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

Dimerization of Serum Mercaptalbumin in Presence of Mercurials. I. Kinetic and Equilibrium Studies with Mercuric Salts[1a,b,c]

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RECEIVED APRIL 13, 1953

Human serum mercaptalbumin (ASH) contains one sulfhydryl group per molecule. In the presence of a mercuric salt HgX_2—where X denotes chloride or acetate—mercury is very rapidly bound to the sulfhydryl group, forming the compound ASHgX. This reacts much more slowly with another molecule of mercaptalbumin to give the mercury dimer, ASHgSA, the reaction probably being $ASH + ASHgX \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ASHgSA + H^+ + X^-$. The velocity constants k_2 and k_{-2} and the "apparent equilibrium constant" $K' = (ASHgSA)/(ASH)(ASHgX)$ have been studied by light scattering measurements as functions of *p*H, temperature and the concentrations of various added anions. The calculation of weight average molecular weights from light scattering in systems containing mercaptalbumin monomer and dimer is discussed, with particular reference to the effects of interactions due to net charge on the protein. Repeated experiments have demonstrated the reversibility of the reaction. Titration of ASH with increasing amounts of HgX_2 shows that dimer formation at equilibrium is a maximum when the molar ratio (Hg)/(ASH) = 0.5; the curve is symmetrical around this point, no dimer being present if (Hg)/(ASH) is zero or unity. The equilibrium constant K' is near 3×10^4 l. mole^{-1} at 25°, and is nearly independent of *p*H from 4.7 to 6; it increases with rise of temperature. The kinetics of dimer formation can be described on the assumption that the reaction is approximately second order in the protein concentration, when equal amounts of ASH and ASHgX are present. The velocity constant of dimerization, k_2, is near 40 l. mole^{-1} min.$^{-1}$ at 25°, for the isoelectric protein at *p*H 4.75; it decreases to 0.7 at *p*H 6, probably owing to the electrostatic repulsion of the negatively charged protein molecules at this *p*H. The energy of activation for dimerization is 18–20 kcal./mole, and the calculated standard entropy of activation is very small, in contrast to the large negative values generally found for reactions in which two molecules are joined. Added halide ions, except fluoride, decrease K', the relative amounts of Cl^-, Br^- and I^- required to produce a given decrease being approximately in the ratio 2000:80:<1. Their effect is primarily to increase the rate of dissociation of the dimer, the halide ion competing with the sulfur atoms of ASHgSA for attachment to the mercury. Addition of excess HgX_2 to ASHgSA causes extremely rapid dissociation of dimer into two molecules of ASHgX. The rapidity of these dissociation reactions indicates the accessibility of the mercury atom in the dimer to ions and molecules in the surrounding medium. No metallic ion other than mercury was found to produce dimerization, but silver ions competitively inhibit dimerization.

It was discovered by Hughes[2] that the major portion of human serum albumin undergoes a reaction with mercuric chloride, and other mercuric salts, which can lead to the formation of a dimer containing two albumin molecules per atom of mercury. The fraction of serum albumin which is capable of undergoing this reaction has been designated as mercaptalbumin, since Hughes[3] has produced decisive evidence that the reaction involves a sulfhydryl group in the protein molecule. Moreover, it was shown by analytical studies that only one such group in the mercaptalbumin molecule is available for reaction with the reagents that are usually found to react with sulfhydryl groups. The mercaptalbumin mercury dimer readily forms crystals containing one atom of mercury for each two molecules of albumin.[2] Optical and morphological properties of these crystals have been studied by Low and Weichel[4]; and Low[5] has made a preliminary X-ray study of the crystals from which some inferences concerning the shapes of the molecules have been drawn. On treatment with such a sulfhydryl derivative as thioglycolate ion, which can then be adsorbed on a suitable ion exchange resin,[6] mercaptalbumin mercury dimer loses its mercury to yield a pure mercaptalbumin monomer. This preparation of the purified mercurial may be used for the study of the dimerization process in the presence of mercuric chloride or other mercuric compounds.

It has been found that the reactions between mercaptalbumin and mercurials are completely reversible; the same equilibrium state may be obtained, under given conditions, by dissociation of the dimer or by association of the monomer molecules in the presence of mercurials. In the present study, velocity and equilibrium constants are reported for the interaction of mercaptalbumin with mercuric salts.

The effects of protein concentration, *p*H, temperature and the specific influences of various added anions have been examined. From the data, tentative inferences have been drawn regarding the nature of the processes occurring during these interactions and the configuration of the

(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. 103 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) Preliminary reports of parts of this investigation have been given by R. Lontie, P. R. Morrison, H. Edelhoch and J. T. Edsall, Abstracts, 114th Meeting, Am. Chem. Soc., 1948, p. 25c; W. L. Hughes, Jr., R. Straessle, H. Edelhoch and J. T. Edsall, Abstracts, 117th Meeting, Am. Chem. Soc., 1950, p. 51c; and by J. T. Edsall, Sixth Spiers Memorial Lecture, *Discs. Faraday Soc.*, **13**, 9 (1953). The first observations of the dimerization reaction by light scattering were made in this Laboratory by P. R. Morrison and R. Lontie in the summer of 1947. (d) To whom inquiries concerning this communication should be addressed.

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

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

(4) B. W. Low and E. J. Weichel, THIS JOURNAL, **73**, 3911 (1951).

(5) B. W. Low, *ibid.*, **74**, 4830 (1952).

(6) H. M. Dintzis, Thesis, Harvard University, 1952. Mimeographed copies of the portion of this thesis dealing with the preparation of mercaptalbumin, and its purification using ion exchange resin columns, may be obtained from this Laboratory on request. These studies will be further reported in a paper, now in preparation, by H. M. Dintzis and J. L. Oncley.

mercaptalbumin molecule in relation to the reactive group. The method of light scattering was found to be particularly well adapted to the determination of the velocity constants for association and dissociation, and also for the determination of equilibrium values. Some comparative studies have also been carried out with the ultracentrifuge.

A later report will deal with the results of similar studies on interaction with a bifunctional organic mercurial.[7] The velocity of dimerization with this mercurial is very much greater than with mercuric chloride or acetate, and the equilibrium also lies more completely on the side of dimer formation.[8]

Materials and Experimental Methods

Materials.—Five times recrystallized mercury–mercaptalbumin dimer prepared according to Hughes[2,3,9] was used. In the earlier studies, the mercury was removed from the mercaptalbumin mercury dimer by treatment with cysteine and subsequent dialysis, to give a solution of mercaptalbumin monomer.

In later work we have employed the procedure of Dintzis,[6] by which a pure aqueous solution of mercaptalbumin monomer was obtained when the mercury of the mercaptalbumin dimer was removed with thioglycolate ion adsorbed on amberlite resin (IRA-400, 20–50 mesh). The cations initially present in the solution were replaced by ammonium ions, employing Amberlite IR-120 on the ammonium ion cycle; the anions initially present were replaced by acetate ions, using IRA-400 on the acetate cycle. Subsequent passage through a mixed bed resin achieved practically complete deionization of the solution. The procedure is described in detail by Dintzis.[6]

Direct titration of the resulting concentrated mercaptalbumin solution with methyl mercury nitrate (*cf.* below) indicated that the mercaptalbumin regenerated contained 0.95–0.98 sulfhydryl groups per molecule, taking the molecular weight as 69,000.

When not in use the mercaptalbumin solution was kept frozen at −20°.

Nitroprusside Titration for SH– Groups.—Sufficient protein to contain approximately 0.5 μmole SH– (*e.g.*, 50 mg. of albumin) is dissolved in 2 cc. of cold guanidine bromide reagent.[10] (Some proteins including serum albumin coagulate as a gel when treated in the dry state with concentrated guanidine salt solutions. In such cases, it has proved simpler to dissolve the protein first in a small amount, 0.5 cc. or less, of water or diluted guanidine reagent.) A drop of 10% $Na_2Fe(CN)_5NO$ is added and the solution is then titrated with 10^{-3} *M* CH_3HgNO_3 to the disappearance of the purple color. The titration is carried out in the cold, near 0°.

Other Reagents.—The mercury salts were standard materials of reagent grade. Potassium fluoride, sodium chloride, bromide and iodide, and the materials used for the preparation of the acetate and phosphate buffers, were also C.P. reagent material.

Apparatus and Methods of Measurement.—Two different forms of light scattering apparatus have been used. Some of the measurements were carried out with the Mueller–Edelhoch–Edsall–Zimm apparatus described previously[11] while the others were carried out with a modified Debye instrument.[12] All measurements were made using the 436 mμ line of an AH4 mercury arc.

The solutions employed for turbidity measurements were freed of particulate impurities by centrifugation in the cold at 16,000 *g* in the high speed head of an International Centrifuge, for 4 to 6 hours. When the reaction of mercaptalbumin with mercurials was studied, the buffer, the mercurial in the buffer and the protein solution in the same buffer were centrifuged. The required amount of each of the purified solutions was pipetted into the cell and weighed. The pipets used were carefully cleaned with triply distilled water. The contents of the cell (2.5–3 ml.) were mixed thoroughly, generally for 4–5 minutes, with a magnetic stirrer before turbidity readings were begun.

The calibration of the light scattering measurements, in the earlier stages of this work, was carried out essentially as described by Edsall, Edelhoch, Lontie and Morrison.[11] Later it was found that the colloidal silica preparation "Ludox," obtained from Dr. R. K. Iler of E. I du Pont de Nemours and Co., furnished an extremely convenient and stable standard for calibrating the measurements on an absolute scale. The preparations employed by us contained approximately spherical silica particles of diameter near 150 Å. Before being made up for use, the original concentrated stock solution (25–30% silica) was diluted 15- or 20-fold, and filtered two or three times through an ultrafine sintered glass filter to remove any larger particles that might be present. A brief period of centrifuging—15–30 minutes at 15,000–20,000 *g*—was sometimes found helpful as a preliminary to filtration.[13] The filtered solution showed almost negligible angular dissymmetry of scattering (the dissymmetry ratio $R_{45°}/R_{135°}$ should be not more than 1.01–1.02) and the depolarization ratio ρ for scattering at 90° was very low, less than 0.02. The turbidity, τ, of the filtered solution was determined in a Beckman spectrophotometer at 436 mμ, using a 10-cm. cell; the concentration of silica was adjusted by dilution with distilled water so that a convenient value of τ was obtained. For our work, a value near 0.025 was found convenient. When τ had been thus determined, another sample of the identical solution was placed in a small rectangular cell, 1 cm. square in cross-section, identical with the cells used in studying the light scattering of the albumin solution. This cell was then sealed at the top to prevent evaporation. The intensity of light scattering from this solution was found to remain constant for many months. Since the turbidity, τ, was known for this solution, and the depolarization and angular dissymmetry of scattering were very small, the reduced intensity of scattering at 90°, R_{90}, could be calculated by the simple equation[14] $\tau = 16\pi R_{90}/3$.

Measurement of R_{90}.—For determination of the absolute reduced intensity at 90° a 1-cm. square Beckman absorption cell, polished on all four sides,[15] was used. As little as 1.5 ml. of solution sufficed for a measurement. The cell was positioned by means of a brass cell holder consisting of uprights and light masks attached to a square brass plate. The cell holder could be removed and replaced reproducibly by means of six-point contact between the base plate and a table in the light scattering compartment. In using this cell, the mirror and photomultiplier were set at 90° and the photocurrent was read directly on a galvanometer with a sensitivity of 10^{-3} μamp./mm. using a 50-cm. scale. The intensity of the transmitted light after passing through the cell, which was essentially identical with that of the incident beam, was read by setting the mirror and photomultiplier at 0°. The resulting photocurrent could be adjusted to a readable value by interposing calibrated filters in the inci-

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

(8) J. T. Edsall, R. B. Simpson, R. H. Maybury and R. Straessle, in preparation.

(9) W. L. Hughes, Jr., in preparation.

