Fractions 9–20 were evaporated to dryness, combined and recrystallized three times from ethanol–water. The final melting point was 159–161°.

Anal. Calcd. for $C_{16}H_{20}N_2O_9$: C, 50.00; H, 5.22; N, 7.29. Found: C, 49.88; H, 5.24; N, 7.27.

Ethyl Thiocladinoside.—Three grams (0.017 mole) of cladinose was dissolved in 60 ml. of dry ethyl mercaptan containing 1.5 g. of dry hydrogen chloride. After the solution had stood at room temperature for one day, it was concentrated under reduced pressure at room temperature. The residue was dissolved in 60 ml. of chloroform, and the solution was washed with 15 ml. of 5% sodium hydroxide followed by 15 ml. of water. The solvent was again removed under reduced pressure and the residue was distilled. The yield of colorless liquid boiling at a bath temperature of 110–120° at 0.3 mm., was 2.8 g. (74%), $n^{25}D$ 1.4901.

Anal. Calcd. for $C_{10}H_{20}O_3S$: C, 54.52; H, 9.15; S, 14.55. Found: C, 54.67; H, 9.50; S, 14.83.

INDIANAPOLIS, INDIANA

[CONTRIBUTION FROM THE UNIVERSITY LABORATORY OF PHYSICAL CHEMISTRY RELATED TO MEDICINE AND PUBLIC HEALTH, HARVARD UNIVERSITY]

Dimerization of Serum Mercaptalbumin in Presence of Mercurials. II. Studies with a Bifunctional Organic Mercurial[1a,b,c]

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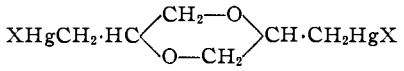
The dimerization of human serum mercaptalbumin (ASH) has been studied with a bifunctional organic mercurial (XHgRHgX) which serves to link two albumin molecules together through the sulfhydryl groups to form a dimer (ASHgRHgSA). When the reagents are mixed in the proportion 0.5 mole mercurial per mole albumin, equilibrium lies almost completely on the side of dimer formation. The dimer dissociates in the presence of an excess of the mercurial or of added cyanide. The equilibrium attained in the latter reaction has provided a method of estimating the equilibrium constant for dimer formation, $(ASHgRHgSA)/(ASHgRHg^+)(As^-)$ as being near $10^{18.2}$ at *p*H 4.75, in acetate buffer at ionic strength 0.05, and 25°. The corresponding equilibrium constant for the mercury dimer (ASHgSA) previously studied is $10^{13.5}$, which is less by a factor of 50,000. The two albumin molecules must approach about 10 Å. closer in ASHgSA than in ASHgRHgSA, and the steric repulsions involved are probably primarily responsible for the difference between the two equilibrium constants. The rate of dimer formation is faster by two to three thousand-fold for ASHgRHgSA formation than for ASHgSA formation. However, both reactions are affected by *p*H to about the same extent. The bimolecular velocity constant for dimer formation with the bifunctional organic mercurial falls from approximately 69,000 l. mole^{-1} min.$^{-1}$ at *p*H 5 and 25° to 4100 at *p*H 6. The temperature coefficient of the velocity constant is large, the energy of activation being of the order of 20 kcal./mole both at *p*H 5.5 and *p*H 6. The standard entropy of activation is large and positive. Possible explanations for this unusual entropy value are discussed.

Equilibrium and kinetic studies on the dimerization of mercaptalbumin in the presence of mercuric salts have already been reported in detail.[2] In the present paper, a briefer report is presented of studies on dimerization with a bifunctional organic mercurial



I

X may be chloride, acetate or nitrate; the acetate was the salt most commonly used in our experiments. When X is nitrate, the resulting compound has been termed by Chatt,[3] 3,6-bis-(nitratomercurimethyl)-dioxane. However, in what follows, we shall refer to it either as XHgRHgX or as "the bifunctional mercurial." A preliminary report on the interaction of this mercurial with mercaptalbumin already has been given.[4] The reactions involving formation and dissociation of the albumin dimer may be formulated by equations which exactly correspond with those previously employed[2] to describe the reaction with mercuric salts.

$$ASH + XHgRHgX \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ASHgRHgX + H^+ + X^- \quad (1)$$

$$ASHgRHgX + ASH \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ASHgRHgSA + H^+ + X^- \quad (2)$$

$$ASHgRHgSA + XHgRHgX \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} 2ASHgRHgX \quad (3)$$

No quantitative evidence is yet available concerning the velocity of reaction 1 but it appears to be extremely rapid, so that reaction 2, here as in our earlier studies, appears to be the rate-controlling step. The velocity constant of this reaction, however, is far greater for dimerization with the organic mercurial than with simple mercuric salts. The temperature coefficient of both reactions is large. The significance of the energy and entropy of activation is considered below in the discussion.

Materials and Methods

Materials.—The albumin preparations were the same as those used in the study[2] of the formation and dissociation of the mercury dimer (ASHgSA). In the earlier experiments preparations of the class previously denoted as type A were employed. The later studies of velocity constants at *p*H 5.5 and *p*H 6 at different temperatures were carried out with a preparation previously denoted as of type C.

The organic mercurial XHgRHgX was prepared in the form of 3,6-bis-(acetatomercurimethyl)-dioxane, essentially

(1) (a) This work has been supported by funds of Harvard University and the Eugene Higgins Trust, by grants from the Rockefeller Foundation and the National Institutes of Health, and by contributions from industry. (b) This paper is No. 104 in the series "Studies on the Plasma Proteins" from blood collected by the American Red Cross, on products developed by the University Laboratory of Physical Chemistry Related to Medicine and Public Health, Harvard University. (c) Some preliminary reports of this investigation have been cited in footnote (1c) of the preceding paper. (See also J. T. Edsall, R. H. Maybury and R. B. Simpson, Abstracts, 124th Meeting, American Chemical Society, 1953, p. 17C).

(2) H. Edelhoch, E. Katchalski, R. H. Maybury, W. L. Hughes, Jr., and J. T. Edsall, THIS JOURNAL, **75**, 5058 (1953).

(3) J. Chatt, *Chem. Revs.*, **48**, 7 (1951).

