova *et al.* (3). The elution profiles of the native and defatted preparations are shown in Fig. 1. There was not an exact volume correspondence between the profiles because the resin columns used in the fractionation varied a little in height from run to run and because the final buffer was added at quite different elution volumes in the various runs. Yet, although there were minor, but real, differences between the native and defatted profiles, the over-all similarity between the elution profiles of the native and defatted albumins clearly indicated that the heterogeneity exhibited in the elution profiles of BSA was not primarily related to fatty acid heterogeneity.

Immediately following elution from the column, samples from individual tubes (or several pooled adjacent tubes if the albumin concentrations were low) were analyzed for their sulfhydryl content (Table III). Starch gel electrophoresis was performed

on samples from each of the tubes which had been sampled for sulfhydryl analysis; the monomer and dimer contents of the corresponding fractions of defatted and native albumin appeared identical except that the defatted Fraction C contained about one-third monomer whereas the native Fraction C contained about one-half monomer. The sulfhydryl and dimer contents of the fractions were determined prior to freeze-drying since it had previously been observed that the sulfhydryl content decreased and the dimer content increased after freeze-drying.

After fractionation had been completed, the tube contents were pooled to give the fractions denoted at the top of Fig. 1. Starch gel electrophoresis was performed on the pooled freeze-dried fractions; again, corresponding native and defatted fractions appeared to have identical monomer and dimer contents with the exception of Fraction C.

Formation of Non-mercaptalbumin Monomers and Further Characterization of Monomer Components

Since non-mercaptalbumin monomers were formed on dialysis and freeze-drying of mercaptalbumin solutions, it was of interest to determine whether or not these monomers corresponded with those present in native albumin. Dilute solutions of mercaptalbumin in water at pH 5.0 were repeatedly exposed to air, dialyzed, freeze-dried, and redissolved to give an approximately 0.3% albumin solution. The sulfhydryl content decreased markedly after the first dialysis and freeze-drying, but subsequently reached a plateau value of about 0.65 mole per mole of albumin. Disc gel electrophoresis indicated small amounts of dimer formation in this process. Fractionation of the treated preparation on DEAE-Sephadex A-50 indicated the formation of the Fraction B2 and C monomers but not of Fraction B1. Disc gel electrophoresis showed that Fraction C was composed of about 50% monomer and 50% dimer, as is normally observed upon fractionation of whole albumin. Similar results were obtained when this procedure was conducted with defatted mercaptalbumin, although slightly less Fraction C monomer was formed. The results of the disc gel electrophoresis of the major fractions of the native and defatted mercaptalbumin preparations are presented in Fig. 2. Parallel studies conducted in the presence of 10^{-3} M EDTA gave results identical with those obtained with the EDTA-free samples. Iodoacetamide-treated mercaptalbumin,

TABLE IV
Fatty acid composition of certain BSA fractions

Nonesterified fatty acid	BSA ^a A	BSA ^a B1	BSA ^a B2 to C	BSA A	BSA B1	BSA B2 to C
	mole/mole fatty acid			mole/mole albumin		
14:0	0.038	0.020	0.017	0.008	0.019	0.017
16:0	0.249	0.210	0.206	0.055	0.201	0.210
16:1	0.051	0.041	0.044	0.011	0.040	0.045
18:0	0.237	0.254	0.229	0.052	0.244	0.234
18:1	0.255	0.338	0.366	0.056	0.324	0.374
18:2	0.126	0.115	0.109	0.028	0.110	0.112
18:3	0.012	0.009	0.009	0.003	0.008	0.009
20:0 ^b	0.008	0.004	0.003	0.002	0.003	0.003
20:1 ^b	0.001	0.002	0.002	0.0002	0.002	0.002
20:2 ^b	0.002	0.002	0.003	0.0006	0.002	0.003
20:3 ^b	0.010	0.003	0.005	0.002	0.003	0.006
20:4 ^b	0.011	0.005	0.006	0.002	0.004	0.006

^a Calculations were based on the total fatty acid content determined by the Dole assay (19).

^b Assignment of these fatty acids was made with less certainty than the major components.

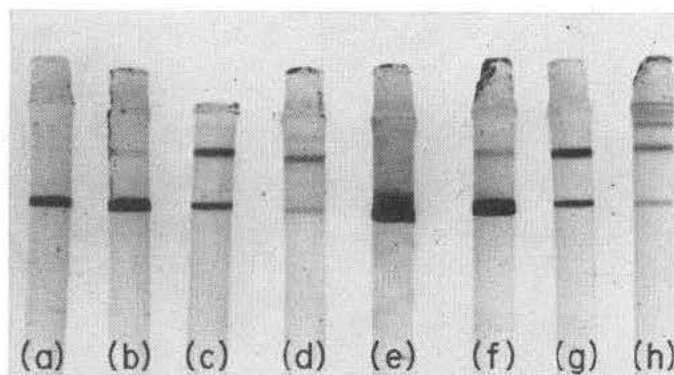


FIG. 2. Disc gel electrophoresis patterns of the major fractions obtained from air-oxidized native and charcoal-defatted mercaptalbumin preparations. Fractions from oxidized native mercaptalbumin: a, BSA-A; b, BSA-B2; c, BSA-C; d, BSA-E. Fractions from oxidized charcoal-defatted mercaptalbumin: e, BSA-A; f, BSA-B2; g, BSA-C; h, BSA-E. Migration was from the top (anode) toward the bottom (cathode) for approximately 50 min at 2.5 ma per tube.

B1, B2, and C monomer preparations remained unchanged after dialysis and freeze-drying.

The detection of unsaturated, readily peroxidizable long chain fatty acids in the gas-liquid chromatographic analysis of BSA raised the possibility of the involvement of lipid peroxides in the observed loss of thiol of the mercaptalbumin. It was therefore decided to examine the effect of adding a peroxidizing lipid to mercaptalbumin. To a small sample of defatted mercaptalbumin (A monomer, 0.95 sulfhydryl group per molecule) in distilled water at pH 5.0 was added 1 mole per mole of linoleic acid. Upon exposure to air linoleic acid readily peroxidizes to give a system of albumin and peroxidizing lipid. The solution was freeze-dried and allowed to stand in the cold room (2°) for 2 weeks. Concurrently, an identical solution of defatted A without added linoleic acid was freeze-dried and allowed to stand in the cold room. After standing for 2 weeks as freeze-dried powders, the sulfhydryl content of the control was 0.9 mole per mole of albumin whereas the sulfhydryl content of the mercaptalbumin-linoleic acid preparation had decreased to 0.76 mole per mole of albumin. The freeze-dried powders were allowed to stand in the cold room for another week and were then fractionated on DEAE-Sephadex A-50 (column size, 1.0 × 12.0 cm). A comparison of the elution profiles is shown in Fig. 3; assignment of the peaks was made by estimates of the buffer concentration at which the peaks were eluted and comparison with the elution profile of native BSA (Fig. 1).

Both preparations consisted mainly of mercaptalbumin and contained about the same proportion of Fraction E. However, the albumin-linoleic acid preparation contained a significant amount of Fractions B2 and C, whereas the control contained smaller amounts of B2 and trace amounts of Fraction C.

Disc gel electrophoresis revealed that very little dimer was formed upon the addition of linoleate. Fraction C, normally 50% monomer and 50% dimer, contained only a trace of dimer. The last eluted fraction (Fraction E) which normally consists mainly of dimers and higher polymers, contained about one-third dimer and two-thirds monomer. The relatively high proportion of monomer in the E fractions was due to the addition of the final buffer somewhat earlier than in the large scale fractionations, so that some late eluted monomer albumin was eluted with Fraction E.

Homogeneity of Mercaptalbumin Sulfhydryl Group—The ease of diminution of the sulfhydryl content of mercaptalbumin to about 0.8 mole per mole and the apparent stability of the re-

maining sulfhydryl groups after the sulfhydryl content had been reduced to about 0.6 mole per mole focused attention on the homogeneity of the albumin sulfhydryl group. Since DTNB reacts slowly with the albumin sulfhydryl group (plateau values are reached in about 30 min), unlike its almost instantaneous reaction with cysteine, it was decided to analyze the rate of reaction to determine whether or not sulfhydryl groups of differing reactivity were present in a mercaptalbumin solution. In the presence of excess DTNB the pseudo-first order rate constant was invariant with time. No heterogeneity in terms of sulfhydryl reactivity was thus observable by this technique.