(10) This was prepared by careful neutralization of C.P. guanidine carbonate with concentrated (40%) colorless HBr. Any insoluble matter is filtered off. The approximately 5 *M* guanidine bromide solution is brought to *p*H 10.0–10.5 by the addition of anhydrous Na_2CO_3 (5 g./100 cc.). Sufficient ethylenediaminetetraacetic acid is added to make the solution 0.01 *M* in this substance.

(11) J. T. Edsall, H. Edelhoch, R. Lontie and P. R. Morrison, THIS JOURNAL, **72**, 4641 (1950).

(12) W. B. Dandliker, *ibid.*, **72**, 5110 (1950).

(13) Recently it has been found that Millipore filters, made by the Lovell Chemical Company, Watertown 72, Massachusetts, are superior to sintered glass filters for clarifying Ludox solutions and removing large particles. Flow of solution through these filters is much more rapid, and the filtrate shows no detectable angular dissymmetry of scattering.

(14) See for instance P. Doty and J. T. Edsall, *Adv. in Protein Chem.*, **6**, 35 (1951). NOTE ADDED IN PROOF.—Further studies have indicated discrepancies between the absolute values of R_{90}, determined by the method described above, and those determined by other methods. The differences amount to several per cent., and may be due to very small amounts of large particles in the Ludox preparations, which decrease the intensity of the transmitted light without appreciably affecting the scattering except at very small angles. Further study of this problem is in progress. However, these difficulties do not impair the use of the Ludox preparations as extremely convenient and stable working standards for light scattering measurements at 90°.

(15) Obtained from Pyrocell Corporation, New York City.

dent beam. Denoting by f_0 the reduction in the intensity of the incident beam due to the interposed filters, and by G_0 the measured deflection on the galvanometer scale, the calculated photocurrent for the unfiltered transmitted beam is $C_0 = G_0/f_0$. Likewise the calculated photocurrent produced by the scattering at 90° was given by $C_{90} = G_{90}/f_{90}$. The filter factor f_{90} was in many cases unity—indicating that no filters were used—but, for solutions giving rather intense scattering, values of f_{90} from 0.2–0.5 were employed. Independent tests showed that the reading on the galvanometer scale was directly proportional to the photocurrent.

R_{90} of the unknown was determined by comparison of its scattering with that of a standard liquid such as silica solution (Ludox), in a similar cell, after applying corrections for stray light, difference in polarization and differences in refractive index. Since the polarization and refractive index values were nearly identical for the silica and for the albumin solution, these corrections were generally negligible. The value of R_{90} for the unknown was then given in terms of that of the calibrated standard by the relation

$$\frac{R_{90}\ \text{unknown}}{R_{90}\ \text{standard}} = \frac{(C_{90}/C_0)\ \text{unknown}}{(C_{90}/C_0)\ \text{standard}}$$

With a standard of a given reduced intensity, the ratio in the denominator of the right-hand side of the equation should be (and was found to be very nearly) a constant of the apparatus and, once determined, did not need to be checked very often. The intensity of the light of the mercury arc was found to be sufficiently constant to permit comparisons of successive readings at 90 and 0°, without significant fluctuations.

Control determinations of R_{90} were made on the pure solvent in which the albumin was dissolved. This value for the pure solvent was subtracted from the values for the albumin solution to obtain the reduced intensity due to the albumin itself. This correction factor amounted to about 1% of the reading for a 1% solution of isoelectric albumin.

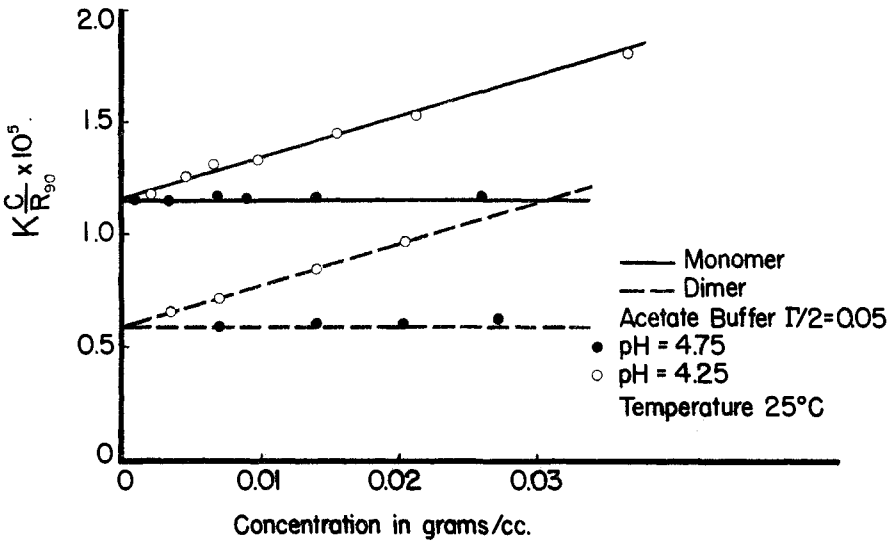

Fig. 1.—Light scattering of mercaptalbumin, showing that the slope of the curve is the same for monomer and dimer at a given pH and ionic strength: solid circles, pH 4.75; open circles, pH 4.25.

The use of Ludox colloidal silica to calibrate light scattering measurements has already been discussed by Mommaerts[15] and more briefly by Tietze and Neurath.[17] The development of the method in this Laboratory was largely carried out by Dr. W. B. Dandliker.

The preparations of mercaptalbumin employed in these studies may be divided into two classes:

(a) Preparations from which the mercury had been removed by treatment by cysteine. These were found to have weight average molecular weights of 78,000 ± 4000. Ultracentrifuge studies on these preparations showed the presence of a small fast shoulder on the main peak, amounting to 5–10% of the total area under the peak. Experiments in which these preparations were used are denoted by the symbol A in the tables of experimental data.

(b) Preparations from which mercury had been removed by means of an ion exchange resin, employing thioglycolate ion according to the procedure of Dintzis.[6] Experiments on the first preparation in which these procedures were employed are denoted by the symbol B. The weight average molecular weight was similar to that of preparation A. A later preparation, purified with particular care so as to remove not only the mercury but nearly all the attached fatty acid molecules from the mercaptalbumin was found to have a weight average molecular weight of approximately 68,000, and the ultracentrifuge diagram showed a single peak with scarcely any trace of a fast component. Experiments on this preparation are denoted by the symbol C in the Tables.

Calculation of the Weight Average Molecular Weight and the Weight Fraction of Dimer in Solution

For a moderately dilute solution of the mercaptalbumin monomer of molecular weight M, the reduced intensity, R_{90}, of the light scattered at 90° is given by

$$\frac{Kc_m}{R_{90,m}} = \frac{1}{M} + 2Bc_m \qquad (1)$$

where the subscript m is used to denote the monomer of mercaptalbumin. Here $K = 2\pi^2 n^2 (dn/dc_m)^2/N\lambda_0^4$, where N is Avogadro's number, n is refractive index, λ_0 is the wave length of the light *in vacuo*, and c is the concentration of the monomer in g./cc. For the derivation of this equation see, for instance, ref. (14). If the protein carries a total net charge Z_m^*, in a solution containing a uni-univalent salt at concentration m_3, the interaction constant B is given by the relation

$$B = \frac{1000}{M^2}\left(\frac{Z_m^{*2}}{2m_3} + \beta_{mm}^*\right) \qquad (2)$$

Here β_{mm}^* denotes $(\partial \ln \gamma_2/\partial m)$, where γ_2 is the activity coefficient of monomer and m its molar concentration. Details of the derivation of (2) have been given.[11]

For a solution of the dimer, of molecular weight $2M$, at weight concentration c_d, an equation of the same form holds

$$\frac{Kc_d}{R_{90,d}} = \frac{1}{2M} + 2Bc_d \qquad (3)$$

Experimentally it was found that B is the same in a given medium for monomer and dimer, as illustrated in Fig. 1. This shows that, while the intercept, Kc/R_{90}, is twice as great for the monomer as for the dimer, the slope $2B$ is zero for both in acetate buffer at pH 4.75, and is approximately 8.3×10^{-5} for both at pH 4.25. Values of B in the various buffers employed in our experiments are listed in Table I.

The identity of B for monomer and dimer means that the interaction of two monomer units—excluding those pairs which are actually linked

(16) W. F. H. M. Mommaerts, *J. Colloid Sci.*, **7**, 71 (1952).

(17) F. Tietze and H. Neurath, *J. Biol. Chem.*, **194**, 1 (1952).

TABLE I

VALUES OF THE INTERACTION CONSTANT, B, IN SOLUTIONS EMPLOYED FOR EQUILIBRIUM AND KINETIC STUDIES

pH	$\Gamma/2$	Buffer	$d\left(\frac{c}{\tau}\right)/dc$	$\frac{BM^2}{1000}$
4.25	0.05	Acetate	16.4	405
4.75	.05	Acetate	0.0	0
5.00	.15	Acetate[a]	9.5	235
5.36	.15	Acetate[a]	10.5	260
5.70	.15	Acetate[a]	12.5	310
6.00	.15	Phosphate[a]	16.5	408

[a] Containing 0.05 M potassium fluoride.

together as parts of the same dimer molecule—is the same whether each exists alone or whether each exists as a portion of a dimer. Therefore, B should also be the same, in a given medium, if one of the interacting units is part of a dimer and the other is free monomer. On this basis one may write the relation between the reduced intensity, $R_{90,s}$, of a solution containing both monomer and dimer, and the corresponding weight average molecular weight $\bar{M}_w$.

$$\frac{Kc}{R_{90,s}} = \frac{1}{\bar{M}_w} + 2Bc \qquad (4)$$

Here c, the total weight concentration of protein, is defined by

$$c = c_m + c_d \qquad (5)$$

The weight average molecular weight[13] is given by the relation (note that M denotes the molecular weight of the *monomer*)

$$\bar{M}_w = \frac{\Sigma c_i M_i}{\Sigma c_i} = \frac{M(c_m + 2c_d)}{c_m + c_d} = \frac{M}{c}(c_m + 2c_d) \qquad (6)$$

The weight fraction, D, of protein which is in the form of dimer is equal to c_d/c; hence $1 - D = c_m/c$. From this definition, (6) immediately leads to the relation

$$D = (\bar{M}_w/M) - 1 \qquad (7)$$

Thus in a solution for which $B = 0$—for instance in 0.05 m acetate at pH 4.75—the weight fraction of dimer is given by comparing the reduced intensity of scattering for the solution, $R_{90,s}$, with the reduced intensity ($R_{90,m}$) for the pure mercaptalbumin monomer in the same medium at the same concentration.

$$D_{(B=0)} = (R_{90,s}/R_{90,m}) - 1 \qquad (8)$$

If B is different from zero, the procedure is slightly more complex. The ratio, $\bar{M}_w/M$, is given by the limiting ratio, extrapolated to $c = 0$, of c to R_{90} for pure monomer to that for the solution containing both monomer and dimer. Hence

$$D = \frac{(c/R_{90,m})_{c=0}}{(c/R_{90,s})_{c=0}} - 1 \qquad (9)$$

The ratio $(c/R_{90,m})_{c=0}$ is determined by extrapolation to $c = 0$ of curves such as those shown in Fig. 1. The value of B is also given from these data. The total protein concentration, c, is always a constant in any one experiment, although the ratio of monomer to dimer is, of course, generally changing with time. An experimental reading of $R_{90,s}$, since c is known, immediately gives the factor $Kc/R_{90,s}$ for the concentration c. The constant term $2Bc$ is then subtracted from this to give the limiting value of $(Kc/R_{90,s})_{c=0}$ for the particular time of measurement. The weight fraction of dimer is then immediately given by equation 9.