(4) R. Straessle, THIS JOURNAL, **73**, 504 (1951).

by the methods previously described by Biilmann, Hofmann and Sand.[5] The first preparations were made by one of us (R. S.); for later preparations we are indebted to Howard M. Dintzis. Equivalent amounts of allyl alcohol and mercuric acetate were dissolved in water, and the solution warmed briefly to about 60°. It was then allowed to cool gradually, and stood at room temperature until the following morning, when it was found that a large amount of crystals had formed. The compound was recrystallized three times from hot water and dried in a vacuum desiccator. Carbon (by Van Slyke wet carbon determination) found 18.2; calcd. for $C_{10}H_{16}O_6Hg_2$, 18.95. Benzoic acid analyzed by the same method gave approximately 98% of the theoretical figure for carbon; so that the correct figure for the mercurial may be nearer to 18.4. If it were assumed that the crystals contain one molecule of water of hydration, this would improve the fit between observed and calculated values; and this possibility is not excluded.

X-Ray studies of the crystals by Dr. Barbara W. Low showed that they were monoclinic. Approximate dimensions for the unit cell have been obtained and will be reported later.

Light Scattering Measurements.—These were carried out by the methods already described.[2] However, in the kinetic studies, since the reaction was extremely rapid, it was necessary to mix the albumin solution and the mercurial as rapidly as possible in the light scattering cell, and then to place the cell in the path of the light beam with a minimum of delay. Ordinarily, therefore, instead of stirring the solution gently with a magnetic stirrer as in the previous experiments, the light scattering cells were covered with a double thickness of carefully cleaned parafilm immediately after mixing the reagents, and the cell was then rapidly inverted several times and set in position for a reading. This procedure involved a risk of carrying dust or other particles from the parafilm into the solution and thereby obtaining falsely high readings in the light scattering measurements. However, control readings on mercury-free albumin solutions and on pure solvent, before and after the cells were similarly inverted, indicated a very small and indeed practically negligible rise in the turbidity due to this treatment. Before the kinetic studies were carried out, the turbidity of the albumin solutions in the absence of mercury was determined as a function of protein concentration, in the same buffers used for the kinetic measurements. The slope of the curve for (c/τ) as a function of c was thus derived and is recorded below in Table I. It was further found desirable, in order to obtain the most accurate possible results, to prepare separate albumin solutions at each protein concentration, and to centrifuge each one separately, for 2 to 4 hours in the refrigerated centrifuge at 20,000 times gravity, to remove any suspended particles. The solutions were then pipetted into the light scattering cells using dust-free pipets. Earlier measurements had commonly involved dilution of the solution within the cell itself by the addition of more buffer from a dust-free pipet, in order to make measurements at lower concentrations. This procedure, although convenient and economical of material, was found to give less dependable and reproducible results than the method of making up each solution separately.

Experimental Results

Equilibrium Measurements.—Determination of the weight fraction of dimer at equilibrium as a function of the molar ratio of mercurial to albumin was carried out at *p*H 4.75 and the results are shown in Fig. 1. Since at this *p*H the reaction was extremely rapid, it was found that the turbidity measurements attain their final steady value within a few minutes after mixing the reagents. The maximum dimer formation, as would be expected from the theoretical discussion given in the preceding paper,[2] (eq. 13 and Figs. 5 and 6), occurs at a molar ratio mercurial/mercaptalbumin of 0.5, taking the molecular weight of the albumin as approximately 69,000. The curve rises to the maximum and descends again much more sharply than the corresponding curve for mercuric chloride (see Fig. 6 in ref. 2). This indicates, in accordance with all our other findings on the bifunctional mercurial, that the equilibrium lies almost completely on the side of dimer formation. However, the absolute magnitude of the peak in the curve corresponds at this *p*H to a weight fraction dimer of approximately 0.86, rather than 1.0. We have as yet found no adequate explanation for this anomaly. The studies reported below on the velocity constant of dimer formation at *p*H 5.5 and *p*H 6 indicated

(5) E. Biilmann, *Ber.*, **33**, 1641 (1900); K. A. Hofmann and J. Sand, *ibid.*, **33**, 2692 (1900); J. Sand, *ibid.*, **34**, 2906 (1901); J. Sand and K. A. Hofmann, *ibid.*, **33**, 1358 (1900).

TABLE I

DIMERIZATION OF HUMAN SERUM MERCAPTALBUMIN WITH BIFUNCTIONAL MERCURIAL

*p*H 5.5; $\Gamma/2$ = 0.05 (acetate); 1% mercaptalbumin; $d(c/\tau)/dc$ = 13; D indicates weight fraction of dimer; see eq. 7.

T = 25°			T = 6°		
Time, min.	D	$D/(1-D)$	Time, min.	D	$D/(1-D)$
1.2	0.52	1.08	2.0	0.36	0.56
1.8	.58	1.38	2.35	.375	.60
2.2	.62	1.63	4.3	.45	.82
2.8	.67	2.03	6.4	.500	1.00
3.5	.71	2.45	8.1	.55	1.22
4.55	.76	3.16	11.0	.61	1.56
5.25	.79	3.76	(12.7	.66	1.94)
6.65	.82	4.55	16.2	.66	1.94
9.35	.89	8.1	17.5	.67	2.03
16.0	.97	32.0	22.5	.71	2.48
29	.98		24.2	.72	2.60
39	.98		27	.74	2.84
64	.99		29.7	.76	2.92
200	1.00		33.7		
300	1.00		52.4	.83	4.9
			55	.85	5.65
			57.6	.85	
			12 hr. later	.99	
Estimated velocity constant k_2 = 9800 l. mole^{-1} min.$^{-1}$			Estimated velocity constant k_2 = 1230 l. mole^{-1} min.$^{-1}$		

*p*H 6.0; $\Gamma/2$ = 0.05 (acetate); 1% mercaptalbumin; $d(c/\tau)/dc$ = 18; D indicates weight fraction of dimer; see eq. 7

T = 26°			T = 12°		
Time, min.	D	$D/(1-D)$	Time, min.	D	$D/(1-D)$
2.5	0.316	0.462	2.5	0.240	0.316
3.75	.416	.714	4.0	.270	.370
4.5	.470	.885	6.0	.330	.492
5.0	.517	1.07	7.5	.355	.550
6.0	.562	1.28	8.8	.373	.595
7.5	.610	1.56	11.0	.423	.733
9.0	.665	1.98	12.5	.423	...
11.0	.705	2.38	14.5	.450	.820
14.5	.765	3.26	17	.488	.954
18.5	.788	3.71	22.5	.543	1.19
23.5	.850	4.00	28	.570	1.32
31	.920	11.5	32	.605	1.53
40	.950		41.5	.630	1.70
55	.975		44	.650	1.85
75	.98		52.5	.702	2.36
101	1.00		2200	.94	
137	1.00		Estimated velocity constant k_2 = 520 l. mole^{-1} min.$^{-1}$		
943	1.00				
Estimated velocity constant k_2 = 2900 l. mole^{-1} min.$^{-1}$					

