probably affected by such factors as the *p*H and salt content.

The iron complex of the red protein is similar in many respects to those of the B_1-metal combining protein[27] (siderophilin, transferrin) from blood plasma and conalbumin.[28] They all show maximum absorbance at 460 to 470 mμ. Absorbance between 310 and 700 mμ of the red protein closely parallels that shown by the iron-transferrin complex of Laurell.[29] Absorptivity at 470 mμ, *p*H 7.0 varied between 0.48 and 0.55 for the iron-transferrin complex and compares with values of 0.49 to 0.55 for the red protein at *p*H 7.9. The red protein contains 0.10 to 0.12% iron, which, compares with a value of 0.126% for the iron-transferrin reported by Laurell.

The absorptivity of 0.62 and iron content of 0.159%[28] for the conalbumin complex, however, are significantly higher. It was also found that the ratio between the absorptivity maximum at 470 mμ to the minimum at 410 mμ is higher for conalbumin[28] and transferrin[29] complex than for the red protein, namely 1.46 and 1.41 compared to 1.1.

Both the conalbumin[28] and the B_1-metal combining protein[27] are found to combine with two molecules of iron per mole of protein, giving molecular weights estimated at 76,600 and 90,000. Using 0.12% iron for the red protein, a minimum molecular weight of 46,500 is calculated, giving a value of 93,000 which compares with a determined value of 86,100.

(27) B. A. Koechlin, THIS JOURNAL, **74**, 2649 (1952).
(28) R. C. Warner, *J. Biol. Chem.*, **191**, 173 (1951).
(29) C. B. Laurell, *Acta Chem. Scand.*, **7**, 1407 (1953).

The iron complex of the red protein appears to be more stable than those of conalbumin and transferrin. Warner[28] removed the iron from conalbumin at *p*H 4.7 with citrate buffer. The red protein complex, however, remained stable in the presence of an iron chelating resin at *p*H 4.0. With the B_1-metal combining complex, Inman[30] reports that EDTA rapidly dissociates the iron complex at *p*H 5.6. The red protein complex was stable under these conditions.

It is interesting to note that Schäfer, *et al.*,[31] found that radioactive iron added to a solution of milk whey proteins produced a pale red color and that the labeled iron moved as a single band in paper electrophoresis with a mobility similar to serum siderophilin. However, it has been reported by Gugler, *et al.*,[32] that the red protein of milk is not present in bovine serum.

Acknowledgments.—I am indebted to Mrs. R. Kelley and Miss L. Scroggins who made moisture, nitrogen, phosphorus and ash determinations, to Miss A. Smith for absorption spectra determinations, to Mr. J. Basch for lactoperoxidase determiuations, to Dr. R. F. Peterson for some ultracentrifuge determinations and to Mr. F. A. Scott and Mr. L. Cerankowski for ultracentrifuge runs. I am grateful to Dr. W. G. Gordon, Mr. N. J. Hipp and Dr. T. L. McMeekin for their suggestions and encouragement.

(30) J. K. Inman, 10th Conference on Plasma Protein, p. 46, January 1956.
(31) K. H. Schäfer, A. M. Brejer, W. Horst, H. Karte and W. Lenz, *Klin. Wochschr.*, **34**, 300 (1956).
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[CONTRIBUTION FROM THE LABORATORIES OF THE ROCKEFELLER INSTITUTE, NEW YORK, N. Y.]

Distribution Studies with Bovine Plasma Albumin[1]

BY T. P. KING, D. A. YPHANTIS AND L. C. CRAIG

RECEIVED DECEMBER 11, 1959

A solvent system composed of ethanol, *n*-propanol, ammonium sulfate and water is described for the countercurrent distribution of bovine plasma albumin. Due to the reactive sulfhydryl group of the albumin, it was found necessary to carry out the distribution with its *p*-mercuribenzoate derivative. The apparent inhomogeneity of the albumin was shown to be due to the presence of dimers and the dimer content could be lowered by treatment with thioglycolate.

Previous studies from this Laboratory have shown[2] that both bovine and human plasma albumin can be studied by countercurrent distribution in an acid system containing 2-butanol, ethanol, water, acetic acid and trichloroacetic acid. Although the *p*H of the system was low, 2.54, stabilization appeared at that time to be achieved by the effect of trichloroacetic acid.

Another interesting observation, apparently also an effect caused by the trichloroacetic acid, was a decided narrowing of the main band in the countercurrent distribution run. This occurred both with bovine and human plasma albumin. However, the partition coefficient (K) value for each of these protein preparations appeared to be very similar.

