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The Relation of Free Sulfhydryl Groups to Chromatographic Heterogeneity and Polymerization of Bovine Plasma Albumin*

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Heterogeneity of bovine plasma albumin has been examined with respect to chromatographic behavior, molecular weight, and free sulfhydryl content. Fractionation of different components of bovine plasma albumin was achieved by taking advantage of their ability to displace one another on DEAE-cellulose. The heterogeneity so disclosed is chiefly related to the well-known heterogeneity of bovine plasma albumin with regard to molecular weight (monomers, dimers, and higher polymers) and free sulfhydryl content (mercaptalbumin and non-mercaptalbumin). There are at least two chromatographically distinct non-mercaptalbumins, the larger fraction being adsorbed more tightly to DEAE-cellulose than mercaptalbumin. The smaller non-mercaptalbumin fraction is the least tightly bound component of all and hence is best demonstrated by frontal analysis. A small fraction of monomer of undetermined sulfhydryl titer was not separable from the dimers and higher polymers, which are bound more tightly than the bulk of the monomers. Further heterogeneity within the more tightly binding non-mercaptalbumin component was revealed by its incomplete reduction to mercaptalbumin by β-mercaptoethanol. Similar heterogeneity was observed in the dimer component, which is only partly reduced to monomer by the same reagent. Within the dimer component produced by ethanol treatment, and probably within that normally occurring, there are chromatographic differences which reflect the heterogeneity of the constituent monomers; these differences are increased by hybridization. While all the ethanol-produced dimers were found to be dissociable by β-mercaptoethanol, free sulfhydryl groups were not required for their formation.

That crystalline preparations of plasma albumin are heterogeneous in several respects has long been recognized. The presence of small amounts of more rapidly sedimenting materials is a common observation, as is the presence of a sulfhydryl titer of less than one equivalent per mole. Heterogeneity has also been reported in chromatographic behavior (Boman and Westlund, 1956; Sober *et al.*, 1956; and Tiselius *et al.*, 1956) and in starch gel electrophoresis (Saifer *et al.*, 1961).

The nature of the intermolecular bond in the normally occurring dimer is not known, nor is it known whether the normally occurring dimer is an artifact of purification or, if it is, to what extent. Some studies have been carried out with synthetic dimers produced by various treatments. Thus, the dimer formed at low pH, most recently studied by Williams and Foster (1960), appears to be held together at first by pH-reversible secondary valences and only with time stabilized by formation of intermolecular disulfides by exchange or oxidation. The initial reversible bond is probably similar to that formed in 3–5 M urea as reported by Gutter *et al.* (1957). The reversible dimerization in the presence of Hg^{++} (Hughes, 1947) or bifunctional organic mercurials (Edsall *et al.*, 1954) is fairly well understood. The formation of a disulfide dimer by oxidation of the sulfhydryl groups on two monomers has been reported by Straessle (1954) on treatment of solid mercury dimer in ethanol with iodine.

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King *et al.* (1960) also describe a process of dimer formation by oxidation of the sulfhydryl monomer by atmospheric O_2 in a solution of the protein in a mixture of ethanol, 1-propanol, water, and ammonium sulfate used in countercurrent distribution studies. Much less clear is the mechanism of the dimerization reaction which takes place (in 15–30% yield) on treatment of the monomer with concentrated methanol (Cohn *et al.*, 1947), ethanol (Straessle, 1954), or a methanol-chloroform mixture (Therriault and Taylor, 1960). These authors report that the dimer formed is split by cysteine, but that the dimerization reaction does not require free sulfhydryl groups. In the present work we have found the same to be true of the dimerization in ethanol.

It should be clear from the above that speculation about the structure of the various types of albumin dimers centers around the nature of the single sulfhydryl group, free or otherwise, of the monomer. This is, of course, an interesting problem in itself, and efforts to interpret the heterogeneity with respect to the sulfhydryl group have led to a number of hypotheses. Most recently, King (1961) has presented evidence that the "missing" sulfhydryl is tied up as mixed disulfide with cysteine and, to a lesser extent, with glutathione.

In the course of a larger study of the mechanisms of interaction of proteins and other biologically interesting polyelectrolytes with the substituted cellulose ion-exchangers, we have investigated the heterogeneity of bovine plasma albumin with respect to adsorption on diethylaminoethyl (DEAE)-cellulose. We have found that most, but not all, of this heterogeneity is directly correlated with molecular weight and sulfhydryl content.

That different proteins can displace one another on DEAE-cellulose under conditions of tight binding has been noted previously, particularly with respect to the colored components of serum (Sober *et al.*, 1956). We have taken advantage of this phenomenon to separate the different components of bovine plasma albumin.

We will refer on several occasions to the tight-binding capacity of the adsorbent. By this is meant that amount of a particular protein which, under particular solvent conditions (salt, pH, etc.), can be bound to the adsorbent while the concentration of that protein in solution remains essentially zero. This is not an unambiguous definition, as some protein can always be removed from the adsorbent by exhaustive washing with the solvent. Operationally it is nevertheless useful, particularly at low salt concentrations, where the tight-binding capacity is high and fairly sharply defined. We will also refer to various albumin fractions as "early" or "late." This refers to their order of appearance in frontal analysis and may be translated roughly as "less tightly bound" or "more tightly bound" respectively.

Materials and Methods

Materials.—The protein used in these experiments was bovine plasma albumin (Armour crystallized Lots T 68412 and V 68802). For a few experiments, as noted, bovine mercaptalbumin (Nutritional Biochemicals, Lot 4499) was used. Protein concentration was measured as optical density at 280 mμ in 1-cm cells in a Beckman DU spectrophotometer. In order to express sulfhydryl titer in terms of moles of monomer, an absorbancy of 0.66 per mg per ml and a molecular weight of 69,000 were used.