One important difficulty arose in the practical application of these equations. In the earlier preparations, denoted in the Tables by the symbols A and B, the weight average molecular weight was in the neighborhood of 80,000; a value which indicated the presence in the solution of appreciable amounts of larger molecules than those of the main component of molecular weight 65,000–69,000. No adequate theoretical basis has yet been formulated for the calculation of the rate of dimerization in such systems, since it is not known whether the small amounts of the heavier components are reactive to the same extent as the principal component in the formation of the mercury dimer. We have calculated the percentage of dimer from the experimental data on the tentative assumption that all species of molecules are equally reactive. Thus, in any given solution, the limiting value of c/R_{90}, before mercury was added, was taken as defining the correct value of this quantity for the pure monomer. The percentage of dimer, D, was then calculated from the increase in the reduced intensity at any given time, relative to the value for the monomer solution, according to eq. 7, 8 and 9. If some or all of the heavier molecules, present in the solution before mercury is added, are inert and do not react to form dimer, it is obvious that this procedure will lead to a calculated percentage of dimer which is lower than the true value. It was indeed found, using preparation C, with a weight average molecular weight of only 68,000, that the calculated weight function of dimer at equilibrium (D_e) was generally somewhat higher than in the earlier preparations. The calculated equilibrium constant for dimer formation—see eq. 11 below—was thus generally found to be 25–30% greater in preparation C than in A and B. However, the variations in rate of reaction and equilibrium constant, due to variations of pH, temperature and other factors, were very consistent from preparation to preparation. Thus the absolute values of the velocity and equilibrium constants reported here may be subject to small revisions later. Recent work has shown that the binding of various anions by the albumin also affects the values of these constants significantly, and the influence of these factors will be considered in detail in a later paper.

Formulation of the Observed Reactions

When mercuric chloride is added to a solution of mercaptalbumin, in the proportion of half a mole of mercury per mole of protein, the turbidity of the solution increases progressively over a considerable time, due to formation of mercaptalbumin mercury dimer. If more mercury salt is added, up to one mole of mercury per mole of albumin or slightly more, the turbidity decreases rapidly to its original value, indicating dissociation of the dimer. If a crystalline preparation of the mercury dimer is dissolved in water—especially if the solution contains halide ions—the turbidity of the solution gradually decreases. These observations suggest that reversible processes are involved, and also indicate that the rates of some at least of the reactions can be conveniently measured by light scattering.

Mercuric ion is capable of reaction with several different groups found in the protein molecule. For example, the association constant of mercury with uncharged amino groups[18] is of the order of 10^9. The affinity constant for imidazole groups is apparently not known, but is probably of the same order of magnitude. However, the affinity constant for sulfhydryl groups is of the order of 10^{18}. Hence, it is to be expected that no other group in the protein molecule will react with mercury to an appreciable extent until the available sulfhydryl groups are saturated.

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

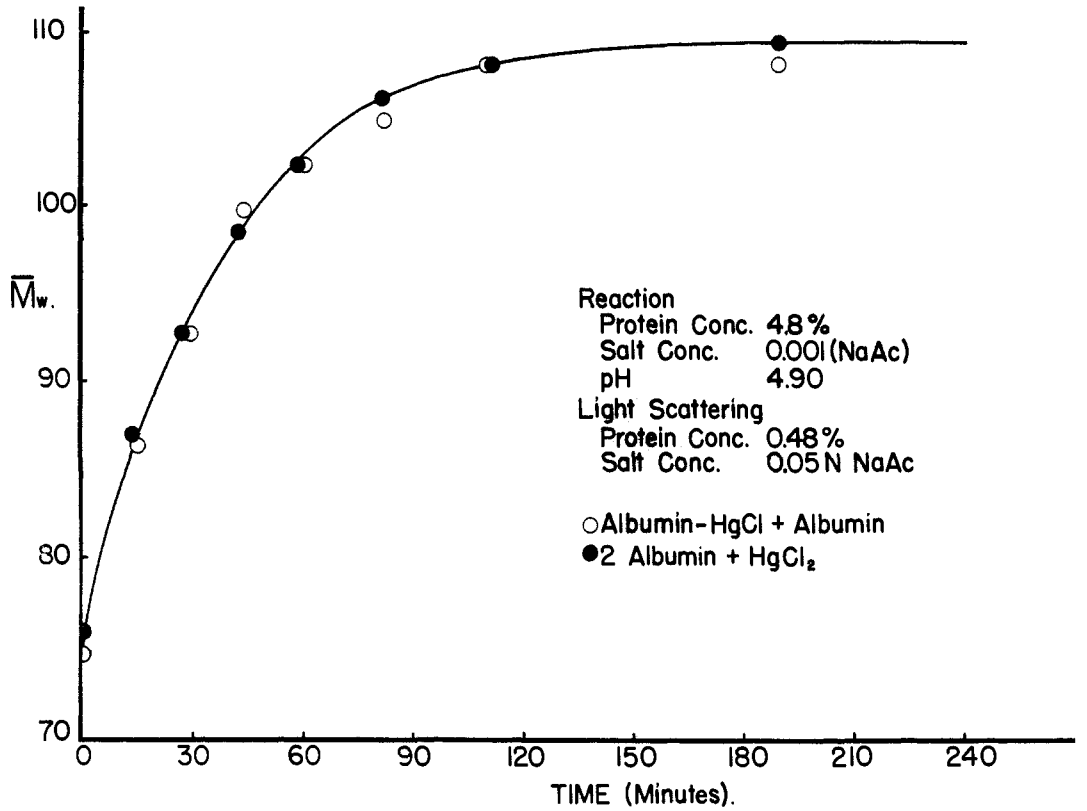

Fig. 2.—Dimerization of mercaptalbumin: open circles, ASHgCl mixed with ASH in equimolar amounts; solid circles, two moles of ASH mixed directly with one of $HgCl_2$. Rate of dimerization is the same in either case.

It has been found that the observed phenomena can be described in terms of three reactions. In formulating these, it will be convenient to denote the mercaptalbumin molecule, with its sulfhydryl group, by the symbol ASH. Its reactions with a mercury salt, HgX_2, may then be written as

$$\mathrm{ASH} + \mathrm{HgX_2} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ASHgX} + \mathrm{H^+} + \mathrm{X^-} \quad (\mathrm{I})$$

$$\mathrm{ASHgX} + \mathrm{ASH} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{ASHgSA} + \mathrm{H^+} + \mathrm{X^-} \quad (\mathrm{II})$$

$$\mathrm{ASHgSA} + \mathrm{HgX_2} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} 2\mathrm{ASHgX} \quad (\mathrm{III})$$

The precise formulation of the reactions as given here is open to discussion. For instance, if the salt added is mercuric chloride, and if the reaction is carried out in acetate buffer at *p*H near 5, the compound denoted here as ASHgX may be ASHgCl, $ASHg(CH_3COO^-)$, ASHgOH or $ASHg^+$. Still other forms, containing one atom of mercury per molecule of protein, may possibly be present in the solution. Estimates of the relative proportions of the different forms will be given later in the discussion. However, reactions I, II and III, as written here, appear to represent a satisfactory first approximation to the facts. The release of hydrogen ions, when reaction I or II proceeds from left to right, has been directly demonstrated by titration studies.

The light scattering measurements cannot, of course, distinguish between the mercaptalbumin molecule ASH and its mercury salt ASHgX, since the ratio of their molecular weights is practically unity. However, the turbidity of an isoelectric solution of the dimer is twice as great as that of a solution of the monomer of the same weight concentration. In a solution which is not isoelectric, this relation holds only at infinite dilution of the protein, but a determination of the slope of the curve for the turbidity concentration ratio as a function of concentration readily permits extrapolation to infinite dilution.

Reaction III, as written, proceeds very rapidly from left to right. Addition of one mole of mercuric chloride per mole of dimer present in solution causes a practically immediate decrease of turbidity to the value characteristic of the monomer ASHgX. The final value is already attained within about a minute—the time necessary to mix the solutions and take a reading of the scattered intensity. Probably the reaction is complete in much less than a minute. Thereafter, the turbidity remains constant for many days, if precautions are taken to prevent bacterial contamination, as by adding fluoride. Thus we may conclude that $k_3 \gg k_{-3}$.

To obtain information concerning the relative velocities of reactions I and II, an experiment was set up which is illustrated in Fig. 2. One mole of mercuric chloride was added per mole of mercaptalbumin, in a solution which contained 0.048 g. albumin/ml. at *p*H 4.9 in acetate buffer containing 0.001 *m* sodium acetate. The solution was left undisturbed for several hours, to permit the reaction between the mercury salt and the albumin to run to completion. Then an equal amount of

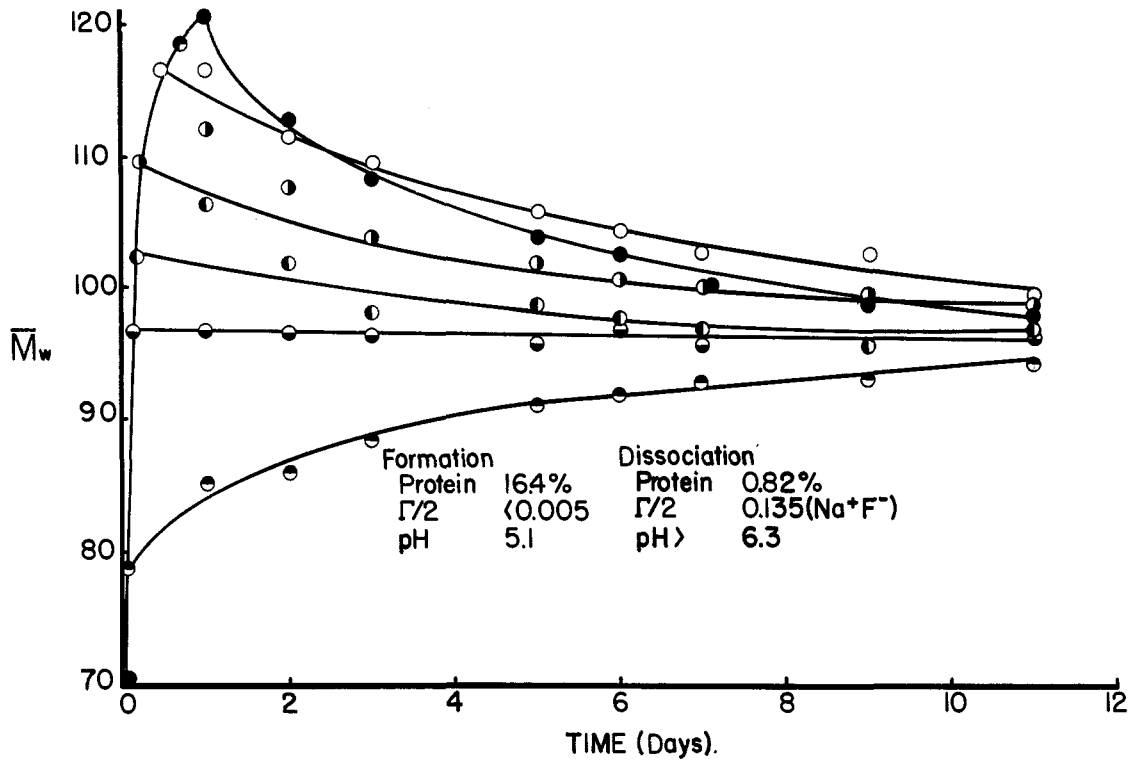

Fig. 3.—Formation and dissociation of mercaptalbumin mercury dimer. Dimer formation at *p*H 5.1, protein 0.164 g./ml., shown by steeply rising curve on left. Subsequent formation or dissociation of dimer, after diluting protein to 0.0082 g./ml., and adjusting *p*H to 6.3, is represented by the family of curves converging gradually toward a common limiting value with time. Temperature was near 23°.

mercaptalbumin was added, and the solution was immediately diluted to 0.0048 g. protein/ml., the concentration of acetate in the final solution being 0.005 *m*. The subsequent increase in scattered intensity as a function of time is shown by the open circles in Fig. 2. The ordinate is given as the weight average molecular weight, which was calculated from the intensity of scattering by eq. 7. In this experiment, reaction I was complete before the second equivalent of albumin was added; hence the measurements indicate the progress of reaction II.