Regenerability of Sulfhydryl Group of BSA-C Monomer—It had previously been shown that B1 non-mercaptalbumin monomer could be reduced under mild conditions (0.05 M thioglycolate, pH 5.5) to give a protein which contained one sulfhydryl group per mole protein, whereas only limited regeneration of the sulfhydryl group of the more retarded B2 non-mercaptalbumin monomer could be achieved by the same treatment (3). Since the regenerability of the C monomer had not been determined, a similar reduction was performed in duplicate on a preparation of Fraction C monomer. The reduced C monomer preparations contained 0.82 and 0.86 mole of sulfhydryl per mole of albumin.

Circular Dichroism and Fluorescence Studies

Preparation of Fractions—Since it was difficult to maintain the mercaptalbumin sulfhydryl group, especially after defatting, whole BSA was alkylated with iodoacetamide to give a sulfhydryl-free albumin preparation. Fractionation of the alkylated native and defatted BSA preparations on DEAE-Sephadex A-50 resulted in elution patterns which showed some retardation in the elution of the alkylated mercaptalbumin from the column; Fraction B1 appeared as a broad shoulder on the late eluted side of the Fraction A peak. The alkylated mercaptalbumin preparations to be used in the CD and fluorescence measurements were therefore obtained by pooling only the early eluted portions of the Fraction A peaks. The fractions eluted after B1 appeared identical with those obtained with native albumin, although rechromatography of the C fractions on Sephadex G-150 showed a somewhat lower percentage of monomer in the alkylated preparations. Since the B1, B2, and C monomers contained no reactive sulfhydryl group and appeared stable in solution, most measurements on these components were made on material obtained from native or defatted BSA. The four monomer fractions all migrated as single apparently identical bands on disc gel electrophoresis.

CD Spectra in pH Range of N-F Transition—The near- and far-ultraviolet CD spectra in the pH range of the N-F transition were determined for several monomer preparations: native A; iodoacetamide-treated A; iodoacetamide-treated, charcoal-defatted A; iodoacetamide-treated, charcoal-defatted A to which 5.2 moles of oleate per mole of protein had been bound; native C; and charcoal-defatted B1, B2, and C. The pH range of the measurements was from about 3 to 5.5 and all solutions were 0.1 M in KCl. More extensive, constant wave length measurements were performed at 209, 220, 262, and 269 nm on iodoacetamide-treated, charcoal-defatted A and charcoal-defatted B1 and B2. Representative spectra for A are shown in Fig. 4; these spectra are qualitatively the same as those obtained with the other monomer preparations.

In the far-ultraviolet (200 to 240 nm) region of the spectrum, BSA exhibits a fork-shaped negative ellipticity band with maxima at 209 and 220 nm which is characteristic of an α helix spectrum (the corresponding α helix maxima are the $n - \pi^-$ band

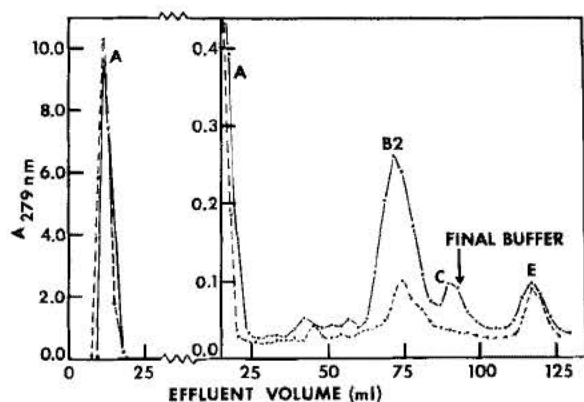


Fig. 3. The elution profiles of freeze-dried charcoal-defatted mercaptalbumin preparations with and without added linoleic acid after fractionation on DEAE Sephadex A-50. —, absorbance of eluate of BSA-A-linoleic acid at 279 nm; ---, absorbance of eluate of BSA-A at 279 nm.

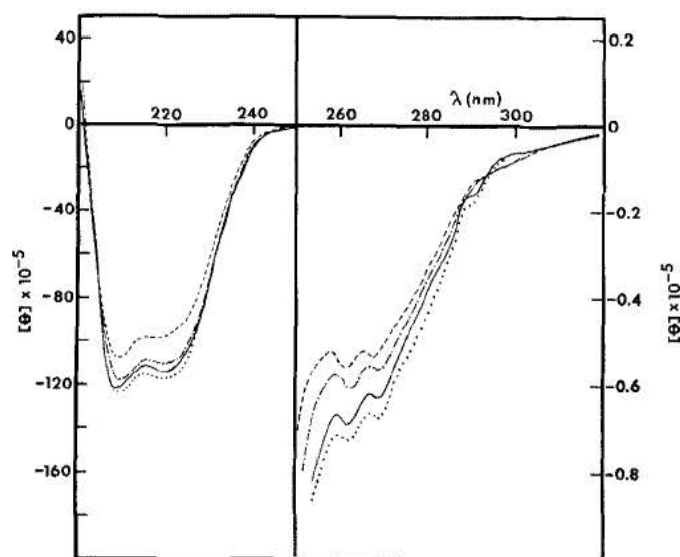


FIG. 4. CD spectra of native BSA-A in 0.1 M KCl at 25°. ---, pH 3.365; - · -, pH 3.81; —, pH 4.13; ···, pH 5.26. $[\theta]$ is the molar ellipticity in degrees·cm²·decimole⁻¹.

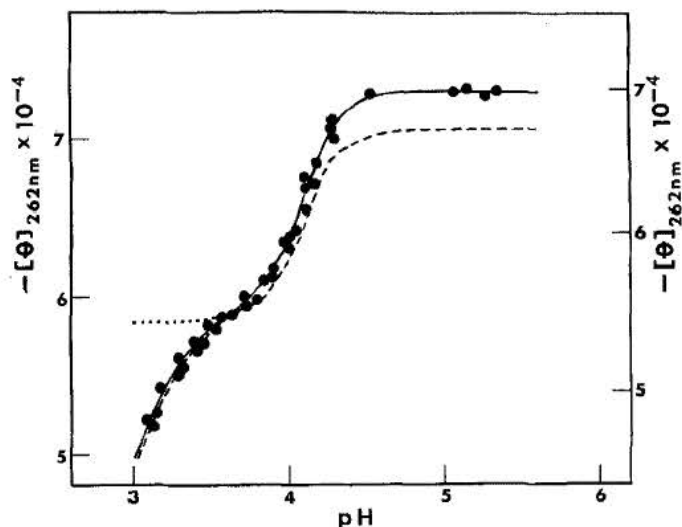


FIG. 5. pH ellipticity profiles of three defatted monomer albumin fractions at 262 nm, 0.1 M KCl, 25°. Left-hand ordinate; ellipticities of BSA-A (45 data points) (—) and BSA-B2 (30 data points) (---). Right-hand ordinate, ellipticities of BSA-B1 (●). ···, estimated ellipticities of the F forms of the defatted albumin monomers.

at 222 nm and the parallel $\pi - \pi^-$ band at 206 nm (28)). For proteins containing no prosthetic group, the optical activity in the near-ultraviolet (240 to 350 nm) region of the spectrum results only from transitions of the aromatic residues or cystine. Within this spectral region, BSA exhibits a broad shoulder of negative ellipticity with maxima at 262 and 269 nm. At pH values greater than 4 there is also a very weak negative ellipticity band centered about 290 nm. The wavelengths of the ellipticity maxima were invariant to albumin fraction and pH in the pH range of the N-F transition.