The DEAE-cellulose was prepared in this Laboratory from Solka-Floc SWB 100–230 mesh, as previously described (Peterson and Sober, 1956). The measured incorporation was 0.9 mEq N/g. Columns were packed under pressure, which was increased gradually during packing from zero to 10 p.s.i.

Eastman white label grade β-mercaptoethanol was used without further purification. All other chemicals were reagent grade.

Ultracentrifugation.—Analytical ultracentrifugation was performed with the Spinco Model E Ultracentrifuge at 59,780 rpm in 0.1 M potassium phosphate pH 7.0.

Sulfhydryl Titrations.—Sulfhydryl titers were measured essentially by the method of Benesch *et al.* (1955), scaled down so that samples of 5 ml could be measured. To each 5-ml sample was added 0.5 ml of a stock solution made up to the following: 2.5 M Tris nitrate, 0.17 M KCl, pH 7.5. The protein concentration was generally 1.5–2.0 mg/ml. The presence of 0.02 M potassium phosphate (pH 7) had no effect on the end-point, so samples from frontal analysis runs could be titrated without intervening dialysis. The titrant was 0.002 M $AgNO_3$ added in 4-μl portions at 1-minute intervals. The current was measured with a Sargent Model XXI Polarograph. At a level of 0.02 μEq of sulfhydryl per ml, reproducibility was within 5% from day to day and generally within 2–3% on consecutive titrations. That systematic errors inherent in the silver titration may introduce larger variance (perhaps 10%) from the true values must be recognized (Cecil and McPhee, 1959), but even such errors would not materially affect our conclusions.

Electrophoresis.—Free boundary electrophoresis was performed with a Spinco Model H Electrophoresis-diffusion apparatus with potassium phosphate buffer pH 7.0, $\mu = 0.1$. Paper electrophoresis was done with the Spinco Model R apparatus with sodium barbital, pH 8.6, $\mu = 0.075$.

Gradient Chromatography.—Gradient elution chromatography was used in this work for analytical comparisons of different preparations, rather than for fractionation. The DEAE-cellulose columns were about 1 cm by 15 cm, and the protein load was 25–50 mg. Samples to be compared were always run identically with equal loads (in terms of volume × OD_{280}) successively on the same column. The column was regenerated between runs with 0.5 M potassium phosphate, pH 7, followed by the starting buffer. Linear salt gradients were used, the pH always being maintained at pH 7.0. Numbering of the tubes was begun at the start of the gradient. For technical reasons, details of the gradient were changed several times in the course of these experiments, but the general shapes of the chromatograms were not significantly altered

thereby. Our present procedure uses 0.02 M and 0.30 M potassium phosphate at pH 7.0 as starting and limit buffers, respectively, the gradient being 1.2×10^{-3} M potassium phosphate/ml. The flow rate is about 0.9 ml/minute and the volume of the fractions 2.5 ml. A load of 25 mg protein is used. With whole bovine plasma albumin, the peak maximum emerges at about 0.06 M potassium phosphate.

In these and in the frontal analyses next described, a constant flow rate was produced by a Sigmamotor finger pump and collections were on a time basis. All chromatography was done at 5°.

Frontal Analysis.—Protein solution was fed into a column continuously, and successive effluent fractions were assayed. Before introduction of the protein, the columns were equilibrated with the buffer, which was generally 0.02 M potassium phosphate, pH 7.0. Under these conditions, the tight-binding capacity of DEAE-cellulose for albumin is about 100 mg/g. Input protein concentrations were as high as was consistent with convenient assay: 1.5–2 mg/ml.

Sectioned Column Analysis.—For this technique the adsorbent columns were packed in polyethylene tubing. The albumin (1.0–10.0 mg/ml) was dialyzed versus a buffer such as that used for frontal analysis. The total amount introduced into the column, however, did not exceed its tight-binding capacity, so that no protein emerged in the effluent. The column was then rinsed exhaustively (50 column volumes) with dialysate to wash all loosely bound protein down to the front. The polyethylene column was then cut into equal segments with a razor and the contents of each segment extruded into the top of a small sintered glass filter, where it was resuspended in water to get even packing. The protein was then eluted in 0.5 M potassium phosphate, pH 7.0, and dialyzed *versus* suitable buffers for assays. At present, this procedure is carried out more easily by the use of small, reusable columns which can be connected in series, with appropriate valves between them for sampling the effluent from each segment.

β-Mercaptoethanol Treatment.—Albumin preparations were treated with β-mercaptoethanol by passing dilute solutions of the reagent through DEAE-cellulose columns to which the protein had been tightly adsorbed. This procedure allows independent variation of reaction time, reagent concentration, and the total amount of reagent to which the protein was exposed. In particular, it permits the exposure of the bovine plasma albumin to large molar excesses of mercaptoethanol while keeping the concentration of the latter low (0.001–0.01 M). All treatments were at 5°.

Ethanol Treatment.—This treatment was carried out, unless otherwise specified, by injecting a concentrated (5–10%) solution of albumin in 0.1 M NaCl into 10 volumes of ethanol with a syringe. The precipitate was centrifuged down and, after removal of the supernatant, dried *in vacuo*.