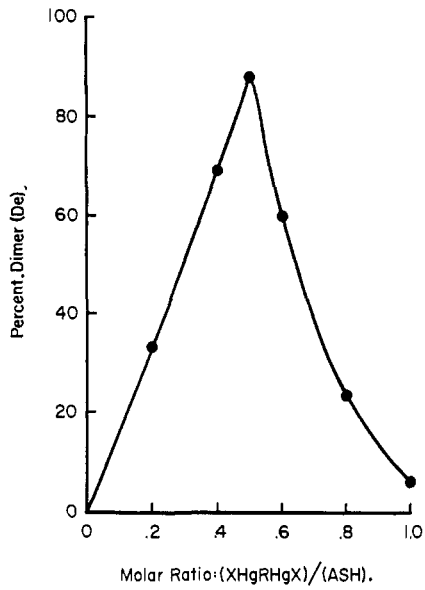


Fig. 1.—Titration of human mercaptalbumin with the bifunctional mercurial, showing percentage dimer at equilibrium as a function of the ratio of total mercurial to total ASH, free and bound: acetate buffer, $\Gamma/2$ 0.05, *p*H 4.75, 0.01 g. protein/ml., temperature 25°.

clearly that the weight fraction of dimer at equilibrium was 1 within the limits of experimental error when equilibrium was attained at these *p*H values.

Further evidence that the equilibrium lies far on the side of dimer formation was obtained from a study of the action of cyanide in dissociating the dimer. A typical experiment is shown in Fig. 2. Addition of one mole of KCN per mole of the dimer (ASHgRHgSA) caused fairly rapid, but only partial, dissociation. Since the reaction was carried out at *p*H 4.75, the cyanide may be taken as being completely in the form of HCN, and the reaction may be written

$$\text{ASHgRHgSA} + \text{HCN} \rightleftarrows \text{ASHgRHgCN} + \text{ASH}$$

In a 1% protein solution, the initial concentration of both dimer and HCN is approximately 7×10^{-5} *M*. The decrease in light scattering shows that the weight fraction of dimer, when the reaction has reached equilibrium, has decreased from approximately 0.95 to the order of 0.56. Thus, approximately 3×10^{-5} mole of dimer per liter had disappeared with an equivalent amount of HCN, leaving approximately 4×10^{-5} mole HCN free in solution. Equivalent amounts of ASH and ASHgRHgCN were formed. Thus, for the equilibrium constant, which we denote as K_{A1}, we obtain roughly

$$K_{A1} = \frac{(\text{ASHgRHgSA})(\text{HCN})}{(\text{ASHgRHgCN})(\text{ASH})} = \frac{0.35 \times 0.4}{0.3 \times 0.3} \cong 1.4$$

Addition of 3 moles KCN per mole dimer (see Fig. 2) reduces the weight fraction of dimer at equilibrium to the order of 0.25, giving an estimate of $K_{A1} \cong 0.9$. Tentatively we take this equilibrium constant as equal to unity. The implications of these experiments are considered further in the discussion.[6]

(6) The discussion given here ignores the possible reaction

$$\text{ASHgRHgCN} + \text{HCN} \rightleftarrows \text{NCHgRHgCN} + \text{ASH}$$

It is probable that this reaction is unimportant except in the presence of a considerable excess of HCN, and that we are, therefore, justified in ignoring it under the conditions of our experiments. The removal of the second mercaptalbumin molecule from the dimer is probably much more difficult than the removal of the first. See eq. 17 and 21 below, and accompanying discussion.

(7) See ref. 2, pp. 5060–5061.

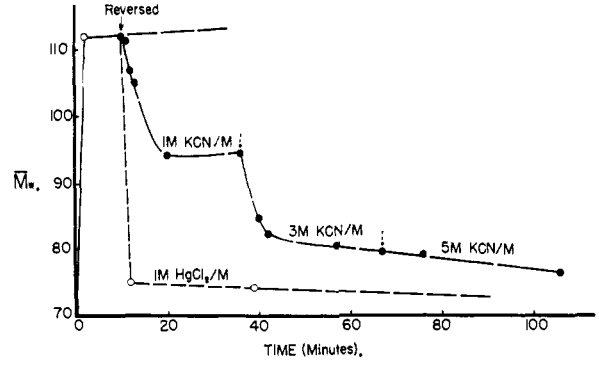


Fig. 2.—Formation of dimer of mercaptalbumin with the bifunctional mercurial, and reversal of dimerization on addition of cyanide. Symbols indicate the molar ratio of cyanide to mercury. The very rapid action of one mole of mercuric chloride in dissociating the dimer is shown for comparison. Ordinate shows weight average molecular weight in thousands but scale should read from 70 to 130 instead of from 70 to 110 as indicated; *p*H 4.75, 1% protein, acetate buffer temperature 25°.

Velocity of Dimer Formation and the Effect of Temperature.—Measurements of the velocity of dimer formation were carried out at *p*H 5.5 and *p*H 6.0 in 0.05 *M* acetate buffer and the results are recorded in Table I. The calculation of the weight fraction of dimer (D) from the measurements was carried out as previously described.[7] It is seen from the table that the weight fraction of dimer at equilibrium was equal to unity within the experimental error, except for the experiment at *p*H 6 and 12°, where it was slightly lower.

We assume that, for these experiments as in those with mercuric salts, the rate-limiting step of the process is reaction 2. The data of Table I indicate that the back reaction of velocity constant k_{-2} (see eq. 2) may be neglected. The second-order velocity constant k_2 for the forward reaction may, therefore, be defined by the equation

$$dx/dt = k_2(\text{ASHgRHgX})(\text{ASH}) \qquad (4)$$

where x again denotes the molar concentration of dimer (ASHgRHgSA) at any moment. Since in these experiments the molar concentration of mercurial (b) was equal to half that of the total initial concentration of mercaptalbumin monomer (a) before mercurial was added, we may write at any stage of the reaction

$$(\text{ASHgRHgX}) = (\text{ASH}) = b - x \qquad (5)$$

Hence

$$dx/dt = k_2(b - x)^2 \qquad (6)$$

Equation 3 is readily integrated and the value of the integral is fixed by the condition that $x = 0$ when $t = 0$. The result may be conveniently expressed in terms of the weight fraction (D) of total albumin present as dimer in the solution at any time. Since $D = 2x/a = x/b$ and the weight concentration of protein c (in g./ml.) $= 2bM/1000$, where M is the molecular weight of ASH, the equa-