Since other studies have indicated that one or both of the maxima at 262 and 269 nm are reflections of disulfide transitions (see "Discussion") and since disulfide heterogeneity has been proposed as an explanation of whole albumin heterogeneity (11), particular attention was paid to the pH-induced ellipticity changes at these two maxima. The molar ellipticities of de-

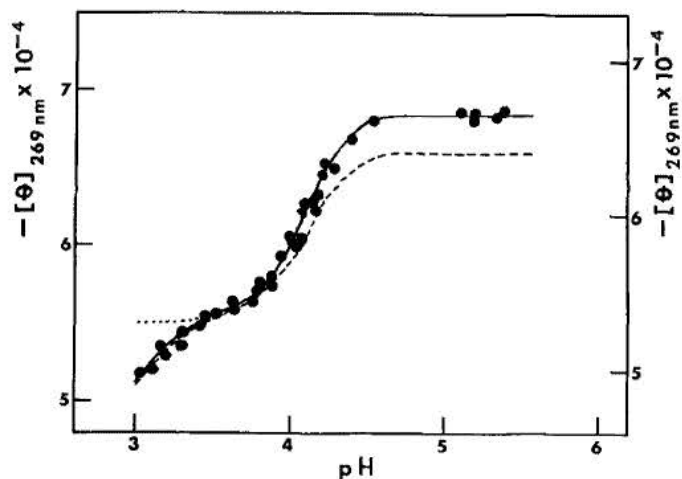


FIG. 6. pH ellipticity profiles of three defatted monomer albumin fractions at 269 nm, 0.1 M KCl, 25°. Same symbols as in Fig. 5.

fatted B1, B2, and iodoacetamide-treated defatted A monomers at 262 and 269 nm as a function of pH are shown in Figs. 5 and 6. These data were obtained by constant wave length measurements on at least three different samples of each monomer. The samples gave identical data within the limits of accuracy of the measurements ($\pm 1\%$). Defatted C monomer gave data which were indistinguishable from those obtained with defatted A.

The curves are reflections of two different types of structural change: the sigmoidal portion of the curves above pH 3.5 is related to the N-F transition while the more linear decrease in ellipticity below pH 3.5 is associated with the over-all acid expansion of the molecule. Since there is some overlap between these two phenomena, the molar ellipticities of the F form of the monomers were obtained by extrapolation of the sigmoidal part of the curve to lower pH values.

Some parameters of the various monomer preparations are given in Table V. $\Delta \text{pH}_{10}^{90}$ is the difference in pH between the ellipticity values representing 90% of $[\theta]_N - [\theta]_F$ and 10% of $[\theta]_N - [\theta]_F$ and is a measure of the breadth of the transition. For a given monomer, the pH values of the 262 and 269 nm transition midpoints were essentially the same, implying that both bands reflect the same configurational change. Within the accuracy of the measurements ($\pm 2\%$), the native and iodoacetamide-treated mercaptalbumin preparations appeared identical. This is in agreement with the findings of Moore and Foster (29), who observed no detectable changes in the optical rotatory dispersion and solubility behavior of native BSA upon alkylation of the sulfhydryl group with iodoacetamide and therefore concluded that the structural integrity of the native protein was unaltered after this chemical modification. This identity validated the use of iodoacetamide-treated, defatted A in the more extensive studies which were performed on the defatted A, B1, and B2 monomers.

The differences in the shape of the transition curves of the various A preparations (except the one containing over 5 moles of oleic acid per mole of protein) and the defatted B1 and C monomers, indicated by differences in $\Delta \text{pH}_{10}^{90}$, were probably within the experimental accuracy of the measurements. The transition curves were adjusted to the same ellipticity magnitude for the N form of the protein and the curves shifted so that the transition midpoint pH values coincided. A best fit curve was drawn through all the data points. The calculated standard deviation of all the data points was 0.0025 and the standard de-

TABLE V
Parameters of N-F transition of BSA monomers as determined by CD measurements at 202, 269, and 209 nm

Monomer	$\lambda = 262 \text{ nm}$				$\lambda = 269 \text{ nm}$				$\lambda = 209 \text{ nm}$			
	$[\theta]_{262} \times 10^{-4}$		Midpoint pH	$\Delta \text{pH}_{10}^{90}$	$[\theta]_{269} \times 10^{-4}$		Midpoint pH	$\Delta \text{pH}_{10}^{90}$	$[\theta]_{209} \times 10^{-6}$		Midpoint pH	$\Delta \text{pH}_{10}^{90}$
	pH 5	pH 3.5			pH 5	pH 3.5			pH 5	pH 3.5		
A native and iodoacetamide-treated.....	7.3	5.8	3.94	0.65	6.7	5.4	3.95	0.68				
A defatted, iodoacetamide-treated.....	7.30	5.84	4.07	0.64	6.85	5.54	4.07	0.67	12.0	10.7	3.95	0.51
B1 defatted.....	7.00	5.54	4.07	0.64	6.65	5.34	4.07	0.67	12.0	10.7	3.95	0.51
B2 defatted.....	7.04	5.82	4.10	0.64	6.60	5.52	4.11	0.66	12.0	10.7	3.95	0.51
C defatted.....	7.3	5.8	4.08	0.61	6.8	5.5	4.08	0.65				

viations of the individual data sets were in the same range (0.0022 to 0.0028). The molar ellipticity differences observed between the defatted B1 and the defatted A monomers are therefore not reflections of any structural change which affects the interactions involved in the N-F transition. They are probably due solely to the conversion of the cysteinyl residue to an asymmetrical disulfide which then contributes to the total ellipticity of the molecule.

However, the ellipticity differences observed with the defatted B2 monomer (lower molar ellipticity of the N form of the molecule; higher transition midpoint pH; and lower magnitude of $[\theta]_N - [\theta]_F$) would indicate that some structural change had occurred which affected the interactions involved in the N-F transition and which destabilized the interacting or N form of the molecule.

Although the native and iodoacetamide-treated mercaptalbumin preparations (0.2 mole of fatty acid per mole of protein) gave pH ellipticity profiles which were indistinguishable in shape from that of the defatted, iodoacetamide-treated mercaptalbumin, the midpoint of the transition was at a lower pH than that of the defatted protein, further evidence of the stabilizing effect of fatty acid.

At higher fatty acid concentrations, native C monomer (1 mole of fatty acid per mole of protein) and alkylated, defatted mercaptalbumin to which over 5 moles of oleate per mole had been bound, the N-F transitions were much broader than those of the defatted proteins with an almost complete loss of the sigmoidal characteristics of the curves. This increased breadth of the pH ellipticity profiles is consistent with the observed increase in electrophoretic heterogeneity upon addition of fatty acid (30) and the observed broadening of the pH solubility curves upon addition of fatty acid (7). While the oleate-A monomer preparation showed a marked stabilization of the N form of the molecule, no such stabilization was observed with native C monomer. Furthermore, although the ellipticity of the N form of the oleate-A monomer preparation decreased to about $-7.0 \times 10^4 \text{ deg cm}^2 \text{ per decimole}$, no such decrease was observed with native C monomer.

Plots of the ellipticity of the defatted, iodoacetamide-treated A and the defatted B1 and B2 monomers at the two far-ultraviolet CD maxima as a function of pH, through the region of the N-F transition, are shown in Fig. 7. Within the limits of accuracy of the measurements ($\pm 2\%$) all three monomers displayed identical pH ellipticity profiles. The 209- and 220-nm profiles differed significantly in shape. The curves at 209 nm appeared sigmoidal in character above pH 3.4 although there was a considerable overlap of the sigmoidal part of the curve

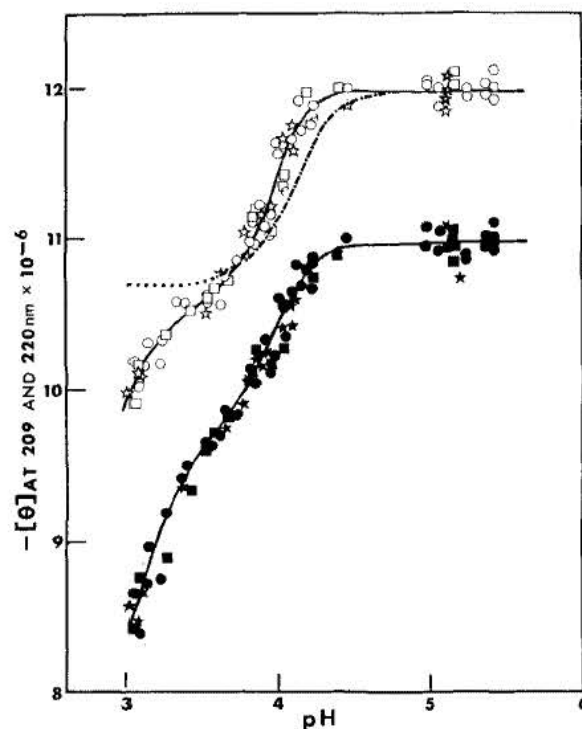


FIG. 7. pH-ellipticity profiles of three defatted monomer albumin fractions at 209 and 222 nm, 0.1 M KCl, 25°. Ellipticities at 209 nm: BSA-A (○), BSA-B1 (□), and BSA-B2 (☆). Ellipticities at 222 nm: BSA-A (●), BSA-B1 (■), and BSA-B2 (★)., estimated ellipticity of the F form of the defatted albumin monomers; - · -, pH ellipticity profile of defatted BSA-A at 262 nm. The pH 5 ellipticity value at 262 nm has been set at that of $[\theta]_{209 \text{ nm}}$ and the other data points normalized to the $[\theta]_N - [\theta]_F$ value at 209 nm.