Results

Frontal Analysis.—The results of a typical frontal analysis are illustrated in Figure 1. In this

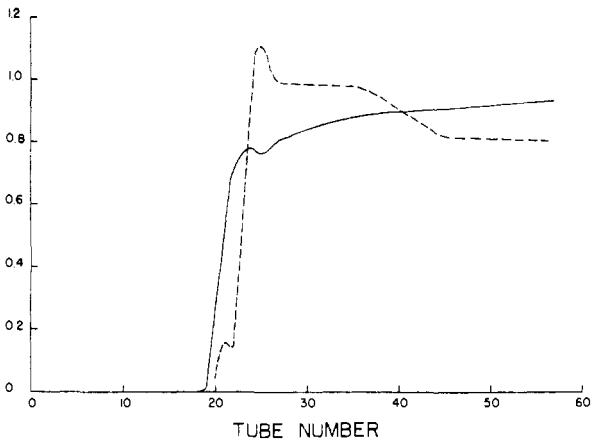

FIG. 1.—Frontal analysis of whole bovine plasma albumin on DEAE-cellulose. The solid line represents the ratio of the optical density at 280 mμ of the effluent to that of the input (1.28). The dashed line represents the sulfhydryl titer of the effluent expressed as sulfhydryl equivalents per 69,000 g.

experiment a 20 × 0.7 cm DEAE-cellulose column was used. The input protein concentration was 1.94 mg/ml in 0.02 M potassium phosphate buffer, pH 7.0. The flow rate was 6.4 ml per hour. Sulfhydryl analysis revealed several distinct molecular varieties. The plateau at 1.0 sulfhydryl equivalent per 69,000 g indicates that almost all of the material which emerged before tube 35 was mercaptalbumin. The presence of nonsulfhydryl albumin in this region is revealed by the anomalous behavior of the leading edge. The first protein to break through had a very low sulfhydryl titer. Although it was identified as monomeric albumin by its electrophoretic mobility and sedimentation coefficient, it was evidently different from the nonsulfhydryl albumin that was bound to the column more tightly than mercaptalbumin. The dip in OD_{280} at tube 25 and the concurrent peak in sulfhydryl titer was not always seen and appears to be due to a nonalbumin contaminant. The dip in OD_{280} implies that this contaminant has a lower molar extinction coefficient than albumin, and a small amount of similar material obtained in a different experiment had a ratio of sulfhydryl titer to OD_{280} about ten times that of albumin.

Between tubes 35 and 45, the breakthrough front for that species (or genus) which comprised most of the nonsulfhydryl albumin is indicated by a drop in sulfhydryl titer from 1.0 sulfhydryl equivalent per 69,000 g to 0.8. The sulfhydryl titer of the input albumin (bovine plasma albumin V68802) was 0.75 sulfhydryl equivalent per 69,000 g.

Sectioned-Column Analysis.—The column used in the experiment illustrated in Figure 2 was packed in 12 mm (I.D.) polyethylene tubing to a length of 110 cm. The input protein solution was in 0.02 M potassium phosphate, pH 7.0, at a concentration of 10.0 mg/ml. The flow rate was 22 ml per hour. After the application of 100 ml of protein solution, 3.8 liters of buffer was put through at the same flow rate to complete development. To achieve clean fractionation it is necessary to move the loosely bound protein down the column in this rinsing

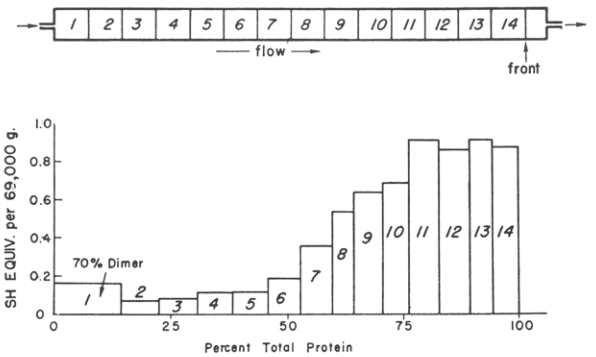

FIG. 2.—Sectioned-column fractionation of whole bovine plasma albumin on DEAE-cellulose. Fraction numbers correspond to the column segments from which they were eluted. Fraction 1 is the most tightly adsorbed.

step. Displacement continues on the tight-binding sites, and the protein front continues to move down the column until the tight-binding capacity of the adsorbent behind the front equals the total amount of protein introduced.

The fourteen segments from which the numbered fractions of Figure 2 were eluted were each 6 cm long. The results show clearly that protein-protein displacement occurred and that the principle difference between albumin monomers in DEAE-adsorption characteristics correlates directly with their sulfhydryl titer. The small nonsulfhydryl fraction which appears on the leading edge in frontal analysis was not resolved here, but a fairly clean fractionation of the larger, more tightly binding nonsulfhydryl fraction was obtained. The dimer and the small fraction of higher polymers, as determined by sedimentation in the ultracentrifuge, were confined to the first, most tightly held fraction. The low sulfhydryl titer of this fraction indicates that most of the dimer, if not all, is lacking free sulfhydryl. On gradient chromatography, the mercaptalbumin and the nonsulfhydryl albumin can be seen to differ (Fig. 3), although such a chromatogram would not have resolved them in the original mixture.

A further examination of the 5–10% that was most tightly bound was carried out by passing 5.0

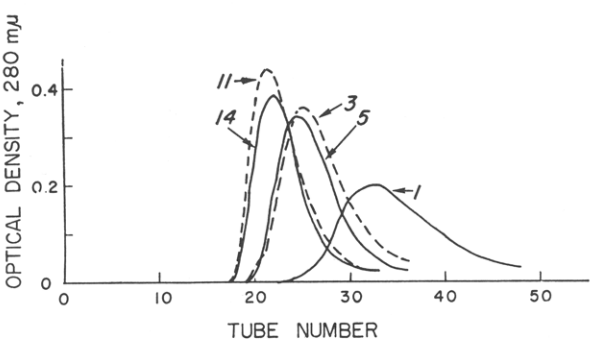

FIG. 3.—Chromatographic analysis of sectioned-column fractions of Figure 2. Column 0.7 × 15 cm packed with DEAE-SF, 100–230 mesh, 0.9 mEq N/g. Protein load 25 mg. Gradient linear from 0.02 M potassium phosphate; slope 1.2×10^{-3} M potassium phosphate/ml; pH 7 throughout. Flow rate 0.9 ml/min. Effluent collected in 2.5-ml fractions.