Shortly after this work was completed, another type of system made from a mixture of ethanol and ammonium sulfate solution[3] which provided a higher *p*H was studied with ribonuclease and lysozyme. The separations accomplished indicated this type of system to have considerable selectivity. More recent work (D. Eaker and T. P. King, unpublished results) has now shown a structural basis for the separation in that one of the ribonucleases separated differs from the main component by lack of the N-terminal lysine. Preliminary studies with this type of system modified slightly by addition of 1-propanol[4] on bovine and human

(1) This investigation was supported in part by a research grant, A-2493 B.B.C., from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, Public Health Service.
(2) W. Hausmann and L. C. Craig, THIS JOURNAL, **80**, 2703 (1958).
(3) T. P. King and L. C. Craig, *ibid.*, **80**, 3366 (1958).
(4) T. P. King, presented at the Fourth International Congress of Biochemistry, Vienna, 1958.

TABLE I

		Composition, % Monomer	Dimer	Polymer	$S_{20,w}$ [c]	$[\alpha]^{23}D$	SH [d]	Hg [e]
Bovine plasma albumin	[a]	89 ± 3	7 ± 3	4 ± 3	4.25 ± 0.08	−62 ± 2	0.91 ± 0.05	
	[b]	86	10	4			.05	0.85 ± 0.10
Cut 1, fig. 1	[b]	71	27	2				.40
Cut 2 fig. 1	[b]							.85
	[a]	86	10	4	4.29	−64	.89	
Cut 3, fig. 1	[b]	75	25	0				.70
Cut 4, fig. 2	[a]	90	5	4	4.44	−63	.87	
Albumin treated with 100 mole of thioglycolate	[a]						1.2	
	[b]	86	11	3				
Albumin treated with 200 mole of thioglycolate	[a]						3.0	
	[b]	75	9	16				
Cut 7, fig. 5	[a]	92	7	1	4.34	−64	1.01	

[a] Analyzed as free albumin. [b] Analyzed as mercurial albumin. [c] For the major component, in Svedbergs ($S = 10^{-13}$ sec.). [d] Expressed as mole of SH per mole of monomeric albumin. [e] Expressed as mole of mercury per 67,000 g. of abumin.

plasma albumins indicated a higher degree of selectivity than the earlier system containing trichloroacetic acid.

Although the first experiments with the ammonium sulfate system indicated that bovine and human plasma albumins could be differentiated readily and that both of them were mixtures, a problem of slow transformation as indicated by a slow shift in the partition ratio of bovine plasma albumin was encountered. This shift was largely suppressed by the presence of 0.09% of sodium caprylate in the system.

The results obtained were particularly interesting in view of the chromatographic experiences of Tiselius, Hjertein and Levine[5] with bovine plasma albumin on hydroxylapatite columns and of Sober, *et al.*,[6] on DEAE-cellulose columns which have also indicated heterogeneity. However, bovine plasma albumin has been shown to possess amino and carboxyl terminal amino acid homogeneity by Thompson[7] and White, *et al.*[8]

Bovine plasma albumin is known to contain seventeen disulfide linkages and one sulfhydryl group.[9] It, therefore, seems likely that the slow transformation noted could stem from the sulfhydryl group and its ability to undergo inter- or intra-molecular interchange. For this reason the studies reported here will be largely concerned with the *p*-mercuribenzoate derivative and the effect of thioglycolate treated derivatives. Since changes in molecular weight might be expected if intermolecular interchange is involved, ultracentrifugal studies have been included.

Experimental

The bovine plasma albumin used was a crystalline sample of Armour Company (Lot No. T68204). Mol. wt. of 67,000 and moisture content of 6% were used for all calculations.

The thioglycolic acid used was purified by vacuum distillation and stored at 4°. The sodium salt of *p*-chloromercuribenzoate was a product of the Sigma Chemical Company. It was found to have a molar extinction coefficient at 234 mμ of 1.74×10^4 at *p*H 4.6, in agreement with the value reported by Boyer.[10]

Sulfhydryl group determinations were made by the amperometric titration method of Benesch, *et al.*[11] The titration medium contained 0.15 μmole of protein in 5 ml. of H_2O, 1.5 ml. of 1 *M* tris nitrate buffer (*p*H 7.4) and 0.1 ml. of 1 *M* KCl. The titrant, 2×10^{-3} *N* $AgNO_3$, was added in 5 μl. aliquots. The values were found to be reproducible within 5%. The data are expressed as mole of sulfhydryl group per mole of monomeric albumin, as it may be assumed that the dimeric and polymeric fractions do not contain sulfhydryl groups.