Another solution was made up, in which mercaptalbumin and mercuric chloride were mixed directly in a molar ratio of 2:1, and made up at once to the same final composition as in the first solution. The increase of turbidity in this system is shown by the solid circles in Fig. 1. In this solution, reactions I and II were proceeding simultaneously, yet the over-all rate of reaction was indistinguishable from that in the first solution. Hence we may conclude that reaction II is the rate determining step, and that $k_1 \gg k_2$. Thus, in the studies to be discussed here, we are concerned with the velocity constants k_2 and k_{-2}.

It could also be shown that, in a solution of fixed total protein concentration, *p*H, mercury and salt concentrations, the same final equilibrium state was attained when the reaction was allowed to proceed in either direction. An example is given in Fig. 3. Mercuric chloride was added to a concentrated mercaptalbumin solution, the final concentration of protein being 0.162 g./ml. and the ratio $HgCl_2$/albumin being 0.45. The concentrated solution was held at 23°, and from time to time aliquots were taken off and diluted 20-fold with sodium fluoride solution, the final fluoride concentration[19] being 0.135 *m* and the final *p*H 6.3–6.4. The light scattering of these diluted solutions was measured immediately and then observed at intervals over a period of more than two weeks. The results of the measurements for the first 12 days are shown in Fig. 3. Although the weight fraction, *D*, of dimer at the moment of dilution varied over a wide range in the aliquots taken off at different times, it is evident that all the solutions were gradually approaching the same equilibrium state, with a value near 0.5 for the weight fraction of dimer at equilibrium. Progress toward equilibrium continued slowly for another period of four days beyond that shown in the diagram, after which the experiment was concluded.

One aliquot of the concentrated solution—the second from the top of the ascending curve at the left of Fig. 3—was treated with additional mercuric chloride, the final concentration ratio being one mole Hg/mole albumin. As soon as a reading could be taken on this solution—about one minute after the mercury was added—the value of R_{90} was found to have decreased to the value characteristic of the monomer solution (see reaction III above) and it remained steadily at this value for the next two weeks.

(19) The use of fluoride served two purposes: (1) since mercuric fluoride is an ionic compound, unlike the other mercury halides, the presence of fluoride ion serves to maintain the ionic strength while at the same time it does not compete with the albumin for mercury, which would inhibit dimer formation; (2) the presence of fluoride inhibits bacterial growth, of which there is a considerabe risk in such prolonged experiments on protein solutions at room temperature.

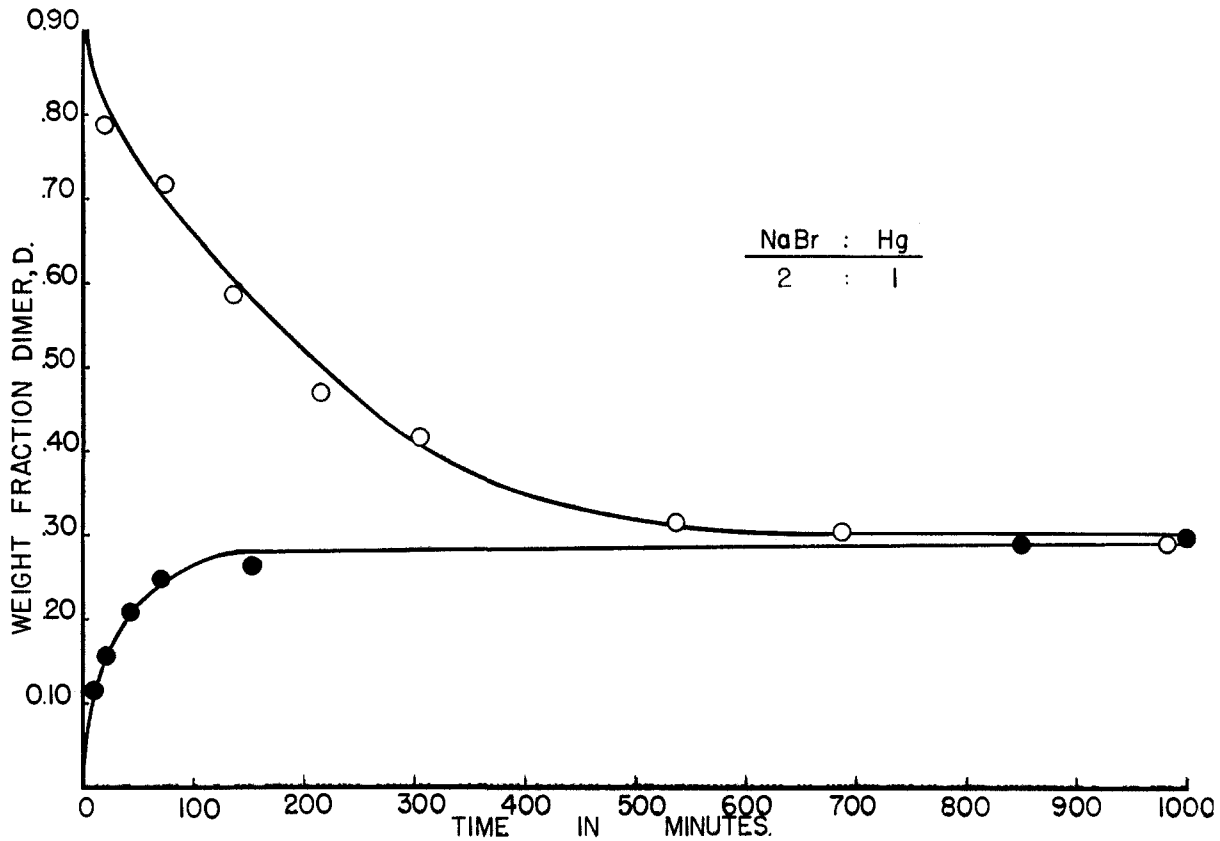

Fig. 4.—Formation and dissociation of mercaptalbumin mercury dimer. Protein concentration 0.01 g./ml., *p*H 4.75, acetate buffer at ionic strength 0.05, temperature 25°. Two moles of bromide ion present per mole of mercury. Upper curve, dissociation of freshly redissolved crystalline dimer; lower curve, association of monomer in the presence of mercuric acetate, one mole Hg to two moles albumin. Note that approach to equilibrium is much more rapid than in the experiment shown in Fig. 3.

The attainment of equilibrium in another experiment, under quite different conditions, is shown in Fig. 4. Here the dissociation of the dimer is compared with the association of the monomer under identical conditions, the total protein concentration being 0.01 g./ml., *p*H 4.75 in acetate buffer at ionic strength 0.05. Sodium bromide was present at 0.00029 *m*—two moles of bromide per mole of mercaptalbumin monomer—and this shifted the equilibrium in favor of dissociation of the dimer, an effect discussed further below. It is clear that the same equilibrium state was attained from either direction; moreover, at this *p*H the two curves had nearly converged in less than 12 hours, whereas over two weeks was required in the experiment at *p*H 6.3 to attain the same degree of convergence.

At constant concentrations of H^+, Cl^- and other ions present, we may formulate the equilibrium attained in reaction II in terms of an apparent equilibrium constant K'

$$K' = \frac{(\text{ASHgSA})}{(\text{ASH})(\text{ASHgX})} = \frac{K_{II}}{(H^+)(Cl^-)} \qquad (10)$$

Symbols in parentheses are used to denote concentrations; activity coefficient corrections, so far as it is possible to evaluate them at present, will be considered later. The evaluation of this apparent equilibrium constant, and the effects of various factors upon it, will now be considered.

Weight Fraction of Dimer at Equilibrium as a Function of $(HgCl_2)/(ASH)$.—At constant *p*H and ionic strength, the yield of mercaptalbumin dimer at equilibrium depends on the molar ratio of the mercuric chloride added to the mercaptalbumin present at the start of the experiment. We assume that all added mercury is bound to albumin -SH groups, provided the ratio $(HgCl_2)$ to initial (ASH) is less than 1. This assumption is justified by the agreement of the consequences deduced from it with the experimental data.

Then we may denote the total molar mercaptalbumin concentration by a

$$a = (\text{ASH}) + (\text{ASHgX}) + 2(\text{ASHgSA})$$

the total molar mercury concentration by b

$$b = (\text{ASHgX}) + (\text{ASHgSA})$$

and the total dimer concentration by x

$$x = (\text{ASHgSA})$$

Hence $(\text{ASHgX}) = b - x$ and $(\text{ASH}) = a - b - x$. Substituting these values in (10), we obtain

$$K' = \frac{x}{(b - x)(a - b - x)} \qquad (11)$$

hence

$$x = \frac{(K'a + 1) - \sqrt{(K'a + 1)^2 - 4K'^2(ab - b^2)}}{2K'} \qquad (12)$$

Since we are interested in the weight fraction of dimer at equilibrium $D_e = 2x/a$ as a function of the

ratio $(HgCl_2)/(ASH)$, it is convenient to introduce two new variables y and z

$$y = K'a \text{ and } z = b/a$$

then (12) becomes

$$D_e = \frac{2x}{a} = \frac{y+1}{y} - \sqrt{\left(\frac{y+1}{y}\right)^2 - 4z(1-z)} \quad (13)$$

Equation 13 shows that if y is constant, while z is varied in the range 0 to 1, $2x/a$ will reach its maximum value at $z = 0.5$. The plot of $2x/a$ *versus* z thus should yield symmetrical curves around the maximum value.

In Fig. 5 the calculated weight fraction of dimer at equilibrium is plotted against the ratio $(HgCl_2)/(ASH)_0$, for several values of the parameter $y = K'a$. Qualitatively these curves are very similar to those given by Michaelis[20] for the formation of semiquinone during an oxidation–reduction titration, as a function of the molar ratio of oxidant or reductant added to that of the substance being titrated.

The result of such a titration of mercaptalbumin with mercuric chloride, at $c = 0.01$ g./ml. ($a = 1.45 \times 10^{-4}$ M) *p*H 4.75 and ionic strength 0.05, is shown in Fig. 6. The experimental data conform fairly closely to the type of curve shown by Fig. 5, if $K'a$ is taken as 4.06, giving $K' = 2.8 \times 10^4$—a value in good agreement with our other determinations under similar conditions. It should be noted that, when the ratio $(HgCl_2)/(ASH)_0 = 1$, the concentration of dimer has fallen to zero, since reaction III has then run to completion. This indicates that, below a value of 1 for this ratio, practically all mercury in the system is bound to albumin, either as ASHgX or as ASHgSA.

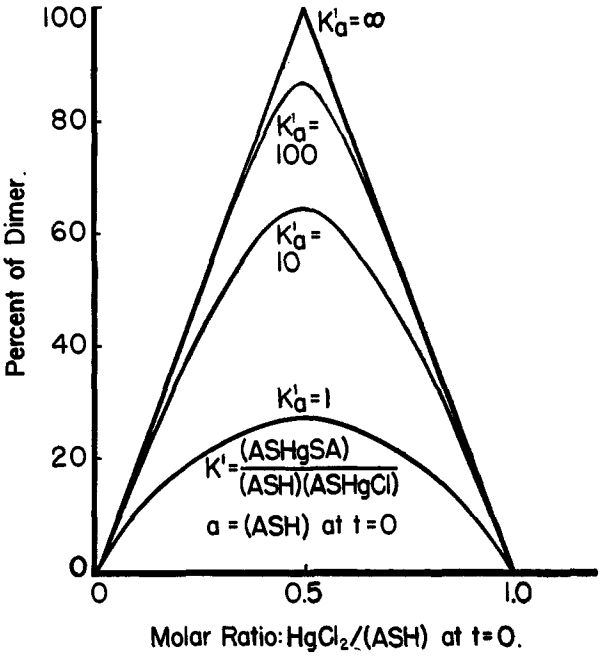

Fig. 5.—Per cent. of dimer formed at equilibrium as a function of the mercury/mercaptalbumin ratio. Each curve is a function of the parameter $K'a$ (apparent association constant multiplied by molar concentration of albumin).