with the lower pH, acid-expansion region. An estimate of the ellipticity of the F form of the molecules was made by inverting the higher pH portion of the curve and best fitting it to the pH region between 4.1 and 3.7. While there was some uncertainty as to the correct value for $[\theta]_F$, the data obtained with upper and lower limit values of -10.8 and $-10.6 \times 10^6 \text{ deg cm}^2 \text{ per decimole}$ did not differ greatly, giving midpoint pH values of 3.97 and 3.93 and $\Delta \text{pH}_{10}^{90}$ values of 0.46 and 0.56, respectively. Data obtained with a mean value of $-10.7 \times 10^6 \text{ deg cm}^2 \text{ per decimole}$ are listed in Table V.

The pH ellipticity profile at 220 nm was almost linear below pH 4; the slight curvature that it displayed was qualitatively the same as those of the other pH ellipticity profiles. No at-

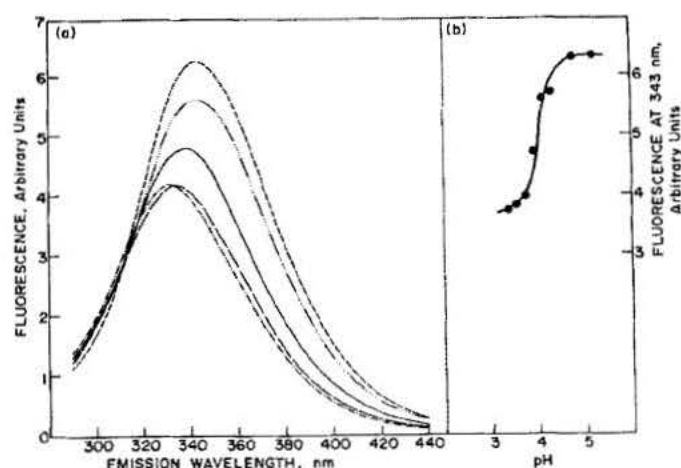


FIG. 8. Fluorescence of mercaptalbumin in 0.1 M KCl at 25°. *a*, fluorescence emission spectra of iodoacetamide-treated, defatted mercaptalbumin. ---, pH 5.30 and pH 4.80; - - - - -, pH 4.14; —, pH 3.95; — — —, pH 3.80; — — — —, pH 3.40 and pH 3.56. $\lambda_{\text{excitation}} = 280$ nm; protein concentration = 0.05 mg per ml. *b*, fluorescence of iodoacetamide-treated, defatted mercaptalbumin at 343 nm as a function of pH. $\lambda_{\text{excitation}} = 280$ nm.

tempt was made to obtain N-F transition data from this profile. The dashed line in Fig. 7 represents the pH ellipticity profile of defatted, iodoacetamide-treated mercaptalbumin at 262 nm. For ease of comparison with the 209-nm data, the 262-nm data have been normalized to the $[\theta]_N - [\theta]_F$ value at 209 nm, 1.3×10^6 deg cm² per decimole, and given the same ellipticity value as the 209-nm data above pH 5.

The intensity of the CD band at 290 nm was so weak that the ellipticity data at this wave length were very inaccurate. However, from the data that could be obtained, it appeared that the pH ellipticity profile was sigmoidal in shape and that the transition midpoint was approximately pH 4.2. The inaccuracy of the data did not permit the detection of any possible differences between the various albumin monomers.

Fluorescence Studies—Fluorescence spectra were obtained for several monomer preparations through the pH range of the N-F transition, with an exciting wave length of 280 nm. The monomer preparations examined were: native A; iodoacetamide-treated A; defatted, iodoacetamide-treated A; defatted B2 and C monomers. More limited studies were conducted on native B2 and C monomers and on defatted B1. Representative fluorescence spectra are shown in Fig. 8.

In general, decreasing the pH from approximately pH 5 to 3.5 resulted in a decrease in the fluorescence intensities and a blue shift in the wavelength of maximum fluorescence, in agreement with previously reported data (31, 32). The wave length of maximum fluorescence occurred at about 343 nm at pH 5 and about 332 nm at pH 3.5. Although the magnitude of the blue shift was somewhat greater than that reported by Spector and John (32) for whole albumin, this disagreement is probably due to the fact that these authors made no measurements below pH 4.

A plot of the fluorescence of charcoal-defatted, iodoacetamide-treated mercaptalbumin at 343 nm as a function of pH is also shown in Fig. 8. Qualitatively similar curves were obtained with the other defatted monomer preparations. It was very difficult to maintain the pH of these very dilute protein solutions in 0.1 M KCl; consequently there was considerable error in the measurements in the pH range of the N-F transition and the transition midpoint pH values could not be determined with

TABLE VI

Fluorescence of N (pH 5; $\lambda_{\text{max}} = 343$ nm) and F (pH 3.5; $\lambda_{\text{max}} = 332$ nm) forms of BSA monomers at emission maxima

Fluorescence (arbitrary units ± 0.1)	Monomer						
	A		B1	B2		C	
	Native	Defatted	Defatted	Native	Defatted	Native	Defatted
<i>nm</i>							
343 (N)	5.9	6.3	5.9	4.8	4.8	4.9	4.8
332 (F)	4.1	4.2		3.8	3.7	3.7	3.8

any great accuracy. All defatted monomers gave midpoint values of 4.1 ± 0.1 .

The fluorescence intensities of the various monomers at pH 5 (343 nm) and pH 3.5 (332 nm) are given in Table VI. The most striking difference between the monomers was the relatively weak fluorescence of the B2 and C monomers at pH 5 as compared with that of mercaptalbumin. Furthermore, while native and iodoacetamide-treated A monomers (~ 0.2 mole per mole of protein prior to defatting) gave identical fluorescence intensities, a small but real increase in intensity was observed at pH 5 upon removal of the fatty acid: no such increase in fluorescence intensity was observed upon defatting the B2 and C monomers (~ 1 mole per mole of protein prior to defatting).

Since tryptophan is readily oxidized, it was thought that the lower fluorescence intensities of the B2 and C monomers might be due to a partial destruction of the tryptophanyl residues. Tryptophan analyses were conducted on a standard tryptophan solution, on whole BSA and on A and B2 monomer fractions with the method of Spies and Chambers (33). Protein concentrations were determined by the microbiuret method. All three protein preparations gave identical spectra in the assay and gave tryptophan to albumin molar ratios of 2:1 ($\pm 3\%$).

In a further attempt to find an explanation for the lower fluorescence intensities of the B2 and C monomers, a series of experiments on the quenching of the fluorescence by iodide was undertaken (16, 34, 35). In the first set of experiments, the ionic strength was maintained at 0.4 by the addition of appropriate amounts of a standard NaCl solution and the pH was maintained at 5.5 ± 0.2 . The quenching of tryptophan fluorescence of defatted, iodoacetamide-treated mercaptalbumin and defatted B2 monomer under these conditions is shown in Figs. 9 and 10. Since both data plots are nonlinear, it is apparent that the simple Stern-Volmer relationship (Equation 2) does not hold. While the curvature of the plots could be due to the presence of tryptophans of greatly differing K_q values, the well documented binding of iodide by albumin (36) suggested that this was a more likely explanation of the data and that static quenching (Equation 1) was involved, especially at the high iodide, low chloride concentrations. To test this hypothesis some experiments were conducted at pH 5.5 in which iodide, chloride, phosphate (which is bound relatively weakly by albumin), and total ionic strength were all variables. The greatly increased quenching by low iodide concentrations in the presence of low chloride concentrations or phosphate showed that iodide binding was contributing to the fluorescence quenching. At high chloride concentrations, little or no variation was observed in the amount of quenching produced by the presence of a given low iodide concentration.