Mercury analyses were made with the dithizone method.[12] A sample of the protein derivative (*ca.* 8 mg., 0.11 μmole) was digested for 4 hr. in 1 ml. of a mixture of equal parts of concentrated nitric and sulfuric acids. After digestion the solution was diluted to 10 ml. A 2.0 ml. aliquot was extracted with 5.0 ml. of 20 μ*M* dithizone in chloroform, and the optical density increase at 500 mμ of the chloroform phase was immediately determined. Due to the presence of some unknown agents, the color was unstable. With *p*-chloromercuribenzoate, the recovery was only 90%. It was assumed that the mercurial albumin would also give the same low yield. The correction factor, 1/0.9, was therefore used for the calculation of the mercury content of the protein samples.

Sedimentation analyses were carried out in a Spinco Model E ultracentrifuge at 50,740 r.p.m. using double channel cells. The solutions were 0.8% with respect to the protein concentration in a solution of NaCl + NaOAc + HOAc, 0.15, 0.02 and 0.03 *N* with respect to each (*p*H 4.4). The amounts of monomer and dimer were estimated from the exposures taken at 100 to 120 minutes after attaining a speed of 50,740 r.p.m., assuming the monomer schlieren peak to be symmetrical and neglecting any Johnston–Ogston effect. Material with a sedimentation coefficient greater than 4.3 was taken as the dimer fraction. A synthetic boundary cell run at 12,590 r.p.m. gave the total protein concentration, and the polymer content referred to that fraction of the sample absent in the high-speed runs.

Rotatory dispersion curves (between 350 and 650 mμ) were determined in a Rudolph oscillating spectrophotometric polarimeter with a Xenon light source. The protein solutions were of 0.5% concentration in water. The $[\alpha]D$ values reported here were evaluated from the Lowry plot of $1/[\alpha]$ against λ^2 thus giving a somewhat more precise and reliable value.

The analytical results of the various fractions are summarized in Table I.

The *p*-mercuribenzoate derivative of bovine plasma albumin was prepared by adding a 2 ml. solution of sodium *p*-chloromercuribenzoate (11.4 mg., 30 μmole) and ammonium hydroxide (30 μmole) to 600 mg. of bovine plasma albumin (8.4 μmole) dissolved in 5 ml. of acetate buffer (*p*H 4.6, $\Gamma/2 = 0.15$). After 1 hr. at 25°, the solution was passed

(5) A. Tiselius, J. H. Hjertein and O. Levine, *Arch. Biochem.*, **65**, 132 (1956).

(6) H. A. Sober, E. J. Sutter, M. M. Wyckoff and E. A. Peterson, THIS JOURNAL, **78**, 756 (1956).

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(8) W. F. White, J. Shields and K. C. Robbins, THIS JOURNAL, **77**, 1267 (1955).

(9) W. L. Hughes in "The Proteins," H. Neurath and K. Bailey, Vol. II B, Academic Press, Inc., New York, N. Y., 1954, p. 663.

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

(11) R. E. Benesch, H. A. Lardy and R. Benesch, *J. Biol. Chem.*, **216**, 663 (1955).

(12) E. B. Sandell, "Colorimetric Determination of Traces of Metals," Interscience Publishers, Inc., New York, N. Y., 1944, p. 321.

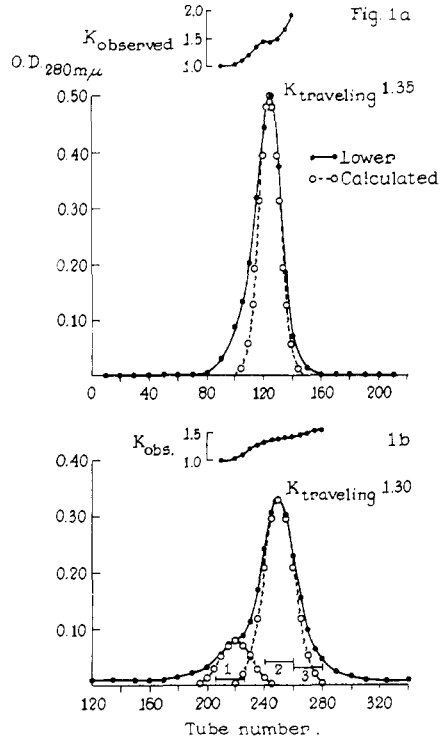

Fig. 1.—Distribution of the mercurial derivative of bovine plasma albumin, (a) 213 transfers and (b) 442 transfers.