Velocity Constants for Rate of Dimer Formation (k_2) and Dimer Dissociation (k'_{-2}).—From the variation with time of the weight fraction of dimer (D), we may evaluate the velocity constants for formation and dissociation of the dimer. The latter constant, denoted by k_{-2} in the chemical equation II, is proportional not only to the concentration of dimer ASHgSA, but also to (H^+) and to the concentration of the anion (or anions) X^-. Since, in any single experiment, (H^+) and (X^-) can generally be taken as essentially constant, it is convenient to define an apparent velocity constant for dissociation, $k'_{-2} = k_{-2}/(H^+)(X^-) = k_2/K'$. Employing the notation of eq. 11 and the preceding discussion, we may write

$$\frac{dx}{dt} = k_2(b-x)(a-b-x) - k'_{-2}(x) = k_2(b-x)(a-b-x) - (x)/K' \quad (14)$$

Equation 14 is readily integrated for any given values of a and b. Here we are concerned only with experiments in which $a = 2b$ (*i.e.*, the molar ratio z of HgX_2 to the sulfhydryl sulfur of mercaptalbumin is 0.5); hence (14) becomes

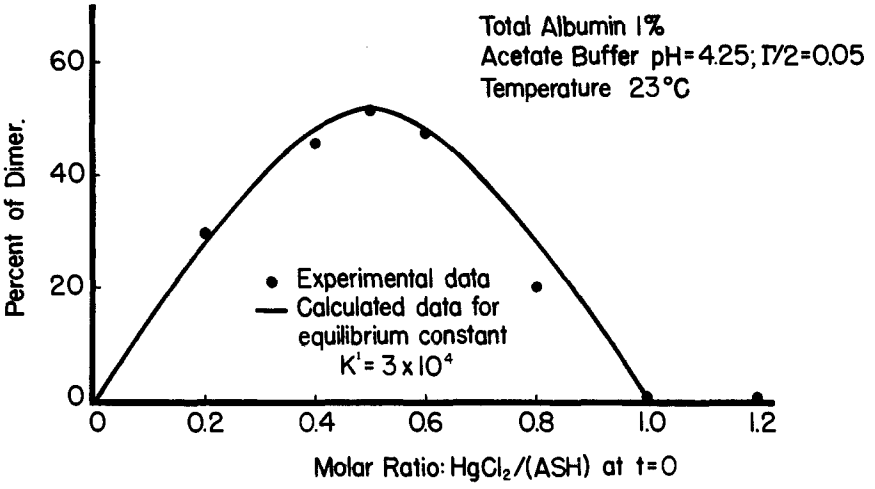

Fig. 6.—Formation of dimer at equilibrium as a function of the mercury/mercaptalbumin ratio. Compare with theoretical curves of Fig. 5. Protein 0.01 g./ml., *p*H 4.25, acetate buffer, ionic strength 0.05. The curve as drawn is for $K' = 3 \times 10^4$; a slightly better fit to the data was given by a curve assuming $K' = 2.8 \times 10^4$.

$$\frac{dx}{dt} = k_2(b-x)^2 - k'_{-2}(x) = k_2(b-x)^2 - x/K' \quad (14a)$$

The integrated form of (14a) has been frequently presented[21]; for our purposes it is conveniently

(20) L. Michaelis, *Chem. Revs.*, **16**, 243 (1935); see especially Fig. 4 on p. 254.

(21) See for instance K. J. Laidler, "Chemical Kinetics," McGraw-Hill Book Co., Inc., New York, N. Y., 1950, p. 21. There is a misprint in Laidler's equation 78, which should read

$$k_{-1} = \frac{x_e}{t(a^2 - x_e^2)} \ln \frac{x_e(a^2 - xx_e)}{a^2(x_e - x)}$$

Note that Laidler's a is identical with our b as given in eq. 11, 14 and 14a above.

expressed in terms of the quantities most directly related to the experimental measurements—namely, the weight concentration of protein $c = 2bM/1000$, the weight fraction, $D = 2x/a = x/b$, of dimer formed after time, t, and the weight fraction $D_e = x_e/b$ of dimer at equilibrium

$$k_2 = \frac{2.303}{t}\left(\frac{2M}{1000c}\right)\left(\frac{D_e}{1 - D_e^2}\right)\left[\log\frac{D_e(1 - D_eD)}{D_e - D}\right] \quad (15)$$

If, on the other hand, we start with a solution of the dimer at molar concentration b, and let x represent the concentration of ASH—and also of ASHgX—formed after time t, we have the equation

$$\frac{dx}{dt} = k'_{-2}(b - x) - k_2(x)^2 \quad (16)$$

In this case $D = 1 - (x/b)$, $D_e = 1 - (x_e/b)$, and the integrated form of (16) becomes

$$k'_{-2} = \frac{2.303}{t}\left(\frac{1 - D_e}{1 + D_e}\right)\log\left(\frac{1 - D_eD}{D - D_e}\right) \quad (17)$$

In all our studies, t was expressed in minutes, so that k_2 has the dimensions l. mole^{-1} min.$^{-1}$ and k'_{-2} the dimensions min.$^{-1}$.

Experimental Results

Values of the Apparent Equilibrium Constant K'.—Values obtained for this constant, as defined in eq. 11, are listed in Tables II and IV. The experiments summarized in Table II were carried out at three different albumin concentrations. At each concentration, equilibrium was approached from both sides, in the one direction by the addition of mercuric chloride to mercury-free mercaptalbumin in a molar ratio of 1:2; in the other by dissolving crystals of pure mercaptalbumin mercury dimer in the same solvent and following the progress of the dissociation. These experiments give extensive added confirmation of the reversibility of the reaction. They also show that the value of K' is somewhat decreased by increase in the concentration of chloride ions—an effect further discussed below. These experiments were all carried out using albumin preparations of type A, which had been treated with cysteine in order to remove the mercury after crystallization. Experiments 1, 2 and 3, in Table IV, were also carried out with this preparation.

TABLE II

REVERSIBILITY OF DIMER FORMATION. APPROACH TO EQUILIBRIUM FROM BOTH DIRECTIONS

Mercaptalbumin 35Hg 5 X (preparation A).

Exp. No.	Albumin concn., C	NaCl, m	$K' \times 10^{-4}$ Forward	Reverse	$(Cl^-)/(Hg)$
I. Experiments at pH 4.75, sodium acetate, $\Gamma/2 = 0.05$, 23–25°					
101	0.0060	0.003	2.1	1.5	65
102	.0118	.003	1.6	1.2	35
103	.0178	.003	1.6	1.0	23
104	.0058	0	3.9	6.3	2
105	.0118	0	2.9	3.1	2
106	.0175	0	2.5	3.2	2
II. Experiments at pH 4.25; sodium acetate, $\Gamma/2 = 0.05$					
111	0.0086	0.003	2.1	1.7	45
112	.0173	.003	1.2	1.3	23
113	.0257	.003	0.94	1.1	14

The remaining experiments of Table IV were carried out with preparations of type B, which had been purified by treatment with thioglycolate and passage over an exchange resin (see under Materials and Experimental Methods). The values of K' for these preparations showed no marked difference from those with the earlier preparation.[22]

In general the data show no significant trend in the value of K' with increased concentration, indicating that the relative activity coefficients of the two types of monomer and of the dimer are not greatly altered by change in concentration. It is more surprising to note that no systematic trend of the K' values with increase of pH was noted over the pH range 4.25–6 as shown by experiments 6–12, inclusive, and 15. It would be predicted from eq. 10 that K' should vary inversely as the hydrogen ion activity. The experimental data, however, give no indication of such an effect. Here it is probable that there is a compensating change in the relative activity coefficients of dimer and monomer, since the electrostatic work of bringing together two molecules, each with a charge Z, to form a single molecule of charge $2Z$, obviously increases with the value of Z.

Equation 10 also predicts that K' should vary inversely with the activity of chloride ion; indeed when the relations involved are stated in more general terms, K' should be decreased by the addition of any ion which competes with mercaptalbumin (ASH) for the ion $ASHg^+$. Such competitors include cyanide and all halogen ions except fluoride. Acetate ion also competes, but more weakly. It is probably significant that the value found for K' in experiment 4 is 5.4×10^4, in 0.01 M sodium acetate, whereas the value found in 0.05 M acetate is approximately half as great as this.

The effects of increasing concentrations of chloride, bromide and iodide on K' are shown in Table V. Here the competition is strong and increases rapidly with increasing size of the halogen ion. A fuller treatment of these phenomena is given in the Discussion.

The effect of temperature on K' may be seen from experiments 13 and 14 in conjunction with experiment 15 and a number of other experiments which were carried out at room temperature. It is interesting to note that rise of temperature shifts the equilibrium in favor of dimer formation. The observed values of K', increasing from 1.3 at 4° to 5.5 at 42°, indicate that reaction II is endothermic, and that $\Delta H°$ is of the order of 7 kcal./mole. This value may be an overestimate, since it is not certain that equilibrium was reached in the very slow reaction at 4°. It should be noted that reaction II involves the release of a hydrogen and a chloride ion (or other anions) if the formulation given is the correct one.

Experimental Values of k_2, the Velocity Constant of Dimerization.—Values of this velocity constant, which is defined by eq. 14 and 15, are given in Tables II and IV. A detailed protocol of one ex-

(22) Still more recent preparations of type C, which had received very thorough treatment with exchange resins in order to remove fatty acid molecules, showed slightly higher K' values than the others although the order of magnitude remained the same. A detailed report on these studies will be given in a later paper.

periment (experiment 9 in Table IV) is given in Table III to illustrate procedures of calculation and the order of magnitude of the accuracy of the results obtained. The computed value of the velocity constant is evidently subject to uncertainties of the order of at least ±15%. However, the data appear to be quite compatible with the assumption that the reaction is of the second order. A further test of this point is shown in Fig. 7, which represents a plot of the data from which the velocity constants in experiments 1, 2 and 3 of Table IV were computed. The data, when plotted in the indicated manner, should be linear with the time for a second-order reaction. There are significant deviations from this linear relation near the beginning and end of the top curve of Fig. 7, but the other two curves are on the whole satisfactorily linear. The calculated velocity constant for the highest concentration in Fig. 7 is slightly more than twice as great as for either of the other two concentrations which gave essentially identical values of k_2 (26 l. mole^{-1} min.$^{-1}$). Similar variations will be noted in experiments 6, 7 and 8 of Table IV which were carried out at *p*H 4.25. In general the velocity constants determined at several different concentrations, in a medium of given *p*H and ionic strength, have been found to differ by as much as a factor of 2. No systematic trend with concentration has, however, been discovered.

TABLE III

DIMERIZATION OF HUMAN MERCAPTALBUMIN AT *p*H 6.0 IN PHOSPHATE BUFFER (c = 0.02 G. PROTEIN/ML.) (EXPERIMENT OF FEB. 2, 1952)

Total ionic strength of solvent was 0.15; this included sodium phosphate buffer of ionic strength 0.10 and 0.05 M potassium fluoride, added to inhibit possible bacterial growth.

Initial solutions: (a) 2.5% human mercaptalbumin, dissolved in the indicated solvent (b) mercuric chloride solution, 7.25×10^{-4} m, dissolved in the same solvent. Both solutions were centrifuged 5 hours at 16,000 times gravity. Albumin solution (2 ml.) was then pipetted carefully into the light scattering cell, using a clean dust-free pipet. The turbidity τ, referred to a Ludox standard (τ = 0.022 at 436 mμ), was 0.0169 cm.$^{-1}$. To this solution was added 0.5 ml. of the mercuric chloride solution (one mol Hg per 2 mols albumin, taking molecular weight as 69,000), with subsequent stirring for 4 minutes with a magnetic stirrer. Subsequent turbidity readings, and the quantities calculated from them, were as follows. Times in minutes after time of addition of mercuric chloride. The weight fraction of dimer at equilibrium (D_e) was taken as 0.60.