To overcome the static quenching by iodide a series of experiments were conducted at an ionic strength of 2.0, the ionic strength being maintained by the addition of NaCl. The

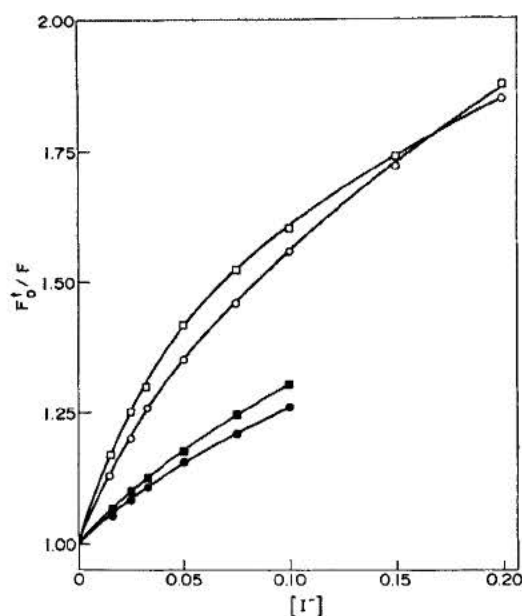


FIG. 9. Stern-Volmer plot of the quenching of monomer albumin fluorescence by iodide. Iodoacetamide-treated, defatted mercaptalbumin, pH 5.5, ionic strength = 0.4 (○) and ionic strength = 2.0 (●); defatted B2 monomer, pH 5.5, ionic strength = 0.4 (□) and ionic strength = 2.0 (■). Albumin concentration = 0.15 mg per ml; $\lambda_{\text{excitation}} = 295 \text{ nm}$; $\lambda_{\text{emission}} = 343 \text{ nm}$, 25°.

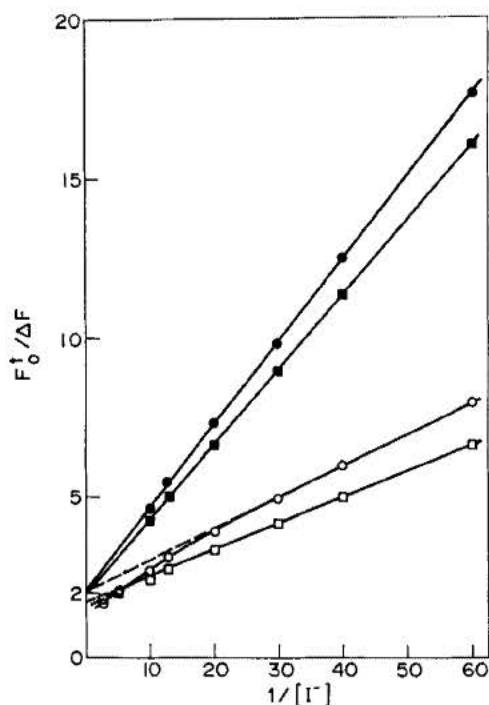


FIG. 10. Modified Stern-Volmer plot of the quenching of monomer albumin fluorescence by iodide. Same symbols and data as in Fig. 9.

iodide concentration was never greater than 0.1 M. Under these conditions, iodide binding should be negligible. The data obtained in these experiments are plotted in Figs. 9 and 10. While the F'_0/F versus $[I^-]$ plots show some curvature, the $F'_0/\Delta F$ versus $1/[I^-]$ plots are linear and extrapolate to a value close to 2 at $1/[I^-] = 0$. When the data were standardized to a concentration of 10^{-6} M , it was observed that the ΔF values of the mercaptalbumin and the B2 monomer were identical within experi-

TABLE VII
BSA monomer fluorescence parameters as determined by iodide quenching of tryptophan fluorescence

Ionic strength	Monomer (defatted)	F'_0	F'_0	F''_0	K'_Q
		arbitrary units			M^{-1}
0.4	A	155 ± 3	74 ± 5	81	21 ± 2
	B2	129 ± 3	73 ± 5	46	21 ± 2
2.0	A	155 ± 2	74 ± 2	81	8.1 ± 0.2
	B2	143 ± 2	74 ± 2	69	8.2 ± 0.2

mental error (± 3 arbitrary units). The data were analyzed by the use of Equation 4 since it appeared that one of the tryptophanyl residues was unavailable to iodide. Using adjacent pairs in the calculations, no progressive change in the values of F'_0 and K'_Q could be detected as the iodide concentration increased. The values were the same as those obtained by graphical extrapolation of the data points in Fig. 10. The data are summarized in Table VII. Also included in this table are data obtained from the three lowest iodide concentrations at an ionic strength of 0.4. As can be seen from Fig. 10 the data plots are linear at these iodide concentrations.

From the data in Table VII it can be seen that the quenchable tryptophanyl residue exhibits the same fluorescence behavior in both monomers but that the fluorescence of the unquenched tryptophanyl residue is greatly decreased in the case of the B2 monomer and that this residue is entirely responsible for the difference in the total fluorescence of the two monomers.

In the course of these experiments it was observed that the ratio $F'_0(\text{B2}):F'_0(\text{A})$ increased as the ionic strength increased. In 0.1 M NaCl the ratio was 0.75, in 0.4 M NaCl it was 0.84, and in 2 M NaCl it was slightly over 0.9. Since the total fluorescence intensity of the mercaptalbumin and the fluorescence intensities of the quenchable tryptophanyl residues of both A and B2 monomers showed no marked increase as the ionic strength was increased from 0.1 to 2.0, the variation in the ratios of the unquenched fluorescence as a function of ionic strength was due to an ionic strength-dependent increase in the fluorescence of the unavailable B2 tryptophanyl residue.

In the presence of 6 M urea, 0.4 M NaCl, pH 5.5, the F'_0/F versus $[I^-]$ and $F'_0/\Delta F$ versus $1/[I^-]$ plots were linear and extrapolated to an ordinate value of 1. The K_Q calculated from the reciprocal plot was 3.6 M^{-1} .

A few experiments were conducted with an exciting wave length of 280 or 305 nm. The data obtained on exciting with 305-nm light gave F'_0/F versus $[I^-]$ and $F'_0/\Delta F$ versus $1/[I^-]$ plots which were indistinguishable from those obtained with an exciting wave length of 295 nm. The data obtained on excitation with 280-nm light resulted in a reciprocal plot in which small but real differences in slope and intercept values were obtained. The near-identity of the 280- and 295-nm data provides further evidence for the very minor role of tyrosyl residues in the fluorescence of albumin at pH 5.

A few experiments were also conducted on defatted B1 monomer and defatted HSA. The B1 monomer gave fluorescence intensities in both 0.4 and 2 M NaCl that were about 95% that of the mercaptalbumin. Once again, it appeared that the slightly lower value was due to a decrease in the fluorescence intensity of the unavailable tryptophanyl residue. The F'_0 and K'_Q values were indistinguishable from those of mercaptalbumin. The lone tryptophanyl residue of HSA was quenched in a manner

very similar to that of the quenchable tryptophanyl residue of the bovine protein. The fluorescence intensity of the mercaptalbumin at $\lambda_{\max}$ was however 2.6 to 2.7 times that of the HSA at its slightly lower $\lambda_{\max}$ of 338 nm in agreement with the findings of Steinhardt *et al.* (37).

DISCUSSION

Fatty Acid Heterogeneity

Although, on the basis of their observations upon addition of oleic acid to mercaptalbumin, Petersen and Sober (10) suggested that complexes of albumin with fatty acids and other small molecules may account for observed chromatographic heterogeneity, the results presented here have clearly indicated that the heterogeneity exhibited in the elution patterns of native BSA on DEAE-Sephadex A-50 is not due to fatty acid heterogeneity. Not only were the same elution peaks obtained upon fractionation of both the native and corresponding defatted proteins, but the sulfhydryl and dimer contents of the fresh defatted albumin fractions remained essentially the same as those of the corresponding fractions of native protein. The discrepancy between the results of Petersen and Sober and those of the present communication may be due to the presence of much lower fatty acid concentrations (less than 2 moles per mole) in the present studies.