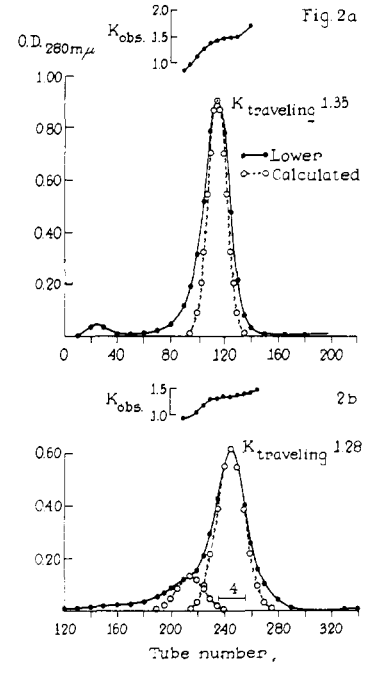

Fig. 2.—Distribution of the mercurial derivative of bovine plasma albumin after seven days standing at 25°, (a) 200 transfers and (b) 437 transfers.

through a 25 × 1.6 cm. column of mixed bed resin, Amberlite MB-1 (16–50 mesh). The flow rate was 30 ml. per hour. The solution was then lyophilized to give 570 mg. of the desired derivative.

Distributions were carried out at 25° in a 220-tube machine with a 10 ml. capacity per lower phase in each tube.[13] The solvent system was composed of ethanol, 1-propanol, ammonium sulfate solution (40 g. in 100 ml. of water) and water with volume ratios of 1.1:0.9:2.0:2.0. The *p*H of the resulting upper and lower phases were 5.7 and 5.4, respectively, as measured with the glass electrode.

For the distribution patterns presented here, samples in the range of about 300 to 900 mg. of the mercurial derivative of albumin were used. The protein derivative was dissolved in 20 ml. of water and 20 ml. of the ammonium sulfate solution. After cooling to 20–22°, a mixture of ethanol (11 ml.) and 1-propanol (9 ml.) was added. After shaking, both phases were adjusted to 40 ml. with pre-equilibrated phases of the solvent system. The solution was then placed in the first four tubes of the distribution train. The settling time was initially about 8 to 9 minutes but gradually decreased to 3 to 4 minutes. Six complete tips were found sufficient to reach equilibrium. The protein concentrations after the distribution were determined by ultraviolet absorption at 280 mμ in a cell with a 1 cm. optical path. The solutions tended to become cloudy during reading but could be cleared by addition of a tiny drop of water.

The fractions were recovered by concentrating each fraction to one fifth of its volume in a rotatory evaporator at 8 to 10° and 2 mm. After standing overnight the precipitated protein was collected by centrifugation. The precipitate was dissolved in water, dialyzed against water at 4° for 40 hr. and, after centrifugation, the solution was lyophilized. The dialysis was made in Visking #23/32 cellophane tubing which had been washed and soaked in 2×10^{-3} *M* disodium versenate overnight.

To remove the mercurial group, the albumin derivative was passed through a thioglycolate column as described by Dintzis.[14]

The pattern in Fig. 1 was obtained with 467 mg. of the mercurial derivative of crystalline albumin. From tubes 240–260 (Cut 2), 210 mg. of albumin were recovered and 36 mg. from tubes 206–226 (Cut 1).

The pattern in Fig. 2 was obtained with 900 mg. of the mercurial derivative of crystalline albumin placed in eight tubes. The protein was dissolved in 80 ml. of upper phase and 80 ml. of lower phase. The distribution was begun after standing for seven days at 25°. A small amount of precipitate which had formed was removed before the distribution was started. From Cut 4, tubes 235–255, 400 mg. of protein were recovered.

Thioglycolate reductions at *p*H 5.9 were carried out as follows: To a solution of bovine plasma albumin (710 mg., 10 μmole) and sodium bicarbonate (40 μmole) in 5.6 ml. of water at 0° was added a 3 ml. solution of thioglycolic acid (184 mg., 2000 μmole) and sodium bicarbonate (168 mg., 2000 μmole). The mixture had a *p*H of 5.9 at 0°. After 18 hr., the pink solution was passed through a 23 × 1.9 cm. column of mixed bed resin at a flow rate of 30 ml. per hour, then lyophilized to give 580 mg. of product. When 1000 μmole of sodium thioglycolate were used, a similar recovery was obtained. The samples were converted to the *p*-mercuribenzoate derivative for distribution.

Distribution of 426 mg. of 100 mole excess thioglycolic acid reduced albumin gave the patterns in Fig. 3. From tubes 236 to 260 (Cut 5), 222 mg. were recovered.