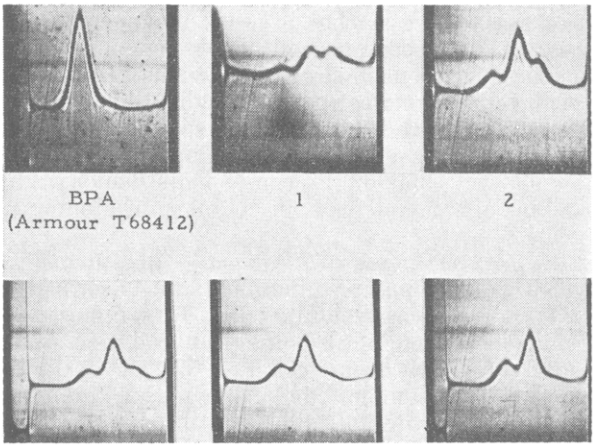

FIG. 4.—Concentration of polymers into the most tightly adsorbed sectioned-column fractions. Numbering is from input end of column, fraction 1 being the most tightly adsorbed. The first and second peaks from the left have the sedimentation coefficients of albumin monomer (4S) and dimer (6S) respectively. Potassium phosphate buffer, pH 7, $\mu = 0.1$, 80 minutes at 59,780 rpm.

g protein (in 500 ml) through a column the tight-binding capacity of which was only 250 mg under the conditions employed. This column was packed to 39 cm in 6 mm (I.D.) polyethylene tubing. After being rinsed with several hundred ml of buffer (that more exhaustive rinsing might have given better resolution was not appreciated at the time of this experiment), the column was cut into five segments for analysis. These fractions correspond roughly to serial subfractions of fraction 1 of Figure 2, the most strongly adsorbed fraction. Figures 4 and 5 show the results of ultracentrifugal and gradient chromatographic analyses. It can be seen that, in general, increasing displacing power goes along with increasing molecular weight, although discrimination between higher polymers is

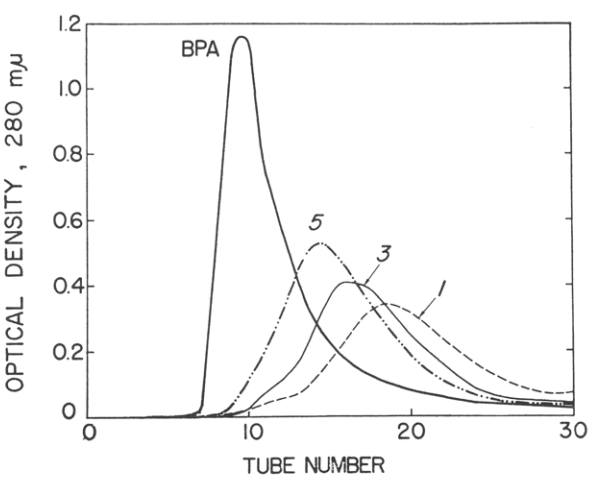

FIG. 5.—Chromatographic analysis of most tightly adsorbed sectioned-column fractions. Numbered as in Figure 4. Curve labeled BPA (bovine plasma albumin) is unfractionated original mixture. Column 1.0 × 25 cm. Protein load 40 mg (50 mg for whole bovine plasma albumin). Gradient linear from 0.05 M sodium phosphate, slope 0.43×10^{-3} M sodium phosphate/ml, pH 7 throughout. Effluent collected in 10-ml fractions. Remainder as in Figure 3.

not as effective as that between monomer and dimer. The appearance of sizable monomer peaks in these fractions in spite of the virtual elimination of the chief monomer peak in gradient analysis suggests that there are monomeric species which bind as tightly as some polymers. Paper electrophoresis showed that all the components demonstrated in this experiment had the mobility of albumin at pH 8.6.

β-Mercaptoethanol Treatment.—Treatment of whole bovine plasma albumin (5 g), adsorbed on a DEAE-cellulose column, with 0.01 M β-mercaptoethanol (500 ml) for 3 hours produced a change in sulfhydryl titer from 0.68 to 1.07. After similar treatment, a sample of bovine mercaptalbumin had a sulfhydryl titer of 0.99. As this material contained mercury before the sulfhydryl treatment, we did not attempt to verify its presumed initial titer of 1.0. However, the sulfhydryl titer of a sample of mercaptalbumin prepared in a sectioned-column experiment was not changed by β-mercaptoethanol (Table I). These results clearly suggest that the

TABLE I

EFFECT OF MERCAPTOETHANOL TREATMENT ON THE SULFHYDRYL TITER OF VARIOUS ALBUMIN PREPARATIONS

Material	Initial SH Titer (SH-equiv./ 69,000 g)	Mercapto-ethanol Concn. (M)	Length of Treatment (Hr.)	SH Titer After Treatment (SH-equiv./ 69,000 g)
Bovine plasma albumin (T68412)	0.68	0.01	3	1.07
Bovine mercaptalbumin (4499)		0.01	3	0.99
Mercaptalbumin from a sectioned column	0.99	0.01	3	0.94
Early non-SH bovine plasma albumin obtained by frontal analysis	0.09	0.01	12	0.94
Late non-SH bovine plasma albumin from a sectioned column (fine cut, fraction No. 5, Fig. 2)	0.12	0.01	4	0.71
Late low-SH fraction from a large-scale sectioned column (coarse cut)	0.15	0.001	0.25	0.30
		0.001	1	0.36
		0.001	12	0.59
		0.01	1	0.47
		0.01	12	0.45
		0.01	18	0.56

increase in the sulfhydryl titer of whole albumin is due not to the appearance of sulfhydryl groups at sites unrelated to the normal albumin sulfhydryl but to a specific conversion of nonsulfhydryl albumin to mercaptalbumin.