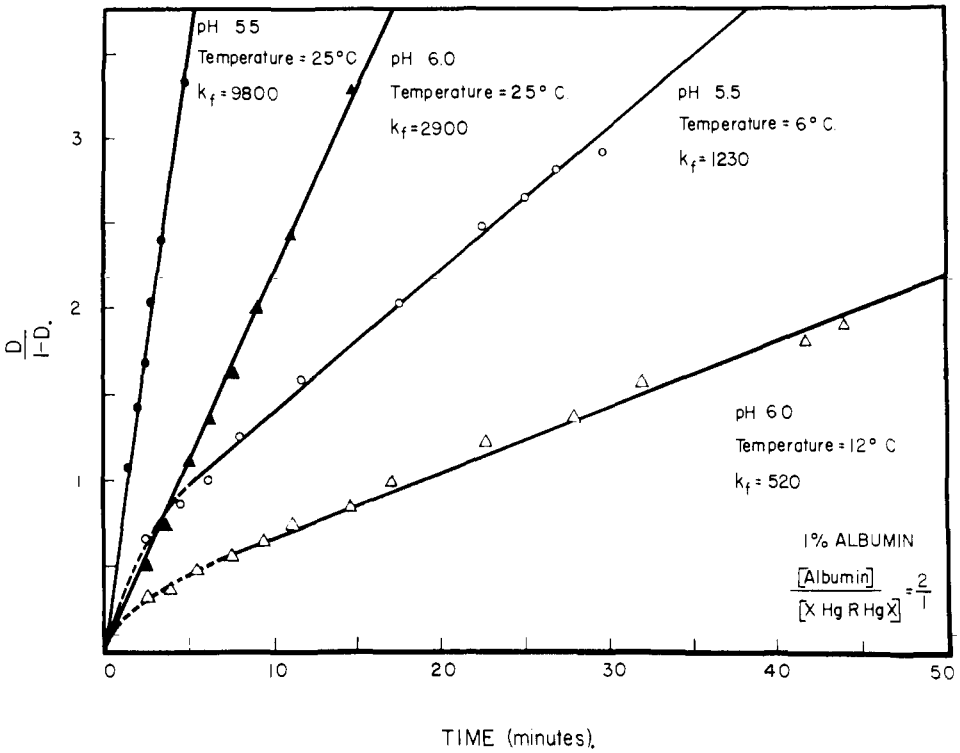


Fig. 3.—Rate of dimer formation with bifunctional mercurial at two *p*H values and two temperatures. The ratio $D/(1 - D)$ is plotted as a function of time (see eq. 7). Note that the symbol k_f has been used in this diagram to denote the quantity denoted by k_2 in the text.

tion for the velocity constant finally assumes the form

$$k_2 = \frac{1}{t}\left(\frac{2M}{1000c}\right)\left(\frac{D}{1 - D}\right) \qquad (7)$$

In evaluating the velocity constant, therefore, it is convenient to plot $D/(1 - D)$ against the time elapsed from the beginning of the reaction. The four experiments recorded in Table I are represented by this method in Fig. 3 for time intervals up to 50 minutes and for values of $D/(1 - D)$ between 0 and 3.5. The values of $D/(1 - D)$ in the two experiments at 25° are linear in the time within the experimental error and lead to values of k_2 of 9800 l. mole^{-1} min.$^{-1}$ at *p*H 5.5 and 2900 at *p*H 6.[8] The measurements on the two solutions at lower temperatures are approximately linear after an initial period of the order of 6 minutes, but the initial slopes in the first few minutes are considerably greater than the slopes of the later linear portions of the curves. We believe that the higher initial slopes were due to difficulties in cooling the solutions during the initial mixing and the placement of the cells in the light scattering apparatus surrounded by dust-free water at constant temperature. In calculating the velocity constants of these reactions, therefore, we have used the slopes of the later portions of the curves, after the solutions had come to thermal equilibrium with the surrounding water-bath.

(8) The molecular weight (M) of the monomer has been taken as 69,000 in calculating k_2 from $D/(1 - D)$ in eq. 7. It is probable—see for instance Low[9]—that the true M is somewhat lower, but we have continued to use the value 69,000 to make the present k_2 value comparable to those reported earlier.[2]

(9) B. W. Low, THIS JOURNAL, **74**, 4830 (1952).

Three major conclusions may be drawn from an inspection of these curves in comparison with those obtained with mercuric chloride or acetate: (1) the velocity constant k_2 is of the order of 2000 to 3000 times as great, at any given *p*H, for dimer formation with XHgRHgX as for the corresponding reaction with HgX_2. (2) The decrease of velocity constant with increasing *p*H is similar for both types of dimerization. The velocity constant of the former reaction decreases by a factor somewhat greater than 3—from 9800 to 2900—at 25° when the *p*H is increased from 5.5 to 6.0. The corresponding decrease in the velocity constant of dimer formation with HgX_2 is from 5.0 to 0.7. (3) The temperature coefficient of the velocity constant is large, the energy of activation being of the order of 20 kcal./mole for both types of dimerization.

In an earlier series of studies, the velocity constant of dimerization with XHgRHgX was determined at four different *p*H values between 5.0 and 6.0, at somewhat higher ionic strength than for the measurements of Table I. The results are summarized in Table II. The probable error of the values given in this table was considerably larger than for

TABLE II

VELOCITY CONSTANTS FOR DIMERIZATION OF HUMAN MERCAPTALBUMIN WITH BIFUNCTIONAL MERCURIAL

Temperature 23–25°

*p*H	Γ/2	Buffer	k_2 (l. mole^{-1} min.$^{-1}$) × 10^{-4}
6.0	0.20	Phosphate	0.25–0.41
5.7	.15	Acetate	0.40–0.57
5.4	.10	Acetate	1.4–1.8
5.0	.10	Acetate	4.3–6.9

those reported in Table I and Fig. 1. The order of magnitude of the figures, however, and the progressive decrease of k_2 with increasing pH agree very closely with the other measurements.