Time	τ	c/τ	D	$\log_{10} Q$	$(\log Q)/t \times 10^4$
11	0.01432	1.396	0.003		
30	.01435	1.396	.004		
60	.0143	1.396	.003	0.001	(0.165)
120	.0146	1.369	.031	.015	(1.25)
520	.01495	1.338	.062	.031	0.60
1109	.0153	1.307	.095	.049	.44
2507	.0161	1.241	.173	.100	.40
3167	.0162	1.235	.182	.107	.34
3941	.0166	1.203	.227	.130	.33
4431	.01715	1.165	.282	.196	.44
5802	.0176	1.139	.322	.242	.42
7240	.0180	1.109	.373	.312	.43
8773	.01905	1.050	.485	.590	(.67)
11701	.0196	1.021	.549		
14416	.02015	0.995	.61		
Av. (values in parentheses omitted in taking average)					0.46

The weight fraction of dimer (D) was calculated from the turbidity by extrapolating the ratio c/τ to c = 0 according to the equation $(c/\tau) = x + 16.5c$ (see Table I), the factor 16.5 having been determined by measurements on pure mercaptalbumin monomer in the same solvent as a function of concentration. The value of x for the pure monomer (x_0) was determined as 1.07.[a] Then D was given by the relation (see eq. 7, 8 and 9)

$$D = (x_0/x) - 1 = (1.07/x) - 1$$

log Q is an abbreviation for log $[D_e(1 - D_eD)/(D_e - D)]$ (see eq. 15). From the average value of $(\log Q)/t = 0.46 \times 10^{-4}$, the velocity constant k_2 was calculated as 0.69 l./mole^{-1} min^{-1} by eq. 15. The equilibrium constant, K' (eq. 11) was calculated as 2.6×10^4 from $D_e = 0.60$.

[a] This value of x_0 corresponds to a weight average molecular weight near 89,000. Later calibrations have indicated that the true value of $\bar{M}_w$ was approximately 10% lower than this. However, as recalibration would also change all the values of x by the same factor, the calculated values of D, and of the velocity and equilibrium constants, would remain unaltered.

In some experiments the "apparent value" of k_2 appeared to decrease in the later stages of the reaction, although a plot such as that of Fig. 7 was linear in the earlier stages of the reaction, and again linear in the later stages but with a slope approximately half as great. This was found to be true for instance in experiments 7 and 8 of Table IV. The values recorded in the table, in such cases, are those for the earlier part of the reaction. This complication, and the factors influencing it, will be further discussed in a later paper of this series.

Effect of *p*H upon k_2.—The data of experiments 6–12, inclusive, and 15, in Table IV, show the very pronounced effect of *p*H on this velocity constant. Dimerization was extremely slow at *p*H 6; at this *p*H observations were carried out over a period of nearly 8000 minutes in order to obtain a reliable velocity constant, and for another period of equal length in order to determine the position of equilibrium (see Table III). Potassium fluoride, 0.05 M, was present in the reaction medium to act as a preservative against bacterial contamination over this long time; it was also added in the experiments at other *p*H values down to *p*H 5 in order to make the conditions comparable in all these experiments. The velocity constant increased approximately 60-fold, from 0.7 to about 40, as the *p*H was varied from 6 to 4.75. If we accept the results of experiments 7 and 8, it appears to rise still further, by a factor of approximately 2, on decreasing the *p*H to 4.25. Thus k_2 appears to increase steadily with decreasing *p*H; there is no indication of a maximum at the isoelectric point.

Effect of Temperature upon k_2.—Experiments 14 and 15, at 4 and 42°, respectively, may be compared with experiment 16 at 24° and a series of other experiments at approximately this temperature, all at *p*H values of 4.75 in the presence of 0.05 M acetate. It is immediately apparent that there is a very rapid rise of velocity constant with temperature. The results of experiments 13, 14 and 15 are shown in detail in Fig. 8. The experimental points for the most part lie well on the calculated curves for second order reactions. However, the first few experimental points at 4° lie considerably above the calculated curve. There

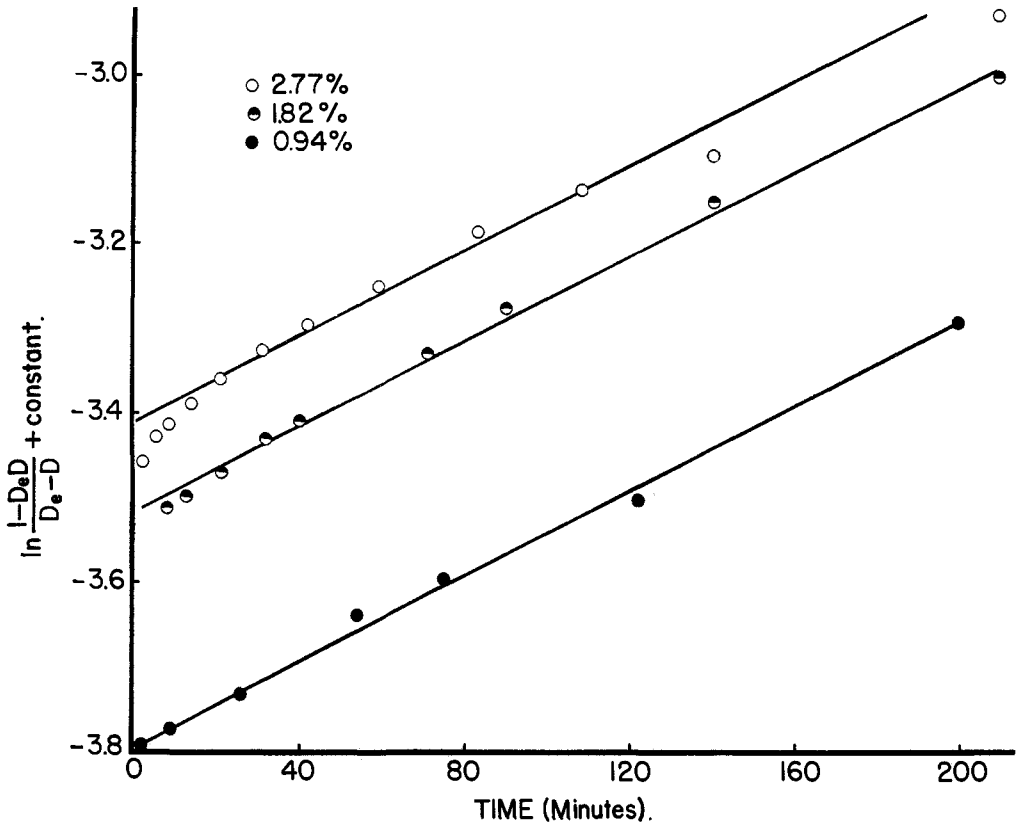

Fig. 7.—Dimerization at three different protein concentrations. The function indicated on the ordinate axis should be linear in the time if the reaction is second order. *p*H 4.75, acetate buffer, ionic strength 0.05.

were technical difficulties in carrying out measurements at this low temperature, since the light scattering apparatus itself was not thermostated; the cell containing the albumin solution was kept in a cold room at 4° and withdrawn occasionally for a minute or two to make a light scattering measurement in the apparatus, being surrounded by an outer jacket of cold water during this brief period. No definite explanation can be offered for these anomalous points. The later points determined in this experiment all lie very close to the calculated curve for the second-order reaction, and we tentatively accept them as determining the true velocity constant, which is found from these data to be 5 l.-mole^{-1} min.$^{-1}$. The general character of the three curves shown in Fig. 8 accords well with that of a similar set of experiments which had been carried out with a preparation of type A three years earlier. In this set of experiments the reaction took place in a considerably more concen-

TABLE IV

VELOCITY AND EQUILIBRIUM CONSTANTS FOR DIMER FORMATION

Experiments 1, 2 and 3 were carried out in 1948, using a preparation of type A. All the other experiments in this table were carried out in 1951–1952 with preparations of type B.

Experiment no.	c, g./ml.	*p*H	Γ/2	Salts present: Na acetate	KF	NaCl	*t*, °C.	k_2 l./mole/min.	$K' \times 10^{-4}$
1	0.0094	4.75	0.05	0.05	0.00	0.003	23	57	2.8
2	.0182	4.75	.05	.05	.00	.003	23	26	1.5
3	.0277	4.75	.05	.05	.00	.003	23	26	1.9
4	.0095	4.75	.01	.01	.00	.00	23	31	5.4
5	.0100	4.75	.05	.05	.00	.00	23	....	2.8
6	.0289	4.25	.05	.05	.00	.00	22	39	3.25
7	.0183	4.25	.05	.05	.00	.00	22	72	4.0
8	.0090	4.25	.05	.05	.00	.00	22	85	3.1
9[a]	.0200	6.0	.15	.00	.05	.00	22.5	0.69	2.6
10	.0200	5.7	.15	.10	.05	.00	23	2.3	2.3
11	.0200	5.36	.15	.10	.05	.00	23	5.0	2.3
12	.0200	5.0	.15	.10	.05	.00	23	15.2	2.7
13	.0100	4.75	.05	.05	.00	.00	4	5	(1.3)
14	.0100	4.75	.05	.05	.00	.00	42	260	5.5
15	.0100	4.75	.05	.05	.00	.00	24	41	2.9
16	.0100	4.75	.05	.05	.00	.00	25–26	47	2.76

[a] This solution contained sodium phosphate buffer ionic strength 0.10.

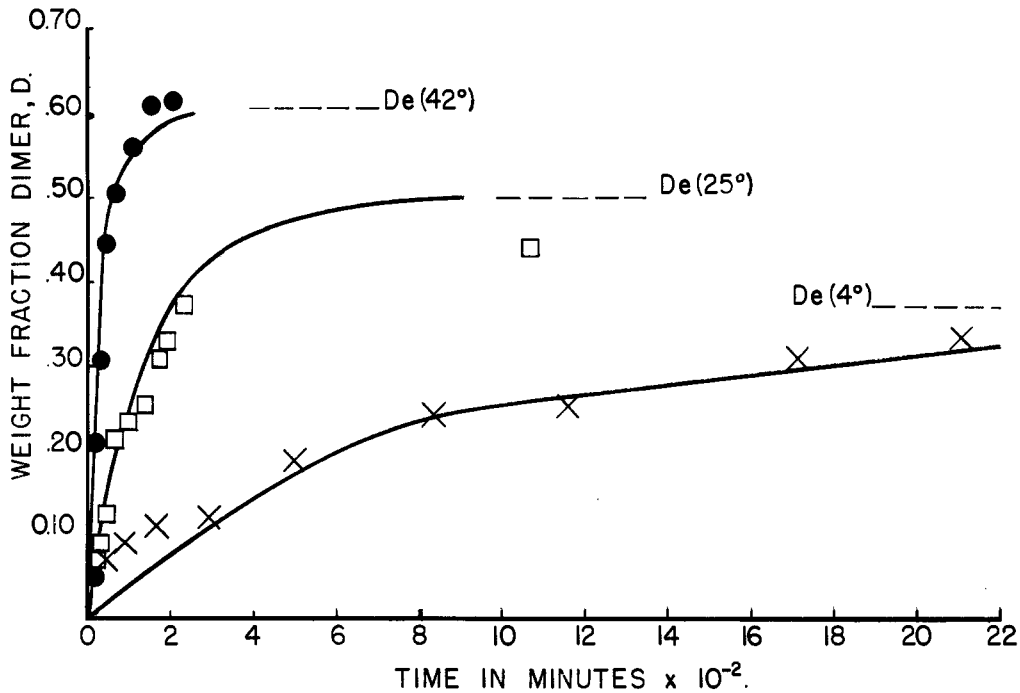

Fig. 8.—Dimerization of mercaptalbumin at three temperatures. Albumin 0.01 g./ml., *p*H 4.75, acetate buffer, ionic strength 0.05. The solid lines represent calculated curves, assuming second-order velocity constants k_2 = 5 l. mole^{-1} min.$^{-1}$ at 4°, 40 at 25° and 260 at 42°. The value of D_e at 4° is based on readings taken 3725 minutes after mixing albumin and mercury, but it is probably lower than the true equilibrium value at this temperature.

trated protein solution (0.056 g./ml.), aliquot samples being removed at intervals, diluted tenfold, and observed in the light scattering apparatus immediately after dilution. The resulting values of k_2 were close to 8, 100 and 500 l. mole^{-1} min.$^{-1}$ at 0, 25 and 37°, respectively. Here again the results at the lowest temperature were the least consistent. The calculated energy of activation, from these and other experiments, was in the range 17–21 kcal./mole.