In view of the errors involved in our titrations, the proximity of these titers to unity may be misleading. Indeed, studies with isolated low-sulfhydryl fractions reveal that not all nonsulfhydryl albumin monomers are equally reducible, and some are not reduced by the 3-hour treatment described above. A large-scale experiment with use of the sectioned column supplied us with about 1 g of low-sulfhydryl material. Just how this material compared with that of fractions 2 to 7 in Figure 2 is not known, as a much coarser cut was taken, but it contained no dimer and had a sulfhydryl titer of 0.15 sulfhydryl equivalent per mole. Table I summarizes the results obtained by β-mercaptoethanol treatment of this and other material, as described.

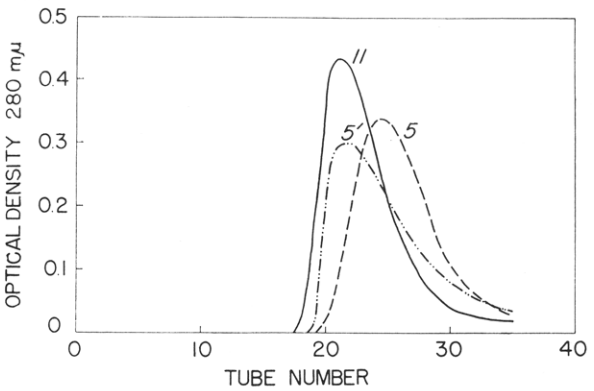

FIG. 6.—Effect of β-mercaptoethanol on nonsulfhydryl albumin. Curves 5 (late nonsulfhydryl albumin) and 11 (mercaptalbumin) are the same as those of Figure 3. 5′ shows the behavior of fraction 5 after β-mercaptoethanol treatment. Chromatography as in Figure 3.

The rise in sulfhydryl titer to about 0.5 sulfhydryl equivalent per mole with no further significant rise with time certainly suggests heterogeneity within the *late* nonsulfhydryl fraction.

Figure 6 shows the change in chromatographic behavior which accompanies the rise in sulfhydryl titer. It appears that the new sulfhydryl albumin in this sample is now chromatographically indistinguishable from the normal mercaptalbumin.

Figure 7 illustrates the change this treatment brought about in a late-displacement fraction rich in dimer. About half the dimer was reduced to monomer in 3 hours with 0.01 M β-mercaptoethanol. Further treatment had no additional effect. Thus there are at least two types of dimer in crystalline bovine plasma albumin. In experiments with fractions containing more of the higher polymers (*e.g.*, fraction 1, Fig. 4) it was found that peaks representing polymers higher than the dimer disappear completely.

Dimerization in Ethanol.—Treatment of bovine plasma albumin with methanol, as first reported by Cohn *et al.* (1947), or with ethanol (Straessle, 1954) will produce some 15–30% dimer and smaller amounts of higher polymers. Figure 8 shows a typical result of such a treatment as viewed in the ultracentrifuge and by chromatography. In the

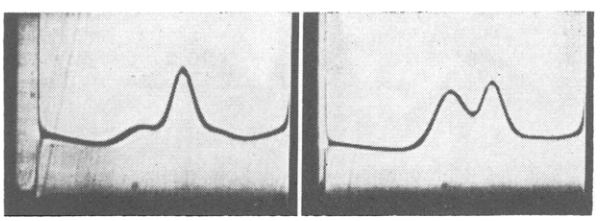

FIG. 7.—Effect of β-mercaptoethanol on late bovine plasma albumin fraction rich in dimer. Ultracentrifugation as in Figure 4. Before (*left*): 80 minutes; after (*right*): 96 minutes.

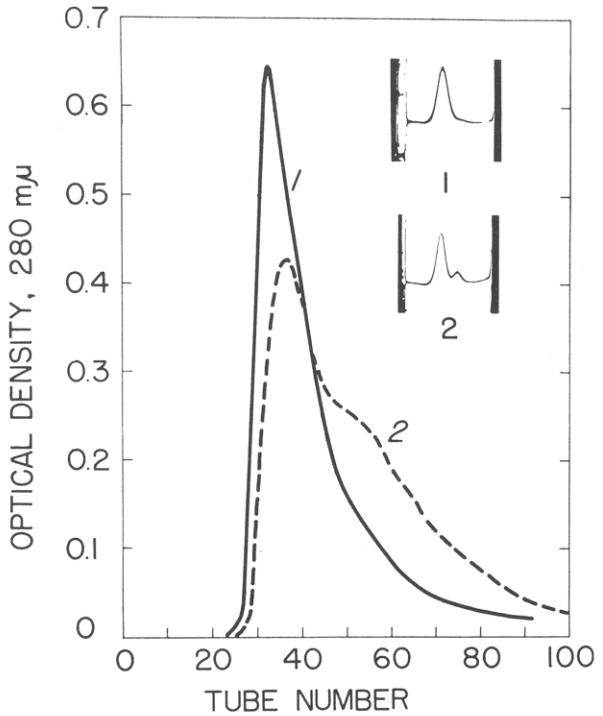

FIG. 8.—Effect of ethanol treatment on whole bovine plasma albumin. 1, untreated; 2, treated. Ultracentrifugation as in Figure 4. Chromatography as in Figure 3, except effluent collected in 1.3-ml fractions.

chromatogram the dimer appears as a shoulder on the descending slope of the monomer peak.

The sedimentation pattern of a late-displacement fraction of a similar preparation, representing the most tightly binding 3–4%, is shown is Figure 9 along with the effect of β-mercaptoethanol on such a fraction. Bovine mercaptalbumin was used in the experiment illustrated, but similar results were obtained with whole bovine plasma albumin. Mercury present in the starting material had been removed by treatment on a DEAE-cellulose column with ethylenediaminetetraacetic acid. The ethanol-produced dimers and polymers were completely reduced to monomer by β-mercaptoethanol. The small dimer peak remaining was undoubtedly the β-mercaptoethanol-resistant dimer that was present in the starting material. It was concentrated, relative to monomer, along with the other dimers.