Discussion

Equilibrium Constants.—A preliminary estimate of the equilibrium constant for the fundamental reaction

$$\mathrm{ASHgRHg^+ + AS^- \rightleftarrows ASHgRHgSA}$$

may be derived from the experiment portrayed in Fig. 2 and analyzed in the accompanying discussion. The equilibrium constant, K_A, for this reaction written above can be formulated as the product of several constants, each of which is accessible to experimental determination or can be estimated approximately by analogy with other related reactions

$$K_A = \frac{(\mathrm{ASHgRHgSA})}{(\mathrm{ASHgRHg^+})(\mathrm{AS^-})} = K_{A1}K_{A2}K_{ASH}/K_{HCN} \quad (8)$$

Here we have

$$K_{A1} = \frac{(\mathrm{ASHgRHgSA})(\mathrm{HCN})}{(\mathrm{ASHgRHgCN})(\mathrm{ASH})} \cong 1 \quad (9)$$

$$K_{A2} = \frac{(\mathrm{ASHgRHgCN})}{(\mathrm{ASHgRHg^+})(\mathrm{CN^-})} \cong 10^{17.5} \quad (10)$$

$$K_{ASH} = \frac{(\mathrm{ASH})}{(\mathrm{AS^-})(\mathrm{H^+})} \cong 10^{10} \quad (11)$$

$$K_{HCN} = \frac{(\mathrm{HCN})}{(\mathrm{H^+})(\mathrm{CN^-})} = 10^{9.3} \quad (12)$$

The evidence for the assumption that $K_{A1} \cong 1$ has already been discussed in connection with Fig. 2. The value of K_{A2} is estimated by analogy, on the tentative assumption that it can be taken equal to the constant $(\mathrm{HgCN^+})/(\mathrm{Hg^{++}})(\mathrm{CN^-})$, or for $[\mathrm{Hg(CN)_2}]/(\mathrm{HgCN^+})(\mathrm{CN^-})$, the values for which are taken from the tables given by Bjerrum.[10] This assumption is obviously uncertain, since the presence of the sulfur atom attached to the mercury, as well as the other adjoining groups in the albumin molecule, may alter the affinity of mercury for $\mathrm{CN^-}$ ion. The value of K_{ASH} is a rough estimate, based on the figures given by Cohn and Edsall,[11] and on the later studies of Ryklan and Schmidt,[12a] and Stricks and Kolthoff.[12b] The figure for K_{HCN} is that given by Sidgwick.[13] From eq. 8–12, inc., we then obtain $K_A = 10^{18.2}$, with a probable uncertainty of at least one power of 10, due chiefly to the uncertainties in K_{A2} and K_{ASH}. Stricks and Kolthoff[12b] give association constants for the reaction

$$\mathrm{Hg^{++} + 2RS^- \rightleftarrows (RS)_2Hg}$$

at various pH values, where RSH is cysteine or glutathione. These association constants, in the case of cysteine, vary from $10^{40.3}$ to $10^{43.6}$ at 25°, according to the state of ionization of the amino groups. For glutathione at 25°, the corresponding range is from $10^{41.0}$ to $10^{41.6}$. Our constant K_A should be comparable to the square root of these values of Stricks and Kolthoff, which would lie between $10^{20.2}$ and $10^{21.8}$. Our estimate of $K_A = 10^{18.2}$ is thus considerably smaller, as might be expected on grounds of steric hindrance, but it is of a reasonable order of magnitude. In any case, it should be taken only as a rough estimate.

A similar estimate of K_A for the mercury dimer, ASHgSA, can be made from the data reported in the preceding paper[2] concerning the dissociation of the dimer by added chloride, bromide, or iodide.

$$K_A = \frac{(\mathrm{ASHgSA})}{(\mathrm{ASHg^+})(\mathrm{As^-})} = K_{A1}K_{A2}K_{ASH} \quad (13)$$

Here we have, for bromide (see reference 2, Table V)

$$K_{A1} = \frac{(\mathrm{ASHgSA})(\mathrm{H^+})(\mathrm{Br^-})}{(\mathrm{ASHgBr})(\mathrm{ASH})} = 10^{-4.9} \quad (14)$$

at pH 4.75, total bromide 1.4×10^{-4}; and K_{ASH} has

$$K_{A2} = \frac{(\mathrm{ASHgBr})}{(\mathrm{ASHg^+})(\mathrm{Br^-})} = 10^{8.3} \quad (15)$$

already been defined in eq. 11. At pH 4.75, total bromide $\cong 1.4 \times 10^{-4}$ M and total albumin 1 g./100 ml. ($= 1.4 \times 10^{-4}$ M ASH before addition of mercury) the weight fraction of dimer (D_e) is 0.30 at equilibrium. Total bromide[14] equals (ASHgBr) plus $(\mathrm{Br^-})$. From these data, we obtain $10^{-4.9}$ for K_{A1} in eq. 14. The value of $K_{A2} = 10^{8.3}$ for bromide ion is assumed to be the same as the value for the reaction $\mathrm{HgBr^+ + Br^- \rightleftarrows HgBr_2}$, as given by Sillén.[15] The same uncertainty resides in this assumption by analogy as in the corresponding assumption for cyanide ion in eq. 10, except that Sillén's value is probably more precise. Using all these values as listed, we obtain K_A for ASHgSA as $10^{13.4}$. An almost identical value ($10^{13.6}$) is obtained from the data of ref. 2, Table V, for systems containing chloride ion. For instance it is found that $D_e = 0.32$ when $(\mathrm{Cl^-}) = 0.007$ M (100 $\mathrm{Cl^-}$ ions per mole Hg) and pH 4.75. Here we have used Sillén's value of $10^{6.5}$ for the association constant $\mathrm{HgCl^+ + Cl^- \rightleftarrows HgCl_2}$.

From eq. 8 and 13, and the values of K_A derived from them, we may calculate directly the equilibrium constant

$$\frac{(\mathrm{ASHgRHgSA})(\mathrm{ASHg^+})}{(\mathrm{ASHgRHg^+})(\mathrm{ASHgSA})} = 10^{(18.2-13.5)} = 10^{4.7} \cong 50{,}000 \quad (16)$$

The value for this constant should be more reliable than that for either of the two constants from which it is derived, since the same assumptions are made in deriving both, and the resulting uncertainties should cancel in taking their ratio. This equilibrium constant shows quantitatively the increase in stability of the dimer ASHgRHgSA compared to that of ASHgSA.

We may also derive an estimate of the association constant K_A^1 for the binding of the first molecule of mercaptalbumin to the mercurial $\mathrm{XHgRHg^+}$

$$K_A^1 = (\mathrm{XHgRHgSA})/(\mathrm{XHgRHg^+})(\mathrm{AS^-}) \quad (17)$$

(10) J. Bjerrum, *Chem. Revs.*, **46**, 381 (1950).

(11) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publ. Corp., New York, N. Y., 1943, Chap. 4.

(12) (a) L. R. Ryklan and C. L. A. Schmidt, *Arch. Biochem.*, **5**, 89 (1944); (b) W. Stricks and I. M. Kolthoff, THIS JOURNAL, **75**, 5673 (1953).