Distribution of 491 mg. of 200 mole excess thioglycolic acid reduced albumin gave the patterns in Fig. 4. Here there was noted a small amount of insoluble material which was removed before distribution. Cut 6 from tubes 230 to 255 gave 210 mg. of recovered material.

Cuts 5 and 6 were combined for redistribution (357 mg. used). This gave the patterns shown in Fig. 5. Cut 7 from tubes 238 to 264 gave 201 mg. of recovered protein. After removal of the mercurial group, the albumin had a diffusion coefficient value of $D_{20,w} = 5.90 \pm 0.12$ F. (Fick = 10^{-7} cm.^{2} sec.$^{-1}$). The diffusion coefficient was determined from the synthetic boundary cell run in the ultracentrifuge, using the height-area method. A mol. wt. of 67,000 ± 3000 was calculated from the diffusion and sedimentation coefficients and the value of 0.734 for the partial specific volume.[15] Equilibrium ultracentrifugation in ultrashort columns[16] gave a value of 67,700 ± 2100 for the mol. wt.

(13) L. C. Craig, W. Hausmann, E. H. Ahrens, Jr., and E. J. Harfenist, *Anal. Chem.*, **23**, 1236 (1945).

(14) H. Dintzis, Ph.D. Dissertation, Harvard University, 1952.

(15) J. T. Edsall in "The Proteins," H. Neurath and K. Bailey, Vol. I B, Academic Press, Inc., New York, N. Y., 1954, p. 636.

(16) K. E. Van Holde and R. L. Baldwin, *J. Phys. Chem.*, **62**, 734 (1958).

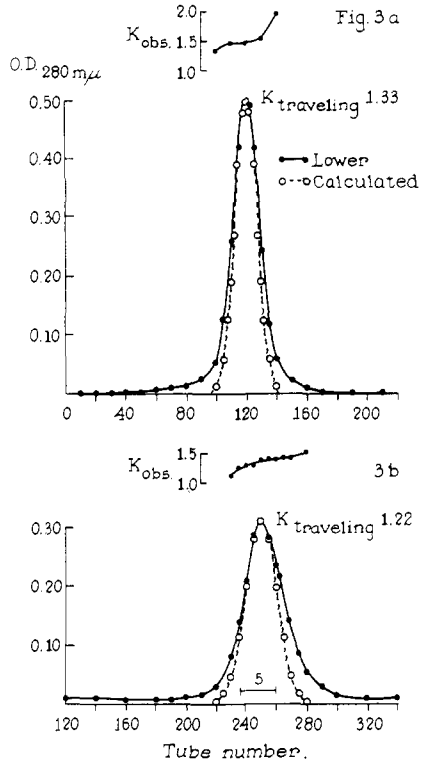

Fig. 3.—Distribution of the mercurial derivative of bovine plasma albumin treated at *p*H 5.9 with 100 mole excess of thioglycolate, (a) 210 transfers and (b) 455 transfers.

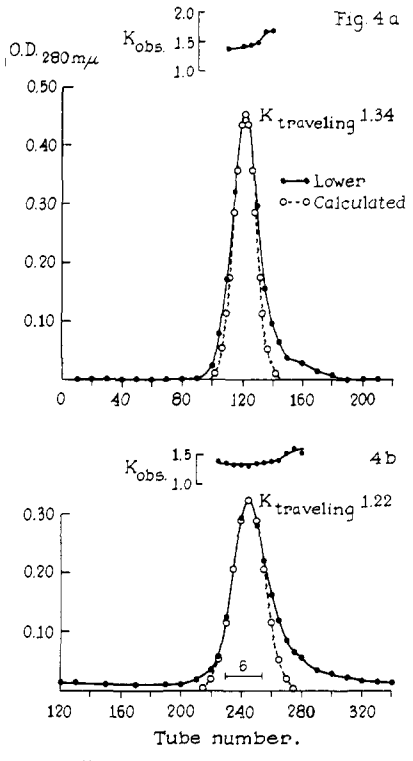

Fig. 4.—Distribution of the mercurial derivative of bovine plasma albumin treated at *p*H 5.9 with 200 mole excess of thioglycolate, (a) 213 transfers and (b) 446 transfers.