Values of the Dissociation Constant k'_{-2}; Effects of Added Halogen Ions.—In the absence of added chloride, bromide or iodide ions or other ions with a high affinity for mercury, the velocity of dimer dissociation is very slow. The velocity constant k'_{-2} was measured directly in a number of experiments by dissolving the crystalline dimer in an appropriate buffer and observing the rate of dissociation (Table V). In other cases it could be calculated from the measured values of K' and k_2 given in Table IV. At *p*H 4.75 in 0.05 *M* acetate buffer, in which the albumin is approximately isoelectric, the calculated k'_{-2} is in the range 0.001–0.002 min.$^{-1}$ at 23°. It is notable, however, that k'_{-2}, like k_2, must be greatly decreased by increase of *p*H, since $K' = k_2/k'_{-2}$ is essentially independent of *p*H, and k_2 decreases rapidly as the *p*H increases. Thus the calculated value of k'_{-2} at *p*H 6, from experiment 9, is approximately 2.5 × 10^{-4} min.$^{-1}$. All our experience accords generally with this finding; the dimer is extremely stable at *p*H values above 6 in the presence of phosphate or fluoride ions, provided that the presence of other halogen ions is avoided. Such solutions are, therefore, suitable for observations on the dissolved dimer when it is desired to carry out a prolonged experiment to study the properties of dimer solutions containing very little monomer.

TABLE V

EFFECTS OF HALOGEN IONS ON FORMATION AND DISSOCIATION OF MERCAPTALBUMIN MERCURY DIMER[a]

All solutions in sodium acetate buffer, *p*H 4.75, Γ/2 0.05, total protein 0.01 g./ml. (1.45 × 10^{-4} *M* if entirely in form of monomer), 0.5 mole Hg/mole of albumin SH groups, temperature 25 ± 2°.

Experiment no.	Molar ratio, (X^-)/Hg	D_e	K'(obsd.) × 10^{-4}	k_2, l. mole^{-1} min.$^{-1}$	k'_{-2}, min.$^{-1}$
		Sodium chloride solutions			
18	0	0.50	2.8	47	0.0017
19	10*	.48	2.8	49	
20	50	.37	1.3	75	
21	100	.32	0.96		.0043
22	200	.28	0.74		
23	2000	.04		(0)	.039
		Sodium bromide solutions			
24	2	0.30	0.85	(120)	0.0034
25	10	.05	.08		.009
26	20	.05	.08		
27	25	.04	.05		.025
28	100	(.00)	(.00)		.040
		Sodium iodide solutions			
29	1	0.0	0.0		0.045
30	5	0.0	0.0		0.10

[a] All the results reported in this table were made on a preparation of type C during 1952. (See under Materials and Experimental Methods.) Practically identical results had been obtained three years earlier in experiments with type A.

The rate of increase in dissociation constant with temperature may be calculated from the data of experiments 13, 14 and 15 for k_2 and K'; the cal-

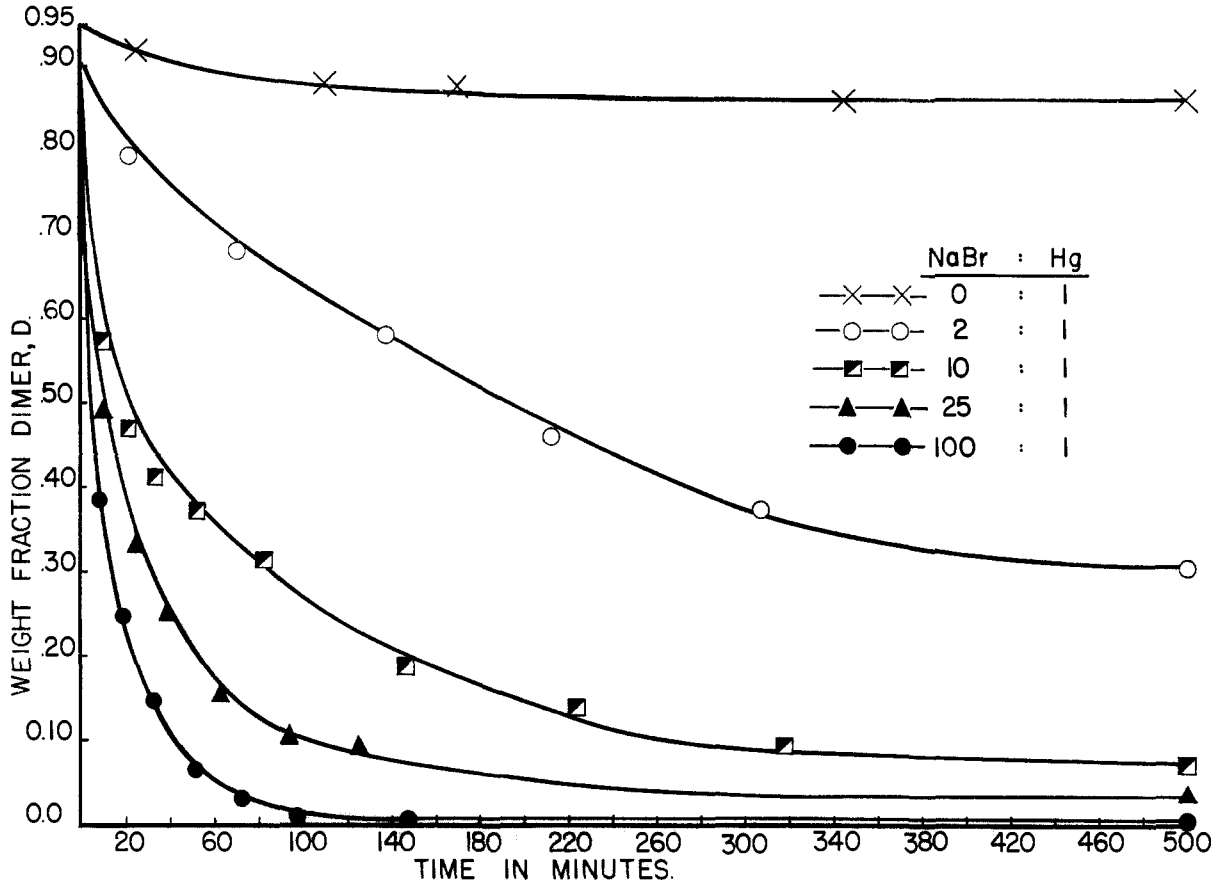

Fig. 9.—Dissociation of mercaptalbumin mercury dimer in the presence of bromide ion. Albumin 0.01 g./ml., *p*H 4.75, acetate buffer at ionic strength 0.05. Molar ratios of bromide ion to mercury are indicated for the various curves.

culated energy of activation is between 10 and 15 kcal./mole.

The addition of halogen ions, particularly of bromide and iodide ions, to the solution leads to a rapid increase in the rate of dissociation of the dimer. This is illustrated in Fig. 9 for several different concentration ratios of bromide ion to mercury. The very slow rate of dissociation in the absence of added bromide should be noted. Increase of chloride ion concentration has a similar effect to that of bromide, but much higher concentrations are required before the effect is apparent. On the other hand, a single iodide ion per atom of mercury in the solution produces an effect nearly as large as that of 2000 chloride ions. The significance of these observations is treated further below in the Discussion.

Discussion

Effect of Charge on Protein on Velocity of Dimerization.—The regular decrease in the velocity constant k_2, as the *p*H increases from the isoelectric value of 4.75[23] to *p*H 6 is undoubtedly correlated with the increased electrostatic work involved in bringing two albumin monomer molecules together to form a dimer, as the net charge on each of the monomer molecules increases.

If, for purposes of calculation, we take the albumin molecule as a sphere of radius $b = 30 \times 10^{-8}$ cm.,[24] then its electrostatic free energy, $\bar{F}_e$, and its activity coefficient, γ_e, when the molecule carries a net charge Z, distributed uniformly over the surface of the sphere, are given by the Debye–Hückel theory as

$$\ln \gamma_e = \frac{\bar{F}_e}{kT} = \frac{\epsilon^2 Z^2}{2DkT}\left(\frac{1}{b} - \frac{\kappa}{1+\kappa a}\right) = Z^2 w \quad (18)$$

Here ϵ is the proton charge, **D** is the dielectric constant of the medium, k is Boltzmann's constant; κ, in aqueous solution at 25°, at ionic strength $\Gamma/2$, is equal to $0.327 \times 10^8 \sqrt{\Gamma/2}$; and w is defined by the equation.[25] If two ions of charge Z_a and Z_b react to form a critical complex of charge $Z_a + Z_b$, and if the velocity of the total process is proportional to the concentration of this complex, then the rate of the reaction will be retarded by increase of the values of Z_a and Z_b, if both charges are of the same sign. According to the theory developed by Brönsted,[26] Christiansen[27] and Scatchard,[28] the change in the velocity constant of the reaction, compared to the velocity constant k_0 obtained when the net charges are zero, is given to a good approximation by the expression

$$\ln k = \ln k_0 - 2Z_a Z_b w \quad (19)$$

(23) The solution is isoelectric at this *p*H in 0.05 *M* acetate buffer, due to the binding of acetate ions, which is sufficient to counterbalance the net charge due to binding of protons at this *p*H. The electrophoretic measurements of L. G. Longsworth and C. F. Jacobsen, *J. Phys. Colloid Chem.*, **53**, 126 (1949), on bovine serum albumin in acetate buffers furnish additional evidence that this isoelectric point is approximately correct. The isoionic point of serum albumin, at which the net binding of protons is zero, lies above *p*H 5 and is a function of the number of anions bound by the protein. See for instance, G. Scatchard and E. S. Black, *ibid.*, **53**, 88 (1949).

(24) G. Scatchard, A. C. Batchelder and A. Brown, THIS JOURNAL, **68**, 2320 (1946).

(25) The definition of the activity coefficient in eq. 18 refers to a standard state in which the protein molecules carry zero net charge.

(26) J. N. Brönsted, *Z. physik. Chem.*, **102**, 169 (1922).

(27) J. A. Christiansen, *ibid.*, **113**, 85 (1924).

(28) G. Scatchard, *Chem. Revs.*, **10**, 229 (1932).

if we make the rough approximation that the radius of the critical complex is the same as that of the reactants.