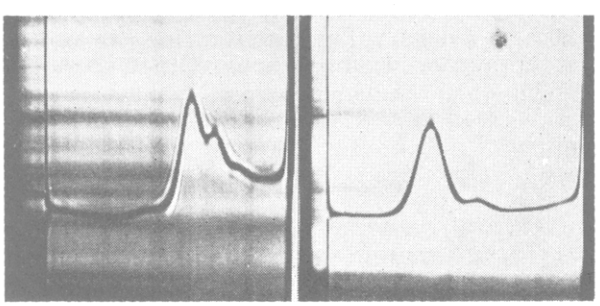

FIG. 9.—Effect of β-mercaptoethanol on polymers fractionated from ethanol-treated bovine plasma albumin. Before, left; after, right. Ultracentrifugation as in Figure 4.

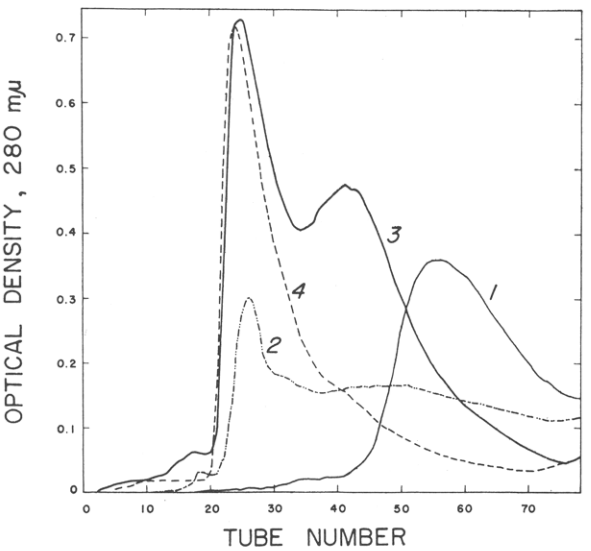

FIG. 10.—Effect of β-mercaptoethanol on early and late dimers. 1. Very late sectioned-column fraction of ethanol-treated bovine mercaptalbumin. 2. Material of curve 1 after β-mercaptoethanol treatment. 3. Ethanol-treated early monomer fraction of bovine mercaptalbumin. 4. Same material as 3 after β-mercaptoethanol treatment. Column as in Figure 5. Protein load 50 mg (approximately 80 mg for curve 3). Gradient linear from 0.3 M sodium phosphate, slope 0.6×10^{-3} M sodium phosphate/ml, pH 7 throughout. Effluent collected in 2.5-ml fractions.

In Figure 10 the gradient chromatographic behavior of these same preparations (curves 1 and 2) illustrates further heterogeneity encountered in bovine plasma albumin. Also shown are the results of identical runs (except for an inadvertently higher load in the "before" experiment, curve 3) on an an ethanol-treated early fraction before and after reduction by β-mercaptoethanol (curves 3 and 4). In addition to separating dimers from monomers, the fractionation process has divided the dimers into two groups, as evidenced by the different positions of the peak of curve 1 and the dimer peak of curve 3. From curve 2 it is obvious that these late dimers owe their position to the character of their constituent monomers. Note that the material of curve 2 was 95% monomer.

As observed above, treatment in concentrated ethanol produces dimers and higher polymers which can be reduced to monomers by β-mercaptoethanol. The main dimerization reaction, however, does not require free sulfhydryl groups. A low-sulfhydryl sample from the sectioned-column experiment of Figure 2 (fraction 3), when treated with ethanol, produced only slightly less dimer than did whole bovine plasma albumin. A similar amount of dimer was formed by ethanol treatment of whole bovine plasma albumin after its sulfhydryl titer had been reduced to zero by pretreatment with *p*-chloromercuribenzoate (CMB). The extent of this reaction was the same at 5° as at room temperature, while the secondary, sulfhydryl-dependent reaction, which accounted for a small part of the dimer and most if not all of the higher polymers, was considerably reduced at 5°.

The results of several experiments which may be helpful in elucidating the mechanism of the sulfhy-

dryl-independent reaction will be summarized briefly. First, extensive ethanol extraction is not required for the maximum production of dimer. Simply dampening albumin powder with ethanol and drying it is sufficient. Second, the dimerization appears to take place only when the albumin is in the solid phase. The amount of dimer produced with *p*-chloromercuribenzoate–treated material was not affected by the addition of acid or base to the ethanol over a wide range of pH, but if the pH and salt concentration were too low, albumin remained in solution as monomer, as revealed by ultracentrifugation in the ethanol. When the albumin was salted out and recovered in aqueous buffer, the usual 15–20% yield of dimer was found. Third, when monomer isolated from an ethanol-treated preparation was again treated with ethanol, dimer appeared in the usual yield. On the other hand, a fraction of the same preparation containing about 70% dimer was not appreciably affected by ethanol treatment, indicating that the 15–20% dimer yield is not due to a monomer-dimer equilibrium. Fourth, albumin could be defatted while adsorbed on DEAE-cellulose with ethanol, chloroform, and 5% acetic acid in iso-octane, without appreciable dimer appearing when the protein was finally eluted in aqueous solution.