A reduction was also carried out at *p*H 9.0 and 0° with 800 mg. of bovine plasma albumin (11.3 μmole), thioglycolic acid (208 mg., 2260 μmole) and sodium carbonate (162 mg., 1530 μmole) in 10.5 ml. of water. After 6 hr. the pink solution was passed through a mixed bed resin. Some coagulation took place on the resin but 420 mg. of reduced albumin were eluted. Titration of this eluate showed 4.7 mole sulfhydryl groups per 67,000 g. of the reduced albumin. Due to the slight solubility of sodium *p*-chloromercuribenzoate in acetateb uffer (*p*H 4.6), the conversion to the mercurial derivative in this case was carried out in distilled water. The reduced albumin (400 mg., 5.6 μmole) in 5.0 ml. of water was treated with *p*-chloromercuribenzoate (22.8 mg., 60 μmole) and ammonium hydroxide (65 μmole) in 2 ml. of H_2O for thirty minutes. After deionization and lyophilization of the cloudy solution, 390 mg. of the desired derivative was obtained. Distribution of 362 mg. of this derivative gave the pattern in Figure 6. Again a small amount of insoluble material was removed before the distribution. From Cut 8, tubes 100 to 120, 90 mg. were recovered. After removal of the mercurial group, it showed a rotation of $[\alpha]^{23°}D = -65 \pm 2°$ and 1.09 ± 0.05 mole of sulfhydryl group. In comparison with this, fractions recovered from Cut 9, tubes 121 to 140, and Cut 10, tubes 141 to 160, were found to contain 2 to 3 moles of sulfhydryl group.

All the albumin samples recovered were found to contain less than 0.1 mole of bound fatty acid as analyzed by the method described in the following paper. The crystalline bovine plasma albumin from Armour was found to contain 0.93 ± 0.10 mole of fatty acid.

Discussion

In a previous paper dealing with the distribution studies of ribonuclease and lysozyme,[2] it was reported that bovine plasma albumin had an unstable *K* value in a solvent system composed of ethanol, *n*-propanol, ammonium sulfate and water but that the rate of this *K* change was diminished upon incorporation of 0.09% of sodium caprylate into the system.

The suppression of the *K* change was thought to be the stabilization of the albumin against denaturation through caprylate ion binding. Luck and his associates[17,18] first reported that caprylate ions protected plasma albumin against heat and urea denaturation as determined by the "cloud point" and viscosity measurements. From our further studies, we have now concluded from the sulfhydryl and sedimentation analyses that caprylate ions can afford partial but not complete protection against denaturation in the system we have used.

Bovine albumin could be recovered in 80–90% yield after seven days in the solvent system containing caprylate ions even though it had lost 80% of its titratable sulfhydryl group while concurrently the dimer content had increased to about 40%. In contrast, it was possible to recover only 60% of the albumin in a water-soluble form after a similar treatment in the solvent system without the addition of caprylate ions. The soluble portion contained about 60% dimer and 20% of the original sulfhydryl group.

Our earlier consideration of the stabilizing action of trichloroacetic acid on the plasma albumins has also been further investigated. By distribution in the system of trichloroacetic and *sec*-butanol, bovine plasma albumin could be separated into two components after 300 transfers. The major component with a *K* value of 0.8 was found to be rich in monomer (80%), while the minor component

(17) G. A. Ballou, P. D. Boyer, J. M. Luck and F. G. Lum, *J. Biol. Chem.*, **153**, 589 (1944).

(18) P. D. Boyer, *ibid.*, **158**, 715 (1945).

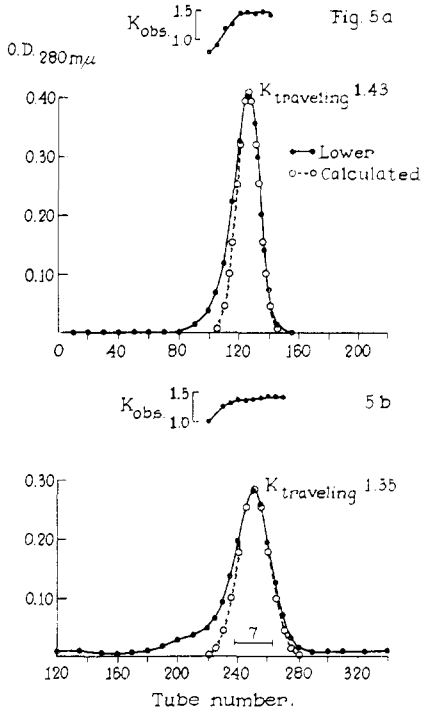

Fig. 5.—Redistribution of Cuts 5 and 6, (a) 214 transfers and (b) 437 transfers.