(13) N. V. Sidgwick, "The Chemical Elements and Their Compounds," Oxford Univ. Press, New York, N. Y., 1950, Vol. I, p. 670.

(14) This neglects $\mathrm{Br^-}$ bound to albumin at sites other than the –SH group, but the error due to neglect of this factor is probably small compared to other uncertainties in these calculations.

(15) L. G. Sillén, *Acta Chem. Scand.*, **3**, 539 (1949).

We consider the state of equilibrium when one mole of the mercurial is present per mole of ASH

$$\text{ASHgRHgSA} + \text{XHgRHgX} \rightleftharpoons \text{ASHgRHgX} + \text{XHgRHgSA}$$

The two molecules on the right-hand side of the equation are, of course, identical. If the residue R were sufficiently elongated, so that the two albumin molecules in the dimer were widely separated, the distribution of AS– and of X– groups on the available mercury sites might in the limiting case be governed by purely statistical factors. If this were true, the equilibrium constant K_B

$$K_B = \frac{(\text{ASHgRHgX})^2}{(\text{ASHgRHgSA})(\text{XHgRHgX})} \qquad (18)$$

would be equal to 4, and the weight fraction of dimer would be 0.5. Actually the data of Fig. 1 show that this is by no means the case; the weight fraction of dimer at equilibrium, at the equivalence point, appears to be of the order of 0.05, possibly as high as 0.1. Taking it as 0.06, we obtain, for total protein = 0.01 g./ml., (ASHgRHgX) = $1.32 \times 10^{-4} M$, (ASHgRHgSA) = (XHgRHgX) = 0.08×10^{-4}, which gives $K_B \cong 270 = 10^{2.4}$ as a very rough approximation. In order to evaluate K_A^1, we must make use also of the constant K_A, already estimated (eq. 8) as of the order of $10^{18.2}$, and of two other equilibrium constants K_C and K_D

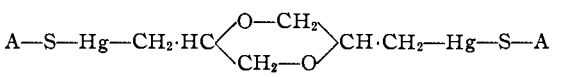
$$K_C = (\text{XHgRHgX})/(\text{XHgRHg}^+)(\text{X}^-) \qquad (19)$$

$$K_D = (\text{ASHgRHgX})/(\text{ASHgRHg}^+)(\text{X}^-) \qquad (20)$$

$$K_A^1 = K_B K_A K_C / K_D = 10^{(2.4+18.2)} = 10^{20.6} \qquad (21)$$

In this estimate of K_A^1, we have assumed $K_C = K_D$, that is, that the affinity of X^- for the group $R'HgRHg^+$ is the same whether R′ is an X or a mercaptalbumin residue. The value of $10^{20.6}$ appears reasonable in the light of the values reported by Stricks and Kolthoff[12b] for cysteine and glutathione, but it is, of course, to be taken only as a very rough estimate.[16] It is plain, however, from the value of K_B that replacing the second –X group by AS–, in XHgRHgX, involves more work than replacing the first, the difference in standard free energy ($RT \ln K_B$) being of the order of 3300 cal./mole. The constant corresponding to K_A^1 for the simple mercury derivative of mercaptalbumin [$K_A^1 = (\text{XHgSA})/(\text{XHg}^+)(\text{AS}^-)$] is probably close to that derived above (eq. 21) for the dimer of the bifunctional mercurial. From the value of $10^{13.6}$ already estimated for K_A we then estimate K_B as approximately 10^7, the corresponding difference in standard free energy of binding, for the two successive AS– groups attached to the Hg^{++} ion, being near 9500 cal./mole. The difference between this and the value of 3300 given above reflects the greater resistance, due presumably to steric factors, to bringing the two albumin molecules so close together that they are linked only by a single mercury atom.

Kinetic Relations.—Both the differences and the similarities in the character of the dimerization reaction with compound I and with mercuric salts are significant. The velocity constant of dimerization k_2 for the formation of ASHgRHgSA is greater by a factor of the order of 3000 than for the formation of ASHgSA. This difference is probably due primarily to a steric factor. If the dimer is formed by reaction with a simple mercury salt, the albumin molecules are linked by a –S–Hg–S– bond and the sulfur atoms of the two albumin sulfhydryl groups must approach within approximately 5 Å. if we take a value of 2.5 Å. for the Hg–S distance.[17] On the other hand, in the dimer ASHgRHgSA, the two sulfur atoms are separated by a much larger distance. The compound may be represented more explicitly by the formula



Examination of a molecular model indicates that the distance between the two sulfur atoms may be as great as 15 Å. In the former case, an extremely close fitting of the albumin molecules in the dimer is required; hence the number of permissible orientations of the two albumin molecules as they approach one another to form the dimer is probably very restricted if reaction is to occur. In the latter case, the coupling between the two albumins is far looser, and a much wider range of possible relative orientations is likely to be compatible with dimer formation. Unfortunately almost nothing is known of the nature of the groups in the mercaptalbumin molecule which adjoin the free cysteine sulfhydryl group, so that no detailed picture of the topography of the molecular surfaces can be given.

It is a striking fact that the relative change of k_2 with pH is virtually the same for the formation of ASHgRHgSA as for ASHgSA, although the absolute value of k_2 at any given pH is so much greater for the former. This is illustrated in Fig. 4, which shows log k_2 as a function of both pH and temperature. The parallelism of the curves for the two dimers, showing decrease of k_2 with increasing pH, on the alkaline side of the isoelectric point, is immediately apparent. An electrostatic interpretation of the effects involved has already been suggested in the preceding paper, and need not be repeated here.

Energy and Entropy of Activation.—The measurements shown in Table I and Fig. 3 lead to a calculated energy of activation $E_{exp.}$ of 18.7 kcal./mole at pH 5.5 and 23 kcal./mole at pH 6. The probable error in both of these figures is large and it is questionable whether the suggested increase in apparent activation energy with pH is significant. Tentatively, however, we may employ these figures in order to calculate a standard entropy of activation ($\Delta S^{\pm}$) for the dimerization process at each of these pH values employing the equation of Glasstone, Laidler and Eyring[18] which

(16) Another factor, not discussed in the text, deserves consideration. The value of free (XHgRHgX) in the expression for K_B, eq. 18, is probably lower than we have figured it here, since some of the mercurial may bind to groups in the albumin other than the sulfhydryl group. Any correction on this basis would give a higher value for K_B, and hence for K_A^1, than that reported here.

(17) See A. F. Wells, "Structural Inorganic Chemistry," 2nd Edition, Oxford, 1950, p. 50. The covalent radius of Hg is given as 1.48 and that of S as 1.04 Å, giving a distance of 2.52. However, this distance may be a little too high; comparing, for instance, the mercury–halogen distances listed by Wells on p. 632.