Although all the freeze-dried albumin fractions exhibited lower sulfhydryl contents and higher dimer contents than the corresponding freshly eluted fractions, the sulfhydryl content of the freeze-dried defatted mercaptalbumin was even lower than that of the corresponding freeze-dried native mercaptalbumin. Water-dialyzed, freeze-dried native A contained about 0.80 sulfhydryl group per molecule, whereas water-dialyzed, freeze-dried, charcoal-defatted A had about 0.7 sulfhydryl group per molecule. These results are in agreement with other reported examples of an increased lability of defatted albumin with respect to heat denaturation (38), urea denaturation (39, 40), the population broadening correlated with microheterogeneity (8, 41) and the postulated disulfide interchange reaction (11).

Interpretation of the fatty acid contents (Table III) of the various BSA fractions in terms of differing binding constants is not justified due to the relatively large amount of fatty acid that was bound by the positively charged Sephadex resin. The data obtained by analysis of whole albumin before and after passage down the resin and by calculation of the average fatty acid content of the summed fractions (moles per mole of whole albumin) show that about 25% of the fatty acid was removed from the albumin by the resin. If the resin-fatty acid interaction is mainly electrostatic in origin (unlike the albumin-fatty acid interaction which contains very large hydrophobic contributions), the resin should compete with the albumin most effectively at low ionic strengths. Hence the low ionic strength, first eluted fraction, the mercaptalbumin, should have a lower fatty acid content than the higher ionic strength, later eluted fractions. Indeed at high ionic strengths, some of the previously bound fatty acid may be displaced from the resin and bound by the later eluted fractions thus increasing their fatty acid contents above their original values. While there may be differences in the fatty acid content of the various species of albumin it is impossible to disentangle them from the large resin-fatty acid effects.

While the varying fatty acid content of the fractions can in large part be explained in terms of resin-fatty acid binding, it is difficult to explain the differences in fatty acid distribution in these terms. Spector *et al.* (27) have shown that the binding

constants of palmitate (16:0), oleate (18:1), linoleate (18:2), and stearate (18:0) for whole BSA primary binding sites at pH 7.4 are 6.8, 4.0, 3.0, and $1.2 \times 10^6 \text{ M}^{-1}$, respectively. No observable correlation exists between these association constants and the observed differences in the distribution of these fatty acids in the three BSA fractions.

Role of Unsaturated Fatty Acids in Formation of Non-mercaptalbumin Monomers

Since the Fraction B2 and C non-mercaptalbumin monomers could be isolated from freshly prepared freeze-dried mercaptalbumin samples in the absence of small molecule thiols, some loss of the cysteinyl thiol group by a pathway other than mixed disulfide formation must be operative. Enhancement of the formation of the Fraction B2 and C monomers upon addition of equimolar amounts of peroxidizing lipid suggested that the mercaptalbumin thiol group is oxidized to form oxidation products higher than disulfide, such as sulfinic, sulfinic, and sulfonic acids. However, the oxidation state of the sulfhydryl group of BSA after oxidation by freeze-drying or treatment with peroxidizing lipid has not been determined. It should also be noted that although the formation of both the B2 and C non-mercaptalbumin monomers was enhanced in the presence of lipid peroxide, the regenerability of the sulfhydryl group is very different in the two monomer fractions. Whether or not the regenerated sulfhydryl group of the C monomer is the one normally present in mercaptalbumin has not yet been determined.

Thiol oxidation by lipid peroxides is well documented. Roubal and Tappel (42) have reported that the presence of a large excess of peroxidizing lipid resulted in extensive oxidation of most of the amino acid residues in BSA. However, Little and O'Brien (43) have demonstrated that low concentrations of lipid peroxide (4:1 molar ratio of thiol to peroxide) resulted in restriction of oxidation to the cysteinyl residues. Relative to the rate of oxidation of small molecule thiols, BSA had a low efficiency of thiol oxidation by linoleic acid hydroperoxide and a low rate of reaction.

It therefore appears that the binding of a long chain fatty acid to bovine albumin can have two opposite effects, depending on the degree of unsaturation of the fatty acid and on the location of the binding site. Binding of a long chain unsaturated (*i.e.* readily peroxidizable) fatty acid close to the reactive sulfhydryl group can result in the oxidation of the sulfhydryl group and, hence, the formation of non-mercaptalbumin monomer. Alternatively, binding of a long chain unsaturated fatty acid at a site far from the reactive sulfhydryl group or binding of a long chain saturated fatty acid results in stabilization of the mercaptalbumin sulfhydryl group.

Assignment of CD Maxima to Protein Residues and Secondary Structures

200 to 250 nm—Although the far-ultraviolet CD spectrum of bovine albumin is characteristic of an α helix spectrum, there is an inversion in the magnitude of the two maxima with respect to those of helical polypeptides. Such an inversion has been noted in many cystine-containing proteins (44, 45) and may be due to overlap of the peptide transitions with those disulfide transitions which can give rise to strong bands at wave lengths just below 200 nm. In addition, tyrosine, tryptophan, phenylalanine, and cystine all show intense pH-independent optical activity near 220 nm, whether free or as amides or esters (45-48). In particular, Coleman and Blout (49) have noted that the presence of a disulfide bond resulted in a red shift in the wave length

of the $n - \pi^-$ peptide Cotton effect as well as a change in the sign and magnitude of the transition. Since BSA contains 2 tryptophanyl residues, 19 tyrosyl residues, and 17 cystinyl residues (50), the $n - \pi^-$ amide transition probably overlaps with the far-ultraviolet residue contributions. Furthermore, since BSA may contain β or other structures in addition to α helical and random coil configurations, the deviation of the far-ultraviolet spectrum from that of α helical polypeptides may be attributed in part to contributions from these other configurations.

The difference in the shapes of the pH ellipticity profiles at 209 and 220 nm affords further proof of the involvement of various residues and perhaps backbone structures other than the α helix in the observed ellipticities. The residue contributions should be greater at 220 nm and may differ in sign and intensity; their presence may thus explain why the ellipticity profiles at this wave length do not exhibit the sigmoidal curve characteristic of the N-F transition. In contrast, the pH ellipticity profiles at 209 nm are sigmoidal with a sharp N-F transition centered about pH 3.95; this may be directly correlated with the abrupt transition in $[\alpha]_{222\text{ nm}}^{22^\circ}$ centered about pH 3.95 which was assumed by Sogami and Foster (41) to indicate the occurrence of the N-F transition.

250 to 275 nm—In a simple aliphatic disulfide, the disulfide dihedral angle is approximately 90° and the barrier to rotation about the disulfide bond has been estimated as 5 to 15 Cal per mole (51–53). Hence any disulfide can exist as either a right- or left-handed screw. While simple aliphatic disulfides exhibit little or no optical activity due to cancellation of the contributions of the two isomeric forms, one isomer may be energetically favored and hence predominate in more complex disulfides. Nearby asymmetrical centers can influence the magnitude of the optical activity of a given disulfide bond, but the sign of the rotation of the ellipticity is determined by the chirality (54). Beychok (45) has suggested that the disulfide bonds of a native protein are not free to rotate. Any particular disulfide bond has thus the same conformation, hence the same chirality and dihedral angle, over the entire population of molecules. The resultant optical activity of the disulfide transitions thus depends on the particular distribution of disulfide conformations in the protein. Recent crystallographic studies on lysozyme (55) and other proteins have shown the disulfide bonds to be rigidly oriented in the native protein.

Studies on L-cystine derivatives and small L-cystine-containing cyclic peptides (49, 56) have shown two transitions directly associated with the disulfide bond; a transition of great rotational strength at about 200 nm and a weaker transition at about 260 to 270 nm. Based on a consideration of rotational strengths (49, 57) and analysis of the corresponding absorption bands (53), the 200-nm disulfide transition appears characteristic of an inherently asymmetrical chromophore and is directly related to the geometry of the disulfide bond (*i.e.* disulfide dihedral angle). Similar considerations indicate that the optical activity of the higher wave length disulfide transition arises through asymmetrical environmental perturbations, resulting from variations in the dihedral angle as well as from other groups in the molecule. Bergson *et al.* (58) have shown that the energy of this disulfide transition is a function of the dihedral angle, so that the *wavelength* of the associated CD band appears to be a better indicator of changes in disulfide bond configuration than is the *rotational strength* of the 200-nm transition. A red shift in the position of the disulfide transition from its normal position near 250 nm has been observed in model compounds when the dihedral angle has been altered from its normal value of approxi-

mately 90° , and such an explanation has been invoked to account for the appearance of disulfide ellipticity bands in cystine derivatives and some proteins at wave lengths greater than 250 nm (59).