(*ca.* 15% of the total sample) with a K value of 0.24 was found to be rich in dimer (60%). Both fractions were found to be devoid of sulfhydryl group. It appears that in the process of distribution or recovery the sulfhydryl group even in the monomer has become of the masked type. A similar disappearance of the sulfhydryl group in acid solutions of the plasma albumins without concomitant formation of dimer has been described by Simpson and Saroff.[19]

In the case of human plasma albumin in the trichloroacetic acid system, a second peak was not separated. An increase of about 5% in dimer content and loss of determinable sulfhydryl group were noted.

The disappearance of the sulfhydryl group and the formation of dimer indicated one important reaction site to be the sulfhydryl group. This is to be expected from the published experiences including the report of Huggins, *et al.*,[20] that this group is the important site for gel formation of albumin in urea. It was hoped that this difficulty would be circumvented by use of the *p*-mercuribenzoate derivative of the albumin. Experimentally at 213 transfers, this derivative of crystalline albumin gave the distribution pattern shown in Fig. 1a and at 442 transfers, in Fig. 1b. Here the main component had a K value of 1.35 ± 0.05, and after removal of its protecting group it was found still to contain 10 ± 3% dimer. Inspection of the analyses in Table I will show that over half of the 10% dimer present in the original sample could be accounted for in the fraction with a K of 1

(19) R. B. Simpson and H. A. Saroff, THIS JOURNAL, **80**, 2129 (1958).

(20) C. Huggins, D. F. Tapley and E. V. Jensen, *Nature*, **167**, 591 (1951).

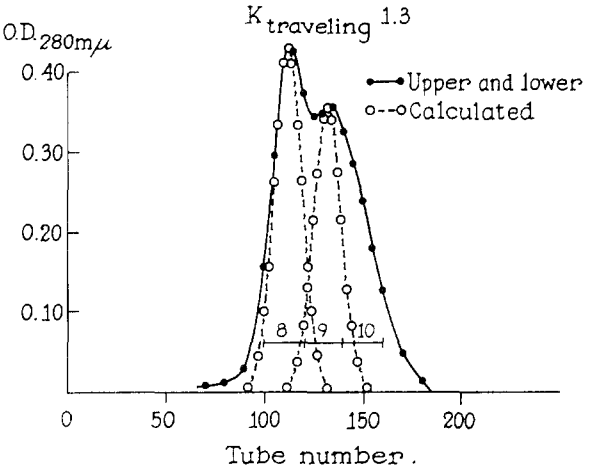

Fig. 6.—Distribution of the mercurial derivative of bovine plasma albumin treated at *p*H 9 with 200 mole excess of thioglycolate, 200 transfers.

and the small shoulder on the right. When the amount of dimer in the fractions was totalled, it was clear that there had been an appreciable increase either during the distribution or the recovery. This could be caused by an inherent instability of the mercurial albumin in the solvent system or more probably could arise from a slight tendency of the derivative to dissociate and then reform the dimers or polymers.

In an experiment to try to decide between these two alternatives, the mercurial albumin was dissolved in the solvent system and permitted to stand for seven days before the distribution. The resulting patterns in Fig. 2 were clearly similar to those in Fig. 1, except for the presence of a small band with a K of 0.1. The results seemed to be in favor of the thesis that there was dissociation of the mercury radical with a resultant slow dimerization of the protein. If such were the case, this would then lead to a broadening of the trailing edge of the main band on prolonged distribution on account of the continuous removal of the formed dimer, as was indeed observed. Under the conditions of distribution, the dissociation reaction would be further assisted by the continuous removal of the mercurial radical, as it had been determined that the *p*-chloromercuribenzoate salt had a K value of 0.65 in the solvent system. Attempts to reverse the dissociation by employing a system containing 2×10^{-5} M *p*-chloromercuribenzoate was impractical on account of the binding of the mercurial compound by albumin and a resultant broadening of the albumin band.

It was next of interest to study the reversibility of the dimer formation by a reducing agent, thioglycolic acid. Katchalski, *et al.*,[21] have reported that bovine or human plasma albumin after treatment with 100 to 700 mole excess of thioglycolic acid between *p*H 5 to 7 gave only one equivalent of sulfhydryl group. In the present investigation, it was found that the amount of sulfhydryl group formed was dependent on the thioglycolate concentration at *p*H 5.9. No increase in sulfhydryl

(21) E. Katchalski, G. S. Benjamin and V. Gross, THIS JOURNAL, **79**, 4096 (1957).

group was noted when the reduction was carried out with a 50-mole excess of thioglycolic acid but with a 100-mole excess the treated albumin had 1.2 mole of sulfhydryl group. With a 200-mole excess, the treated albumin had 3.0 mole of sulfhydryl group and also contained 16% polymer.