(18) S. Glasstone, K. J. Laidler and H. Eyring, "The Theory of Rate Processes," McGraw-Hill Book Co., Inc., New York, N. Y., 1941, p. 199.

may be written in terms of the logarithm of the velocity constant as

$$\ln k_2 = 1 + \ln (kT/h) + \Delta S^{\pm}/R - E_{exp}/RT \quad (22)$$

(Note that the values of k_2 given in this paper must be divided by 60 before being used in eq. 22 in order to convert them from l. mole^{-1} min.$^{-1}$ to l. mole^{-1} sec.$^{-1}$). Using the indicated values of E_{exp} and the values of k_2 at 25° given in Table I, we obtain $\Delta S^{\pm}$ = 14 cal. deg.$^{-1}$ mole^{-1} at *p*H 5.5 and 26 cal. deg.$^{-1}$ mole^{-1} at *p*H 6.

These entropies of activation are extraordinarily large, very much larger than the value near 0, found for dimerization with the mercuric salts[2]; and that value in turn is very much more positive than the reported values of the order of −40 to −50 cal. deg.$^{-1}$ mole^{-1} obtained for many simple reactions involving the linkage of two molecules to form one.[19] Since a dimerization process must always involve a considerable loss in translational and rotational freedom, a large negative contribution to the entropy of activation is expected due to such effects.[20] In order to explain the positive values for the reactions reported in this paper, we must, therefore, consider possible factors which may contribute large positive terms to the entropy of activation. Two clues to the problem may be suggested:

1. It is possible that albumin molecules in their native configuration cannot link through their sulfhydryl groups and form the dimer because of steric hindrance. In this case a partial unfolding of the molecule, perhaps involving a sort of extrusion of the active group outward from the surrounding molecular surface, may be required as a preliminary to the linkage of the two sulfhydryl groups through the mercurial. If this were the correct explanation, the preliminary unfolding might be regarded as a sort of partial denaturation of the molecule. It is well known that denaturation involves a very large positive entropy of activation, due presumably to loosening and unfolding of the structure of the native molecule. The partial unfolding of mercaptalbumin required by this explanation would probably involve a large positive entropy term also. It must be remembered, however, that all our evidence indicates that the dimerization process is entirely reversible and that the albumin molecule can, therefore, return from the unfolded to the original folded configuration when the dimer dissociates.

Since the urea denaturation of bovine serum albumin is accompanied by marked changes in optical rotation,[21] it appeared possible that a change in rotation might also accompany the dimerization reaction. One of us (R. B. S.) has investigated this possibility in conjunction with Dr. H. A. Saroff by studying the dimer dissociation. The lyophilized mercury dimer was dissolved in 0.01 *M* bicarbonate buffer at *p*H 7, at 1.61 g. of dried protein/100 ml., giving $[\alpha]_D$ −58.4° (values for protein and $[\alpha]_D$ not corrected for moisture in the dried sample, which was probably near 5%, so that true $[\alpha]_D$ should be between −60° and −61°). A solution of sodium bromide in acetate buffer was added, the final concentration of bromide being 1.3 moles per mole of albumin, the acetate ion concentration 0.05 *M*, the *p*H 4.8 and the protein concentration 1.58 g./100 ml. There was an immediate change of $[\alpha]_D$ to −60.8°; this could not have been due to dimer dissociation, which is a slow process. There was no further change in $[\alpha]_D$ over a period of two weeks, although the weight fraction of dimer should have decreased to approximately 0.35 from previous measurements under these conditions (see ref. 2, Fig. 9). The absence of change in $[\alpha]_D$ thus indicates that there is no evidence, by this criterion, of molecular folding or unfolding in the change from dimer to monomer. Such unfolding effects, however, may occur in formation of the activated complex, even if not in the final equilibrium state of the dimer.

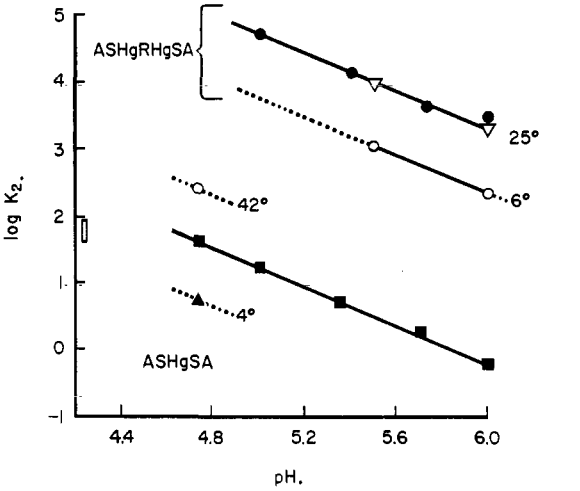


Fig. 4.—The logarithm of the velocity constant of the dimerization reaction (k_2) as a function of *p*H and temperature. Lower curve, marked ASHgSA, is for the formation of the mercury dimer at 25°; for data see ref. 2, Table IV. Points above and below this curve show k_2 values for 4 and 42° at *p*H 4.75. The upper pair of curves represent the velocity constant k_2 for dimer formation with the bifunctional mercurial at two different temperatures. Solid circles are from data of Table II; other symbols refer to data of Table I. The value at *p*H 6 and 6° is extrapolated from the values at 25 and 12° in Table I. Note the general parallelism of the curves. Concerning the solvents used, see the tables in this and the preceding paper (ref. 2).

2. An alternative explanation involves dehydration of some of the charged groups on the albumin molecules when they come together to form the dimer. This is essentially the explanation offered by Doty and Myers[22] to explain their data on the association of insulin in acid solution. Since the process of hydration of electrically charged groups, with accompanying electrostriction, involves a large entropy decrease, dehydration correspondingly gives an increase. Such dehydration might occur if there were a large surface of contact between the two albumin molecules in the dimer

(19) See, for instance, K. J. Laidler, "Chemical Kinetics," McGraw-Hill Book Co., Inc., New York, N. Y., 1950, especially chaps. 3 and 5; also Glasstone, Laidler and Eyring, ref. 18, chaps. I and VIII.

(20) L. Slutsky and S. H. Bauer, THIS JOURNAL, **76**, 270 (1954), have recently shown that the decrease in translational and rotational entropy associated with the dimerization of carboxylic acids, is associated with a numerically smaller *increase* of vibrational entropy. Their data relate to the over-all entropy of the reaction, not to the entropy of activation. Naturally no conclusions can be drawn as yet, for the kinetics of such a complicated system as that which we are studying here, from thermodynamic findings on much simpler dimers. However, consideration of these simpler systems may possibly provide clues to elucidate the behavior of the proteins.