Further evidence on the nature of the residues responsible for the 262- and 269-nm ellipticity maxima of BSA comes from the experiments of Velluz and Legrand (44) and Legrand and Viennet (60) on the reduction of human serum albumin, a protein whose CD spectrum and general physical properties are almost identical with those of BSA. These authors found that the addition of mercaptoethanol to human serum albumin had virtually no effect on the CD spectrum: this is not unexpected since it is known that the disulfide bonds of BSA and HSA are not available for reduction by small molecule thiols at neutral pH values (24). Addition of 0.2 M dodecyl sulfate resulted in the reduction of both the near- and far-ultraviolet ellipticity bands by about 20%. Addition of dodecyl sulfate and mercaptoethanol resulted in an almost complete disappearance of the dichroism in the 250- to 300-nm region of the spectrum without reducing the magnitude of the far-ultraviolet bands below that observed in the presence of dodecyl sulfate alone. While not conclusive, these data strongly suggest that the 262- and 269-nm ellipticity maxima are due to disulfide transitions.

The near-ultraviolet data at 262 and 269 nm should thus primarily reflect the resultant optical activity due to environmental perturbations of the 17 disulfides in albumin with perhaps some unresolved tyrosine contributions. It could be argued that one of the two maxima (preferably the one at 269 nm, although even this wavelength is low for a tyrosyl band) is mainly due to the resultant optical activity of some rigidly oriented tyrosyl residues. However, in view of the great similarity of the N-F transition data at 262 and 269 nm, such an assignment would imply a high degree of parallelism in the pH-dependent perturbations of some tyrosyl and cystinyl residues. Rather than make such an implication in the absence of other confirmatory data we have, *pro tem*, assigned both bands to cystine. The ascription of one maximum to tyrosine would not invalidate any of the conclusions which have been derived from the CD data.

275 to 300 nm—On the basis of studies on amino acids and certain proteins (45, 47), the small long wave length ellipticity band at 290 nm has been assigned to tryptophan. Although no discrete bands which could be attributed to tyrosine were observed in the 270- to 290-nm region of the spectrum, the broad shoulder of negative ellipticity in this region could contain unresolved tyrosine contributions.

CD-Structure Correlations

While side chain optical activity contributes to the ellipticities observed at 209 and 220 nm, the large magnitudes of the ellipticities and the general shape of the spectra are evidence that these transitions are mainly helical in origin. From optical rotatory dispersion studies, Sogami and Foster (41) have estimated the helical content of the N form of BSA to be about 54% and that of the F form to be about 47%; similar results were obtained by Bryan and Nielsen (61) from hydrogen-deuterium exchange studies. The lowering of $[\theta]_{209}$ by about 10% on going from pH 5 to pH 3.5 agrees well with the helix-content data. The 20% decrease in $[\theta]$ at 262 and 269 nm on going from pH 5 to 3.5 suggests a greater decrease in rigidly oriented cystinyl and perhaps tyrosyl residues. The invariance in the wave lengths of the ellipticity maxima through the pH range of the N-F transition is evidence that this isomerization does not involve the formation of new, low pH, rigid structures in which the environments

and hence dihedral angles of the cystinyl residues are altered. The N-F transition ellipticity data can best be interpreted as a change in the over-all structure of the molecule without any great perturbation of the local environments of most of the cystinyl residues and without much loss of helix content. The ellipticity data are thus very compatible with Foster's crevice model for albumin (62).

All defatted monomers exhibited a broader N-F transition at 262 and 269 nm than at 209 nm. Furthermore, the N-F transition midpoint pH values were considerably higher at 262 and 269 nm than at 209 nm. Although it was not possible to determine a midpoint pH for the transition at 222 nm, it was observed that the pH values at which the 209- and 222-nm ellipticities began to decrease from their N form values were the same and were considerably lower than the corresponding pH values at 262 and 269 nm even when the latter values were corrected for differing transition breadth. It would therefore appear that some local disorientation of some cystinyl and perhaps tyrosyl residues precedes the generalized opening up of the crevice as evidenced by the decreased α helix ellipticities. In disagreement with this hypothesis, other studies (7, 63, 64) have suggested that the N-F transition is an all or none process. However, a two-step process has been observed for the N-F isomerization in the presence of SCN^- and ClO_4^- (65). If a much less pronounced two-step process were operative in the presence of 0.1 M KCl, the CD data could be explained satisfactorily, provided the first step did not involve the helical portions of the molecule.

The breadth of the N-F transition profile has been taken as a measure of the heterogeneity of the albumin preparation. The extreme sharpness of the 3.0 M KCl pH solubility profile of charcoal-defatted mercaptalbumin (midpoint pH = 4.2 to 4.3, $\Delta\text{pH}_{10}^{90} = 0.11$ (7, 66)) as compared with the much broader profile of native whole BSA provides a striking example of such relationships. However, although the high ionic strength pH solubility data and classical solubility studies in ammonium sulfate (66) at pH 5.32 and high ionic strength have shown the defatted mercaptalbumin to be homogeneous, reversible boundary spreading (66) and isoelectric focusing measurements (67) at low ionic strengths have shown the protein to be electrically heterogeneous. This microheterogeneity may in part be responsible for the broadness of the CD N-F transition profiles. It is difficult to correlate the N-F transition solubility data with the CD data in view of the differing salt concentrations (a situation of even greater importance in the case of an anion-binding protein like albumin) and of the fact that small structural changes which are clearly reflected in one type of measurement may be of negligible importance in another type of measurement and hence not reflected in the resultant data. The difficulty in relating transition breadth to heterogeneity is highlighted by the CD data in the far- and near-ultraviolet regions of the spectrum: the breadths of the N-F transitions at 262 and 269 nm are considerably greater than that at 209 nm.

The identity of the pH ellipticity profiles of the various defatted monomers at 209 nm and the near-identity of the profiles at 262 and 269 nm comprise compelling evidence for an almost complete identity of structure of the monomers. The only ellipticity profiles which displayed differences that could be ascribed to structural differences were the near-ultraviolet profiles of B2 monomer at 262 and 269 nm. The lower transition magnitude and higher midpoint pH of these profiles indicate some dislocation of cystinyl and perhaps tyrosyl residues in the crevice of the N form of the molecule with a resultant destabilization of the N form of the albumin. Due to lack of material, more

detailed, constant wave length measurements were not conducted on C monomer preparations and some small but real differences could exist between this monomer and mercaptalbumin.

The effect of fatty acid on the N-F transition (stabilization of the N form of the molecules, broadening of the transition) agrees well with other observations (8, 11, 38-41) on the stabilizing effect of fatty acid and affords further proof that at least one of the fatty acid primary binding sites is in or very near the crevice of the N form of the molecule.

Fluorescence-Structure Correlations

The fluorescence emission spectra of human and bovine serum albumin at neutral pH values and those of many other tryptophan-containing proteins are predominantly that of tryptophan (68, 69). Based on the observation that the wavelength of maximum fluorescence of tryptophan underwent a blue shift from 350 to 332 nm upon going from a hydrophilic environment to a hydrophobic one, Teale (69) suggested that the fluorescence intensity and the wave length of maximum emission of tryptophanyl residues in proteins are sensitive functions of their environment. This hypothesis has been confirmed by several investigations on native and denatured proteins (16, 69-72).

Changes in fluorescence which accompany the N-F transition in BSA and HSA have been reported by Steiner and Edelhoch (73), Spector and John (32), and Chen (74). The first two papers report a decrease in the fluorescence intensity and a blue shift in the wave length of the emission maximum of BSA as the pH decreased. The fluorescence-pH profiles were quite similar to N-F conversion-pH profiles obtained with other types of measurement. With an exciting wave length of 275 nm, Chen demonstrated that the pH-dependent changes in the fluorescence emission intensities at 350 nm (tryptophan fluorescence) of HSA solutions correlated almost exactly with the N-F transition with a midpoint at pH 4.1. In contrast the increase in intensity at 310 nm (tyrosine fluorescence) as the pH decreased was more gradual with a midpoint about pH 3.6 to 3.8. By exciting with 295-nm light which is not absorbed by tyrosine, Chen (75) showed that the tryptophan peak of both HSA and BSA was blue-shifted by about 8 nm during the N-F transition.