The treated albumins were converted to mercurial derivatives. Figure 3 was obtained with albumin treated with 100-mole excess of thioglycolic acid, and Fig. 4 was obtained with albumin treated with 200-mole excess of thioglycolic acid. The band with a K of 1 present in the untreated sample had disappeared, but a new band had appeared with a K of 1.6 to 2.0. Material isolated from the region with a K of 1.6 to 2.0 was estimated to be composed of 70% monomer, 14% dimer and 16% polymer.

The albumin recovered from the main bands in Fig. 3 and 4 was combined and redistributed to give the patterns in Fig. 5. Here the small band with a K of 1 reappeared because of the tendency of the mercury derivative to dissociate as discussed above.

When the albumin was treated with thioglycolate at pH 9.0, it showed 4.7 mole of sulfhydryl group The distribution of the mercurial derivative of this sample gave the pattern shown in Fig. 6. Based on the albumin band (K = 1.3) in the distribution pattern, it was calculated that about 15% of the albumin in the reduction mixture was the unaltered albumin but 50% had formed insoluble polymers.

The reduction of the disulfide bonds of the albumin could conceivably occur by two distinct pathways, (a) the susceptible disulfide linkages of all the albumin molecules cleaved one at a time with essentially the same rates or (b) the initial cleavage of one disulfide linkage of an albumin which could then lead to the explosive cleavage of the remaining susceptible linkages present in the same molecule. In the above reduction experiments, the albumin concentration was held at a constant value but the thioglycolate concentrations and pH of the solutions were varied. In all cases, significant amounts of apparently unchanged albumin could be recovered from the reaction mixture. While a detailed analysis of the products was not possible due to formation of polymers, the results suggest that reaction pathway (b) is favored and that cleavage of the first linkage of the albumin introduced new labilities to the protein structure. Another possible reaction with the thioglycolate treatment would be in the formation and equilibration of albumin isomer(s) due to the migration of the sulfhydryl group in the chain. This is suggested by the sulfhydryl group value of one mole per mole of albumin isolated from thioglycolate treated sample in Fig. 5, as compared with the value of approximately 0.9 from untreated albumins in Figs. 1 and 2. Such reaction products would not have been differentiated if the new isomers had K values similar to that of the original albumin. In the case of human plasma albumin as described in the following paper,[22] there seemed to be definite evidence for this type of interchange.

The reduction experiments support the belief that the band with a K of 1 and the shoulder on the right edge of the main band were products of sulfhydryl-disulfide reactions of the albumin, thus indicating that the observed broadness of the albumin bands did not result from some slow disorganization of the secondary structure by the organic solvents. This observation was fully substantiated by the measurements of the rotatory dispersion and sedimentation rate of the recovered albumin which indicates that it had retained its native secondary structure, shape and molecular size. In terms of countercurrent distribution, those observations implied the possibility of fractionation of many proteins in these near-critical point, high-salt content solvent systems if their reactive functional sites could be blocked. It perhaps should be stressed that the sulfhydryl-disulfide groups are not the only sensitive sites in proteins, since chymotrypsinogen, a protein containing no detectable sulfhydryl group, was rapidly denatured in the present solvent system.[3]

Acknowledgments.—The authors are grateful to Mrs. J. O'Brien and Mr. O. Griffith for their technical assistance.

(22) T. P. King, D. A. Yphantis and L. C. Craig, THIS JOURNAL, **82**, 3355 (1960).

[CONTRIBUTION FROM THE LABORATORIES OF THE ROCKEFELLER INSTITUTE, NEW YORK, N. Y.]

Distribution Studies with Human Plasma Albumin[1]

BY T. P. KING, D. A. YPHANTIS AND L. C. CRAIG

RECEIVED DECEMBER 11, 1959

Dissolution of human plasma albumin in a solvent system composed of ethanol, 1-propanol, water and ammonium sulfate leads to the formation of a mixture of dimer and monomer. The two fractions can be separated by countercurrent distribution in this solvent system. The dimer was shown to be formed through an oxidative coupling of two molecules of mercaptalbumin.

Introduction

In the preceding paper[2] concerning the distribution of crystalline bovine plasma albumin, it was reported that the albumin slowly underwent dimer and polymer formation when dissolved in a solvent system of ethanol, n-propanol, water and ammonium sulfate and that the reaction rate was diminished by the presence of sodium caprylate in the solvent system. The present paper will deal with the fractionation of human albumin in the same solvent system.

(1) This investigation was supported in part by a research grant, A-2493 B.B.C., from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, Public Health Service.

(2) T. P. King, D. A. Yphantis and L. C. Craig, THIS JOURNAL, **82**, 3350 (1960).