(21) W. Kauzmann and R. B. Simpson, THIS JOURNAL, **75**, 5154 (1953).

(22) P. M. Doty and G. E. Myers, *Disc. Faraday Soc.*, No. 13, 51 (1953).

with a resulting elimination of water from a number of the charged groups as the surfaces come together.

This explanation is confronted by one obvious difficulty. The entropy of activation is much larger and more positive for dimerization with the bifunctional organic mercurial than for the formation of the simple mercury dimer. On the other hand, the latter requires a much closer approach of the two albumin molecules and, therefore, might be expected to involve a larger surface region of close contact between the two mercaptalbumin molecules than the former. Thus, the magnitude of the dehydration effects should, if anything, be greater for the formation of ASHgSA than for that of ASHgRHgSA, and the entropy of activation in dimer formation should be greater for the former. The experimental facts indicate just the contrary. However, so little is known of the details of the process that this argument cannot be taken as a definite refutation of the explanation of the positive value of $\Delta S^{\pm}$ in terms of dehydration.

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[CONTRIBUTION FROM THE UNIVERSITY LABORATORY OF PHYSICAL CHEMISTRY RELATED TO MEDICINE AND PUBLIC HEALTH, HARVARD UNIVERSITY]

A Disulfide Dimer of Human Mercaptalbumin[1a,b]

BY RUDOLF STRAESSLE[1c]

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Human serum mercaptalbumin (Alb-SH) forms a disulfide dimer (Alb-S-S-Alb) which is prepared by oxidation with iodine of the mercury dimer (Alb-S-Hg-S-Alb). Maximum yield of the disulfide dimer is obtained when the reaction is carried out at low temperature (−8 to −18°), the protein being in the solid state in a system containing a high concentration of ethanol. The maximum yield of disulfide dimer obtained was 75–80%. Measurements of sedimentation constant and light scattering indicated that the disulfide dimer had approximately the same size and shape as the mercury dimer. Fractionation procedures gave some separation of dimer from monomer, but a completely pure disulfide dimer preparation has not yet been achieved. Unlike the mercury dimer, the disulfide dimer is not dissociated by the addition of an excess of a mercuric salt or of iodide ion. Reduction with cysteine, however, reconverts Alb-S-S-Alb to two moles of Alb-SH as shown by the change of sedimentation constant to that of the monomer and by the reappearance of titratable sulfhydryl groups.

Introduction

Human serum mercaptalbumin contains one and only one sulfhydryl group per molecule[2,3] and forms a mercury dimer of the composition $(Alb\text{-}S)_2\text{-}Hg$– the symbol Alb-SH being used to denote mercaptalbumin.[2–4] In the dimer, the two albumin molecules are linked through an S–Hg–S bridge, involving a cysteinyl residue in each molecule.

In the course of studies on the iodination of human serum albumin[5] it was found that when using the mercury dimer of mercaptalbumin instead of the monomer form of human serum albumin as starting material, the formation of two components was observed. Ultracentrifugal analysis indicated that one corresponded to monomer albumin and the other to a component with a sedimentation constant virtually identical with that of the mercury dimer. The double molecule, however, did not dissociate upon addition of one mole of mercuric chloride, although this reagent immediately dissociates the mercury dimer.[4] This suggested that iodine had oxidized the two adjoining sulfhydryl groups of the mercaptalbumin molecule to a disulfide bond with the release of mercuric iodide. While the actual process may proceed in several steps, the over-all reaction should presumably be formulated as

$$\text{Alb-S-Hg-S-Alb} + I_2 = \text{Alb-S-S-Alb} + HgI_2 \quad (1)$$

Oxidation of mercaptides of low molecular weight by halogens generally leads to the formation of disulfides.[6] Under rather special conditions, the formation of the corresponding sulfenyl iodide has been observed.[7] Sulfenyl halides, however, tend to form disulfides in many ways; among others by hydrolysis, alcoholysis and reaction with mercaptans. The over-all reaction, therefore, tends to be the formation of disulfide. The disulfide linkage, once formed, has been reported by Kharasch[6] to be cleaved only by a considerable excess of halogen. Formation of a disulfide–tetraiodide may occur as a side-reaction.

It seemed reasonable, therefore, to assume, as was further suggested by the experimental results, that a disulfide dimer was formed in a side-reaction during the iodination of the mercury albumin dimer. This reaction was the subject of the investigation here presented.

Materials and Methods

Crystallized Mercury Albumin Dimer.—One sample (prep. 178–5x) was crystallized according to Hughes[2] as the mercuric salt from human serum albumin, and subsequently recrystallized three times from ethanol–water mixtures of the composition: EtOH, 7.5%; *p*H 5.1; Γ/2 0.01; *T* −5°; protein concn., 13%. The preparation was analyzed in the ultracentrifuge as a 0.7% solution at *p*H 6.50 and Γ/2 0.15. It consisted of 95% dimer ($s_{20,w}$ = 6.5*S*) and 5% monomer sedimenting with the rate characteristic

(1) (a) This work has been supported by funds of Harvard University and the Eugene Higgins Trust, by grants from the Rockefeller Foundation and the National Institutes of Health, and by contributions from industry. (b) This paper is No. 106 in the series "Studies on the Plasma Proteins" from blood collected by the American Red Cross, on products developed by the University Laboratory of Physical Chemistry Related to Medicine and Public Health, Harvard University. (c) F. Hoffmann–La Roche and Co. A.G., Basle, Switzerland, Research Laboratories.

(2) W. L. Hughes, Jr., THIS JOURNAL, **69**, 1836 (1947).

(3) W. L. Hughes, Jr., Cold Spring Harbor Symp. on Quant. Biol., **14**, 79 (1949).

(4) H. Edelhoch, E. Katchalski, R. H. Maybury, W. L. Hughes, Jr., and J. T. Edsall, THIS JOURNAL, **75**, 5058 (1953).

(5) W. L. Hughes, Jr., and R. Straessle, *ibid.*, **72**, 452 (1950).

(6) N. Kharasch, S. J. Potempa and H. L. Wehrmeister, *Chem. Revs.*, **39**, 269 (1946).

(7) H. Rheinboldt, *et al.*, *Rev. brasil chim.*, (*Saõ Paulo*), **4**, 169 (1937); *C. A.*, **32**, 484 (1938); *Ber.*, **72**, 657, 668 (1939).