In the reaction between ASH and ASHgX to form the mercury dimer, we have $Z_a = Z_b = Z$, which is the total net charge on the albumin determined by the binding of protons and other ions; and the factor Z_aZ_b in eq. 19 becomes equal to Z^2. If the ionic strength is 0.15, as in our experiments at pH 5 and above, the value of κ is 1.26×10^7, and **D** at 25° is 78.4. For a rough calculation, we take the collision diameter $a = 30 \times 10^{-8}$ cm., identical with the radius b. Inserting numerical values, and converting to denary logarithms, we obtain

$$\log k = \log k_0 - \frac{5.00 \times 10^{-2} Z^2}{2.303} = \log k_0 - 0.0217Z^2 \quad (20)$$

Experimentally—see Table IV—we find for k_0 approximately 40 l. mole^{-1} min.$^{-1}$ at pH 4.75, where $Z = 0$, and $k = 0.7$ at pH 6. From eq. 20 we should calculate Z at pH 6 to be approximately −9. From the titration curve of Tanford,[29] it would appear that approximately 10 protons per molecule are removed between pH 4.75 and 6. Partially counterbalancing this, there will be some decrease in the binding of acetate, because of the increased electrostatic repulsion to the binding of anions as the total negative charge on the protein increases. Thus the calculations based on eq. 19 and 20 give results of the right order of magnitude. The model employed involves so many approximations, however, that little significance is to be attached to the close agreement between the values of Z estimated by these two different methods.

The electrostatic theory would predict that the velocity constant should decrease on the acid side of pH 4.75. Actually the reaction proceeds at least as fast, if not faster, at pH 4.25. Here the electrostatic theory seems to give results quite at variance with the facts. However, it should be remembered that the binding of anions by the protein increases with the binding of protons,[30] although certainly not enough to maintain the total net charge at a value of zero. The findings of Tanford[29] concerning the titration curve of serum albumin acid to its isoionic point are also of interest in this connection.

Effect of Charge on Velocity of Dimer Dissociation.—Since k_2 decreases with increasing pH, while K' is nearly independent of pH, it follows that k'_{-2} must vary with pH in almost exactly the same manner as k_2. It is probable that part of the explanation may again be given in electrostatic terms. From the data of Table V, it is evident that the rate of dimer dissociation is greatly increased by the presence of halide ions, and that the effect of these ions in promoting dissociation is directly related to their affinity for mercury (a more detailed analysis of this point is given below). This suggests that the critical complex for dissociation of the dimer involves an anion attached to the mercury, so that the structure might be written as ASHg(X$^-$)-SA, the binding of three groups to the mercury being perhaps analogous to the structure of the HgI_3^- ion. Then, if the protein carries the negative net charge Z, the charge on the anion X$^-$ being −1, eq. 19 could again be applied to the velocity constant of the reaction, except that now the term $2Z_aZ_b$ simply becomes equal to $2Z$, with positive sign. Applying eq. 20, but substituting $2Z$ in place of Z^2, we should calculate a two- to threefold change in reaction velocity when Z changes from 0 to −10, as compared to the actual change by a factor of approximately 60 when the pH varies from 4.75 to 6. Most of the change in k'_{-2}, then, must be ascribed to other causes. It should be remembered that when reaction II, as written, proceeds from right to left, the uptake of a proton is involved. If this proton addition is a part of the rate determining step for dimer dissociation, then the decrease in (H$^+$) by a factor of nearly 20, between pH 4.75 and 6, would be a major factor in determining the decrease of k'_{-2} that occurs over this pH range.

Effect of Charge on the Apparent Equilibrium Constant, K'.—Employing the model defined by eq. 18, the activity coefficient ratio when two molecules of monomer, each of charge Z, combine to form one dimer molecule of charge $2Z$, is $\gamma_d/\gamma_m^2 = e^{4wZ^2}/e^{2wZ^2} = e^{2wZ^2}$. Thus as Z increases by adding charge to the isoelectric protein the *concentration* ratio defined by K' should tend to decrease. On the other hand, since (H$^+$)—see eq. 10—is decreasing as the pH is shifted to the alkaline side of the isoelectric point, this should tend to increase K', more or less counterbalancing the effect of the increased net charge on the protein. The actual result is, as we have already seen, that K' is very nearly independent of pH. The value of w, which has been chosen in evaluating eq. 20, gives too large a value of γ_d/γ_m^2 to fit the experimental facts. It is easy to make adjustments in the value of w, which will give better agreement with experiment, particularly if (as is reasonable) we take w for the dimer as about 25% less than for the monomer on account of its larger size. Detailed presentation of such calculations, however, would be pointless, since there is too much possible latitude in the choice of parameters to make quantitative agreement significant.

Effects of Added Anions on the Apparent Equilibrium Constant, K'.—It is apparent from the data of Table V that added halide ions displace the state of the system at equilibrium in the direction of dissociation of the dimer. This is to be expected, since the addition of halide (X$^-$) ions to the solution of the dimer should displace reaction II to the left, with formation of the mercury containing monomer ASHgX. The resulting decrease in the apparent equilibrium constant (K') for dimer formation can be interpreted as depending on the relative affinity of the ion $ASHg^+$ for the anion X$^-$ and for the anion AS$^-$, which is the conjugate base of mercaptalbumin (ASH). The larger the association constant $K_X = (ASHgX)/(ASHg^+)(X^-)$ the smaller will be the value of K' for a given concentration of X$^-$, if pH, temperature and other variables are fixed. Available data are not sufficient to calculate the concentration of $ASHg^+$, or the absolute value of K_X. However, without attempting a detailed mathematical analysis here, it is reasonable to estimate roughly the *relative* values of K_X for Cl$^-$, Br$^-$ and I$^-$ as being inversely proportional to the concentrations of each of these ions which must be added to reduce K' by a given amount. Thus from the data in Table III, it is seen that K' decreases from 2.8×10^4 to 0.74×10^4 when 200 moles of chloride ion is added per mole of mercury. A slightly smaller decrease is produced by adding 2 moles of bromide ion; hence the ratio K_{Br}/K_{Cl} is somewhat less than 100, probably 80–90. The effect of iodide ion is so great that it is difficult to compare with that of the other two. A single iodide ion per mole of mercury is equivalent to approximately 2000 chloride ions. However, the binding of iodide by mercury is so strong that the ratio of *free* chloride to *free* iodide in the two solutions must be much greater than 2000:1.

These figures may be compared with the relative affinity constants of the HgX^+ ion for X$^-$, where X$^-$ is Cl$^-$, Br$^-$ or I$^-$. These have been evaluated by Sillén[31] and his colleagues at 25° and at ionic strength 0.50, together with the other successive association constants of Hg^{++} with these ions. Sillén reports $\log K = \log [(HgX_2)/(HgX^+)(X^-)]$ as 6.48, 8.28 and 10.95 for X = Cl$^-$, Br$^-$ or I$^-$, respectively (see also Bjerrum[18]). Thus the three K values are in the ratio 1:63:30,000, which is not far from our estimates of K_X as in the ratio of 1 to 80–90 to a number much greater than 2000, for Cl$^-$, Br$^-$ and I$^-$, respectively.

(29) C. Tanford, THIS JOURNAL, **72**, 441 (1950).

(30) G. Scatchard, I. H. Scheinberg and S. H. Armstrong, *ibid.*, **72**, 540 (1950).

(31) See the summary by L. G. Sillén, *Acta Chem. Scand.*, **3**, 539 (1949).

A similar analysis may be applied to the effects of the halide ions on the velocity constants k'_{-2} for dissociation of the dimer. It already has been suggested that the critical complex for the dissociation process may involve a halogen ion bound to the dimer molecule, so that the reaction could be represented by the equations

$$\mathrm{ASHgSA} + \mathrm{X^-} \rightleftharpoons \mathrm{ASHg(X^-)SA} + \mathrm{H^+} \rightleftharpoons \mathrm{ASHgX} + \mathrm{ASH}$$

The possible part played by the hydrogen ion has been discussed already. As regards the halide ion, the rate of the over-all reaction may be expected to depend on the formation of the activated complex $\mathrm{ASHg(X^-)SA}$. The amount of the complex formed must depend on the product of the affinity constant and the concentration of the halide ion. It is seen from Table V that 100 moles of chloride ion have about the same effect in increasing the dissociation constant k'_{-2} as 2 moles of bromide ion; likewise 100 moles of bromide ion have about the same effect as 1 mole of iodide. Thus the ratios of the association constants of the dimer for these halide ions, in the step which precedes dimer dissociation, appear to be very similar to the association constant ratios of the ions $\mathrm{X^-}$ for $\mathrm{ASHg^+}$ or for $\mathrm{HgX^-}$. This system thus represents one of the numerous examples in which a group of substituents exert parallel effects on the velocity constant of one reaction and on the equilibrium constant of a closely related reaction.[32]

The speed with which these dissociation reactions take place demonstrates the accessibility of the mercury atom and the sulfhydryl groups in the dimer to these reagents. With one mole of iodide added per mole of dimer at 25°, dissociation of the dimer is 60% complete within 10 minutes, and 90% complete within half an hour. The effect of additional mercuric chloride in dissociating the dimer (reaction III) is complete within a minute. Hence the reactive center that links the two halves of the dimer together is not shielded from the solvent, but is readily available for attack by molecules and ions in the surrounding liquid.

Energy and Entropy of Activation in the Dimerization Process.—The measurements at different temperatures, listed in Table II, indicate a relatively large energy of activation, E_{exp} for the dimerization, approximately 18 kcal./mole. We may estimate the standard entropy of activation $\Delta S^{\ddagger}$, utilizing this value of E_{exp} and the measured velocity constant of dimerization, k_2 at 25°, which may be taken as 40 l. mole^{-1} min.$^{-1}$ or 0.67 l. mole^{-1} sec.$^{-1}$. Then $\Delta S^{\ddagger}$ may be evaluated from the equation[33]

$$k_2 = e^{kT/h} e^{\Delta S^{\ddagger}/R} e^{-E_{exp}/RT} \qquad (28)$$

where k is Boltzmann's constant and h is Planck's constant. $\Delta S^{\ddagger}$ is found to be slightly negative and is approximately -1.7 cal. deg.$^{-1}$ mole^{-1}. This value, although negative, is surprisingly near zero for a reaction involving the union of two molecules to form one, since $\Delta S^{\ddagger}$ is usually large and negative for such processes. Further discussion of this point will be deferred to a later paper in which a comparison is made with the dimerization reaction involving bifunctional organic mercurials.

Studies with Other Metallic Ions.—To each of a series of 2-cc. samples of 11% salt-free crystalline mercaptalbumin solution was added one-half equivalent of one of the following salts (0.01 *M*): $Zn(CH_3COO)_2$, $ZnCl_2$, $CdCl_2$, $MnCl_2$, $CuCl_2$, $Cu(CH_3COO)_2$, $LaCl_3$, $Pb(NO_3)_2$, $BaCl_2$, $UO(NO_3)_2$, $Fe_2(SO_4)_3$, $Hg(NO_3)_2$ and $HgCl_2$.

After 20 hours at room temperature, 1 ml. of each solution was removed and diluted to 20 cc. with acetate buffer, pH = 4.75; $\Gamma/2$ = 0.05. The specific turbidities (τ/c) of the solutions containing the mercury salts were almost twice those of the others. Finally one-half equivalent of $HgCl_2$ was added to the remaining volume of the 11% mercaptalbumin solutions. After 20 hours, and appropriate dilution with acetate buffer, the specific turbidities of all the solutions other than the $Hg(NO_3)_2$ and $HgCl_2$, had increased almost twofold and the mercury solutions gave results close to their initial values.

It may be concluded from these experiments that none of these ions except Hg^{++} is capable of forming a dimer of mercaptalbumin under these conditions; nor does any one of them competitively inhibit dimerization. Studies with silver nitrate, however, showed that the addition of Ag^+ inhibits dimerization by mercury, a result to be expected in view of the high affinity of the Ag^+ ions for the sulfhydryl group.

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(32) For discussion of other examples, see for instance L. P. Hammett, *Chem. Revs.*, **17**, 125 (1935); "Physical Organic Chemistry," McGraw-Hill Book Co., New York, N. Y., 1940; S. Glasstone, K. J. Laidler and H. Eyring, "The Theory of Rate Processes," McGraw-Hill Book Co., New York, N. Y., 1941, p. 466 ff.

(33) See Glasstone, Laidler and Eyring, ref. 31, p. 199.