While there is some tyrosine contribution to the fluorescence spectrum of HSA ($\lambda_{\text{excitation}} = 275$ or 280 nm) even at pH 5, as evidenced by a pronounced low wave length shoulder, the tyrosine contribution to the BSA fluorescence spectrum appears to be minimal. The BSA spectrum at pH 5 has no detectable low wavelength shoulder and the band half-width of the BSA preparations did not increase as the pH was lowered as might be expected if contributions due to tyrosine fluorescence became important. The BSA fluorescence spectra obtained on exciting pH 5 and 3.5 BSA solutions with 295-nm light were almost identical with those obtained on exciting with 280-nm light. The pH-dependent quenching of the 343-nm fluorescence of the BSA monomer fractions on exciting with 280-nm light can thus be attributed primarily to a pH-dependent change in the environment of one or both of the 2 tryptophanyl residues although some of the quenching may be due to the nonspecific effect of carboxyl neutralization since Steiner and Edelhoch (31) have noted similar although smaller quenching in proteins whose structures are believed invariant to pH in the pH range of the N-F transition. The similarity of the pH ellipticity profiles at 262 and 269 nm to the pH fluorescence profiles provides strong evidence that the changes in the environment of the tryptophanyl residues parallel the course of the N-F transition. While the

accuracy of the fluorescence data was not great enough to detect small differences in the midpoint pH values of the various defatted monomer preparations, the marked differences in the fluorescence intensities of the A and of the B2 and C monomers at pH 5 are indications that the environments of at least one of the tryptophanyl residues are significantly different in the N form of these monomers.

The slight increase in fluorescence upon defatting mercaptalbumin is in agreement with the observation of Spector and John (32) that addition of fatty acid to BSA resulted in a decrease in the fluorescence. These authors have observed that this quenching of fluorescence never exceeded 45% and they have therefore suggested that fatty acid affects the emission of only 1 of the 2 tryptophanyl residues. Fatty acid was found to produce minimal changes in the fluorescence of the lone tryptophanyl residue of HSA. Based on his fluorescence data, Weber (70) has suggested that 1 tryptophanyl residue of BSA is near a protein carboxyl group while the other, and the tryptophanyl residue of HSA, is not. Polet and Steinhardt (76) have concluded from binding-induced alterations in the ultraviolet absorption that at least one of the tryptophanyl residues of BSA is at or near one or more of the primary binding sites for certain hydrocarbons. From recent studies on the optical rotatory dispersion, viscosity, H^+ titration behavior, and fluorescence changes induced by the addition of alkyl sulfates to BSA and HSA, Steinhardt *et al.* (37) have concluded that the fluorescence data can best be explained in terms of a specific tryptophan-perturbing, alkyl ion binding site with a few other equivalent binding sites within 10 Å of the perturbing site. They have also concluded that the tryptophanyl residue at or near the perturbing site is in a relatively polar environment, which is changed to a more apolar one when an alkyl ion is bound.

While the data on the fluorescence of mercaptalbumin in the presence and absence of fatty acid as reported in this paper are consistent with the results cited above, the data obtained with the B2 and C monomers are not. From the identity of the fluorescence intensities of the native B2 and C monomers (~1 mole of fatty acid per mole of albumin) with those of the defatted proteins it would appear that oxidation of the sulfhydryl group to an oxidation state higher than disulfide has resulted in the loss of the tryptophan-perturbing property of this binding site or that the site no longer binds fatty acid. The fluorescence data thus suggest that at least some of the differences in the fatty acid distributions of the monomers may be due to real differences in fatty acid binding at this site.

The results obtained on fluorescence quenching by iodide indicate that the environments of the 2 tryptophanyl residues are quite different with respect to their availability to iodide. The marked collisional quenching of the fluorescence of one of the tryptophanyl residues locates this residue in a part of the molecule which is freely available to solvent. The identity in fluorescence intensity and hence quantum yield of this residue in mercaptalbumin and B2 monomer further locates this residue in a portion of the molecule which is not perturbed on the conversion of mercaptalbumin to the sulfhydryl-free B2 monomer.

The inability of iodide to quench the fluorescence of the other tryptophanyl residue places this residue in a part of the molecule into which iodide cannot freely diffuse. The difference in the fluorescence intensity of this residue in mercaptalbumin and B2 monomer further suggests that this residue is in a restricted environment in reasonably close contact with other protein R groups and that this environment is perturbed on the conversion of mercaptalbumin to B2 monomer. It must therefore

be in the vicinity of the mercaptalbumin sulfhydryl group. The data obtained with the B1 monomer, in which the cysteinyl residue has been converted to a cystinyl residue by condensation with cysteine, are also consistent with this hypothesis. Cystine has been shown to quench tryptophan fluorescence (77). The somewhat lower fluorescence intensity of the unquenched tryptophanyl residue of the B1 monomer (whose pH ellipticity profiles were identical with those of mercaptalbumin) could be due to quenching of the fluorescence of this tryptophanyl residue by the cystinyl residue thus placing both residues in reasonable proximity to one another. Although sequence data on tryptophanyl peptides of BSA have been published (78, 79) it is impossible to correlate the sequence data with any postulated proximity of the sulfhydryl group and one of the tryptophanyl residues.

The marked increase in the fluorescence intensity of the unquenchable tryptophanyl residue of the B2 monomer with increasing ionic strength suggests that, in this monomer, the sulfhydryl group has been converted to an anionic oxidation product which perturbs the environment of the tryptophanyl residue, the perturbation decreasing as the ionic strength increases. The presence of such an anionic group could also interfere with the binding of fatty acid in this vicinity.

While the wave length of the fluorescence maximum and the fluorescence quantum yield of HSA and BSA are different, the quenchable nature of the HSA fluorescence suggests that the tryptophanyl residue in HSA is in an environment that approximates that of the quenchable tryptophanyl residue of BSA. The minimal effect of fatty acid on the fluorescence of HSA thus supports the view that it is the fluorescence of the unquenchable tryptophanyl residue of BSA which is affected by the binding of fatty acid.

Macroheterogeneity-Structure Correlations

The macroheterogeneity of defatted monomer albumin is a function solely of the sulfhydryl group. Iodoacetamide-treated mercaptalbumin does not form other species of monomer. There is considerable evidence that the N form mercaptalbumin sulfhydryl group is in a somewhat restricted environment; for example, although the sulfhydryl group can react freely with small molecules such as iodoacetamide, it reacts very slowly or not at all with larger molecules like DTNB. One of the 2 tryptophanyl residues is also in a restricted environment since its fluorescence is not depressed by collisional quenching by iodide. Furthermore, since oxidation of the sulfhydryl group to oxidation states higher than disulfide causes no observable changes in the gross structure of the molecules as evidenced by ellipticity data at 209 and 220 nm but does markedly quench the fluorescence of the restricted tryptophanyl residue, the sulfhydryl group must be reasonably close to the tryptophanyl residue. A specific primary fatty acid binding site must also be in the vicinity of the sulfhydryl group and the tryptophanyl residue. Unsaturated fatty acid binding at this site potentiates oxidation of the sulfhydryl group. Such oxidation, with perhaps the concomitant introduction of a negative charge, results in a localized perturbation of this area of the protein. The pH 5 ellipticity values at 262 and 269 nm suggest that at least 1 cystinyl residue is also perturbed on oxidation of the sulfhydryl group and thus that a cystinyl residue is also present in this region of the molecule.

The ellipticity and fluorescence pH profiles show that this area of the protein is located in the crevice which is opened up in the course of the N-F transition. Since the crevice of the N form

of the albumin contains many charged groups, the environment in the crevice is probably quite hydrophilic in nature, a situation which would explain the relatively high wavelength of the fluorescence emission maximum of the unquenchable tryptophanyl residue. The higher midpoint pH values of the pH ellipticity profiles at 262 and 269 nm as compared with those at 209 and 220 nm indicate that some limited opening up of this area of the crevice precedes the more extensive structural alterations which involve the helical portions of the molecule. The higher transition midpoint pH of the B2 monomer at 262 and 269 nm is evidence of a decreased stability of the N form of this region of the crevice when the sulfhydryl group has been oxidized to an oxidation state higher than disulfide.

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