Discussion

The separation of albumins differing in sulfhydryl content by the use of DEAE-cellulose has significance with regard to both albumin heterogeneity and the mechanisms involved in protein chromatography on the modified celluloses. The well-known work of Hughes (1947) in separating the two components was based on the specific reaction between Hg^{++} and the sulfhydryl groups. The albumin-S-Hg-S-albumin dimers formed could be isolated by crystallization. In the present work the separation is based on mechanisms (albeit poorly understood) which are of a much more general and nonspecific nature. For at least 90% of the monomer, other variations in surface organization which can affect chromatographic behavior must be small compared to those involving the sulfhydryl parameter. That the latter variations are small seems to us a reasonable assumption, although the exact nature of the difference is still in doubt. Benesch and Benesch (1956) have reported that if 8 M urea is added to Tris buffer, the argentometrically determined sulfhydryl titer rises from $^2/_3$ to 1 sulfhydryl equivalent per mole. Below pH 6 this effect of urea is said to be reversible. Benesch and Benesch suggest that the irreversible increase above pH 6 is due to migration of the "hidden" sulfhydryl group from the interior by sulfhydryl-disulfide exchange. On the other hand, King (1961) has recently reported evidence that the missing sulfhydryl groups are tied up in disulfide linkages to several low-molecular-weight thiols, principally cysteine. The apparent partial conversion of nonsulfhydryl bovine plasma albumin to mercaptalbumin by β-mercaptoethanol would tend to support King's interpretation, but the effect of urea is then not easily explained. It is noteworthy that in the literature on albumin we have found no previous report of heterogeneity correlating directly with the sulfhydryl content except in those properties which can be directly related to the chemistry of the sulfhydryl group. The addition of a dipolar ion such as cysteine might be expected to increase the binding to the positively charged adsorbent, as is observed for most of the nonsulfhydryl bovine plasma albumin. Glutathione, with a net negative charge, should have an even greater effect. A small number of positively charged groups added similarly through a disulfide linkage could account for the nonsulfhydryl bovine plasma albumin that binds less tightly than mercaptalbumin, constituting the leading front in frontal analysis.

The smaller we assume the difference between the sulfhydryl and nonsulfhydryl albumin to be, the more impressive is the selectivity demonstrated by the adsorbent. Detailed speculation on the nature of this selectivity and the general mechanism of the adsorption and displacement processes does not seem profitable at present. It would seem from our experience, however, that the albumin-adsorbent interaction is complicated in such a manner that clean separation of the albumin components by salt gradient elution at pH 7 would be difficult if not impossible. Useful fractionation by single-buffer chromatography is also impeded by this complication. The difficulty is circumvented in the type of protein-protein displacement fractionation we have employed here. No matter how the protein-adsorbent interactions may vary with protein concentration, the difference in the strength of these interactions for two molecules such as a nonsulfhydryl albumin and a mercaptalbumin appears to be maintained at a level adequate for effective displacement. In the sectioned-column type of fractionation, the high selectivity of the adsorbent compensates for the fact that the countercurrent effect is only partially utilized. A serious drawback is that it is not possible, even in principle, to obtain quantitative yields of the pure components. A more conventional chromatographic technique giving clean separations would be preferred were it available. A practical consideration in using the displacement phenomenon for fractionation is that it is time-dependent and the time required for effective fractionation increases as the capacity of the adsorbent is increased with decreasing salt concentration. We have not as yet made any serious attempt to determine optimum values for such variables as flow rate, salt concentration, and protein concentration. Clearly, optimum conditions would depend in part on whether a particular fractionation was being carried out for preparative or for analytical purposes.

While the frontal and sectioned-column analyses are designed to make the best use of the protein-protein displacement phenomenon, this phenomenon is undoubtedly active in gradient chromatography as well. How much of a role it plays in the gradient chromatography of such complicated systems as serum (Sober *et al.*, 1956; Sober and Peterson, 1958), where relatively high flow rates are employed, remains to be determined. It should be noted that the shape of a gradient chromatogram of a mixture as determined by some unspecific assay (*e.g.*, OD_{280}) might be quite insensitive to

increased resolution produced by displacement.

The separation of the dimer and higher polymers from all but a small fraction (perhaps 5%) of the monomer is not so surprising as the sulfhydryl-nonsulfhydryl fractionation, but it is nonetheless satisfying and is of considerable importance. As an instance, the complete removal of polymers will increase the value of albumin as a standard test material in various methods of determining molecular weights of macromolecules. A very sensitive test for the presence of dimers is also afforded. By extraction of a small fraction of the albumin sample on DEAE-cellulose, either by stirring a small amount of the adsorbent with the protein and then filtering or by passing the protein through a small column of the adsorbent, any dimer present can be concentrated and observed ultracentrifugally. A 0.1% dimer contaminant could be readily detected by this technique. On a larger scale, it seems likely that stocks of outdated human albumin might be recovered for clinical use by the removal of age-accumulated polymers (Finlayson *et al.*, 1960). For our own purposes, any fractionation is a step forward, as one of the original aims of this study was the isolation of chromatographically pure albumin for use in studies of the mechanisms of interaction between proteins and the cellulose ion-exchangers.

The fractionation also greatly facilitates the study of the dimers and higher polymers themselves. The dimers normally occurring in preparations of bovine plasma albumin are of at least two varieties, as reflected in their resistance to reduction to monomer by β-mercaptoethanol. While chromatographic heterogeneity of these dimers has not been explicitly demonstrated, it follows almost certainly from the heterogeneity of the monomers and of the dimers produced by ethanol treatment. Indeed, it seems likely that the dimers in the starting materials that are reduced to monomer by β-mercaptoethanol are preparative artifacts and in every way equivalent to those formed by ethanol.

The dimerization reactions occurring in ethanol treatment are of two types, only one of which requires free sulfhydryl groups. That the latter appears to be involved in the formation of higher polymers suggests a disulfide-sulfhydryl interchange rather than direct oxidation of two sulfhydryl groups. Whether disulfide bonds are involved in the β-mercaptoethanol–sensitive dimers formed in the absence of free sulfhydryl groups or in the stable fraction of normally occurring dimer, remains to be demonstrated. The difference between these two is something more than a simple rearrangement of secondary valences. Disruption of the secondary and tertiary structure in 8 M urea, followed by dialysis to remove the urea, resulted in no change in the β-mercaptoethanol resistance of either.

By treatment of an early monomer fraction in ethanol, a monomer-polymer mixture can be obtained for which there is no overlapping of DEAE-cellulose binding properties between monomer and dimer. From such a preparation we have isolated a small (20-mg) sample of relatively pure (95%) dimer. Complete isolation in larger amounts seems feasible and would be most useful in studies of the dimer bond. Such pure dimer preparations would also be useful as auxiliary molecular weight standards and, along with higher polymers which might also be isolated, as members of a homologous series in various types of studies of the physical chemistry of proteins. Among such studies, of course, is the study of their interactions with various adsorbents.

The very late monomer which contaminates polymer fractions obtained from whole albumin might be accounted for in any number of ways, such as differences in primary structure or differences in more or less tightly bound contaminants. In the latter categories, two early suspects should be mentioned. Analysis for long-chain fatty acids by the method of Dole (1956) revealed slight but possibly significant differences in fractions from a sectioned-column experiment, the protein adsorbed at both ends having a somewhat higher fatty acid content than that in between. In displacement experiments the yellow-brown color of albumin is strongly concentrated in the most tightly bound fractions. The early monomer fractions are nearly colorless, even in 10% solution. However, the ethanol treatment we have used for dimerization removes most of both the fatty acid and the color. "Late" monomers nevertheless persist and even appear to form "late" dimers on treatment with ethanol (note curves 1 and 2, Figure 10).

Table II lists the components we have found in crystalline bovine plasma albumin in the order of increasing adsorption. Preliminary experiments with human plasma albumin indicate that a similar picture will be found for that species.

TABLE II
COMPONENTS OF BOVINE PLASMA ALBUMIN

Component	Relative Abundance (%)
(1) Early non-SH albumin	5–10
(2) Mercaptalbumin	50–75
(3) Late non-SH albumin	
(a) reduced by β-mercaptoethanol	10–20
(b) not reduced by β-mercaptoethanol	10–20
(4) Very late monomer (color ?)	<5
(5) Dimers	
(a) β-mercaptoethanol–resistant (early and late)	5–8
(b) β-mercaptoethanol–stable (early and late)	
(6) Higher polymers	<1

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Modification of the Methionine Residues in Ribonuclease*

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To shed further light on the relationship between the enzymic activity and the chemical structure of ribonuclease the methionine residues in the enzyme have been modified by reaction with iodoacetic acid to form the carboxymethylsulfonium derivatives or with hydrogen peroxide to form the sulfoxides. These reactions do not occur at neutrality but take place when the protein is unfolded by urea or by exposure to pH 2–3. Both reactions at pH 2–3 are inhibited markedly by sulfate ions. Chromatographic separation of products with varying degrees of substitution was performed on columns of Amberlite IRC-50. Alkylated derivatives containing more than one carboxymethyl group were inactive. An active monosulfoxide derivative was isolated, but products in which more than one methionine residue had been oxidized were inactive. Inactivation probably results from inability of the altered protein to refold into the native conformation. In addition, methods are described for the determination of the acid-labile carboxymethylsulfonium and methionine sulfoxide residues in a protein. The methods depend upon the facts that sulfonium derivatives are stable to performic acid oxidation and the sulfoxides are stable to alkylation with iodoacetate. Hence, oxidation of alkylated proteins yields the acid-stable methionine sulfone only from unsubstituted methionine residues, whereas exhaustive alkylation followed by oxidation yields sulfone only from methionine sulfoxide residues.

Numerous chemical modifications of ribonuclease have been studied in the past in efforts to learn the nature of the amino acid residues required for the catalytic activity of the enzyme. Relatively few attempts have been made, however, to modify selectively the methionine residues in the molecule, largely owing to a scarcity of suitable reagents. Recently, Gundlach *et al.* (1959a,b) observed that iodoacetic acid reacted readily with methionine to form the carboxymethylsulfonium salt. Although the reaction with the free amino acid was relatively insensitive to pH, alkylation of the methionine residues in ribonuclease, with loss of enzymic activity, was found to occur easily only under acidic conditions (pH 2–3). Apparently, at neutral pH the methionine residues are buried in the interior of the ribonuclease molecule and are unavailable to iodoacetate. Indeed, Stark *et al.* (1961) observed that sulfonium salt formation would occur at neutral pH only if the organized structure of the molecule were first disrupted either by reduction of the disulfide bonds or by exposure to a denaturing agent such as 8 M urea or 4 M guanidinium chloride. From the work of Vithayathil and Richards (1960), the formation of a monosubstituted active derivative might be envisioned. They found that alkylation of the methionine residue in the S-peptide obtained by cleavage with subtilisin decreased greatly the tendency of the S-peptide and the S-protein to combine, but did not alter the enzymic activity of the combination.

Loss of activity following chemical modification of a group in a protein may depend not only upon the group modified but also upon the modification introduced. For this reason, we have explored the possibility, suggested by the work of Toennies and Callan (1939), of using hydrogen peroxide as a selective reagent for the conversion of methionine residues to residues of the sulfoxide. Instances of this conversion are known. Photooxidation of phosphoglucomutase and chymotrypsin in the presence of methylene blue has been found by Ray and Koshland (1960) to transform a methionine residue to the sulfoxide, but alteration of a histidine residue occurred simultaneously. Oxidation of methionine to the sulfoxide by atmospheric oxygen or hydrogen peroxide has recently been shown by Dedman *et al.* (1961) to be responsible for the loss of activity of ACTH earlier observed by Dixon (1956) and by Dedman *et al.*

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