IMMUNOCHEMICAL STUDIES OF THE KINETICS OF MONOMERIZATION OF SERUM ALBUMIN MERCURY DIMER*

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The reaction of human serum mercaptalbumin with mercury salts was described by EDELHOCH, KATCHALSKI, MAYBURY, HUGHES AND EDSALL in the following terms:

$$ASH + HgX_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ASHgX + H^+ + X^- \qquad I$$

$$ASHgX + ASH \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ASHgSA + H^+ + X^- \qquad II$$

$$ASHgSA + HgX_2 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} 2\,ASHgX \qquad III$$

ASH refers to the mercaptalbumin molecule with its sulfhydryl group and X^- to the anion of the mercury salt[1]. Reaction III proceeds too rapidly from left to right for measurement by the light-scattering technics used by these authors[1]. The present report describes an immunologic method for measurement of k_3 and provides information on the effect of antigen aggregation on the precipitin reaction by comparing mercaptalbumin monomer and its mercury dimer as antigens.

MATERIALS AND METHODS

Preparation of antigens

Human serum albumin was crystallized three times as the mercury dimer by the method of HUGHES[2]. Mercaptalbumin monomer was prepared by dialysis of the dimer against an excess of cysteine to remove the mercury and, finally, against several changes of water. Human serum albumin was also crystallized three times with decanol by the method of COHN, HUGHES AND WEARE[3].

Preparation of antisera

Immune sera were prepared in rabbits by the intravenous injection of increasing doses of alum-precipitated antigens on alternate days for five weeks. A total of 60 mg of protein was administered. The rabbits were bled six days after the last injection and the complement in the sera inactivated by heating at 56° C for thirty minutes.

Immunologic and chemical procedures

The quantitative precipitin method of HEIDELBERGER AND KENDALL[4] was followed. All sera and antigens were dialyzed against several changes of the diluent, 0.105 *M* phosphate buffer at pH 7.0, before use in the precipitin analysis. Dimer solutions were prepared from the crystalline material on the day of each experiment and analyses were made in the Spinco analytical ultracentrifuge on the antigens (dimer and monomer), the day after an experiment was set up. This was done to determine the extent of spontaneous monomerization of the dimer solution after 24 hours of incubation at 0° C in phosphate buffer. Usually, no monomerization occurred, although a few determinations showed about 10% or less monomerization. Nitrogen (N) was determined by the MARKHAM[5] modification of the Kjeldahl method. All sera were analyzed for immunologic homogeneity by the OUDIN method[6] using whole human serum as the diffusing antigen.

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RESULTS*

Effect of aggregation on the precipitin reaction

Precipitin curves using antibody to mercaptalbumin reacted with varying amounts of decanol crystallized albumin, ASH or ASHgX were identical within experimental error.

In the experiment described in Fig. 1 and Table I, varying quantities of antigen (albumin monomer or dimer) were reacted at 0° C with a constant quantity of rabbit anti-monomer, stored at 0° C for 48 hours and centrifuged. The precipitates were washed twice with phosphate buffer and analyzed for N. In the region of antigen excess, more total nitrogen was precipitated by albumin dimer than by albumin monomer. In reaction No. 11 (Table I), 164 μg of monomer N precipitated 0.31 μg total N while in No. 11', 160 μg of dimer N removed 108 μg N. These differences are not due to differences in rate of forming the immune precipitate in antigen excess since reaction mixtures analyzed after 2, 3, and 4 days at 0° C showed the same amount of N in the precipitate. In analyzing the precipitin supernates for the presence of excess antigen or excess antibody, three μg N of each antigen were added. Supernatant solutions from tubes 3 and 4' show the presence of antibody when tested with dimer but not when tested with monomer. This is not antibody-specific for dimer but a reflection of decreased solubility in antigen excess with dimer. The 3 μg of monomer N were in complete antigen-excess inhibition with the amount of antibody present in this supernate, while 3 μg of dimer N with this amount of antibody were not completely soluble. This interpretation is consistent with the fact that the total amount of antibody N in the serum is identical regardless of whether monomer or dimer is used as antigen.

The decreased solubility of the dimer precipitate in the antigen-excess zone might be due to increase in the number of available antigenic sites per molecule or to increase in the molecular weight of the antigen. The mixed mercury dimer of bovine serum albumin (BSA) and HSA should provide information on this problem. Equimolar amounts of BSA-SHg** and HSA-SH brought to pH 5.2 and almost to the point of amorphous precipitation with ethanol were reacted. Small crystals appeared after about 18 hours. They were washed with 15% alcohol. A solution of the crystals, examined in the ultracentrifuge, appeared to be about 80% dimer and 20% monomer. When reacted with antibody to HSA, differences comparable to those in Table I were observed.

To estimate the qualitative nature of the dimer, that is, was the dimer formed BSA-S-Hg-S-BSA, HSA-S-Hg-S-HSA, or BSA-S-Hg-S-HSA, this preparation was reacted in another experiment with antibody to BSA in one set of reactions and to HSA in another set. The supernates of each reacted with the antibody opposite to that used in the initial reaction. The resulting data suggested that these molecules may have exchanged before and during crystallization, forming some BSA-S-Hg-S-BSA, and HSA-S-Hg-S-HSA molecules. The interpretation of these experiments is made more difficult by the cross-reactivity of HSA with antibody to BSA[7]. However, a washed precipitate, formed with the preparation and antibody to BSA and solu-

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TABLE I

PRECIPITIN STUDIES WITH HSA MONOMER AND DIMER

Sample	Antiserum to HSA	Monomer N added	Dimer N added	Supernate analyses: Excess antibody		Excess antigen	Total N precipitated	0.5 ml: Average antibody N precipitated	Ratio antibody N to antigen N
				3 µg Monomer N	3 µg Dimer N				
	ml	mg	mg				mg	mg	
1	1.0	0.016		+	+	−	0.251 0.262	0.120	16.0
2	0.5	0.025		+	+	−	0.251 0.251	0.226	9.1
3	0.5	0.029		+	+	−	0.271 0.265	0.239	8.2
4	0.5	0.033		+	+	−	0.285 0.290	0.254	7.7
5	0.5	0.037		+	−	−	0.307 0.303	0.268	7.2
6	0.5	0.041		+	−	−	0.317 0.321	0.278	6.8
7	0.5	0.045		−	+	−	0.309 0.303	(0.261)	5.8
8	0.5	0.049		+	−	+	0.286 0.289		
9	0.5	0.057		−	+	+	0.258 0.256		
10	0.5	0.082		−	−	+	0.163 lost		
11	0.5	0.164		−	+	+	0.035 0.028		
1′	1.0		0.016	+	+	−	0.228 0.221	0.104	13.0
2′	0.5		0.024	+	+	−	0.231 0.230	0.206	8.6
3′	0.5		0.028	+	+	−	0.244 0.243	0.215	7.7
4′	0.5		0.032	−	+	−	0.260 0.260	0.228	7.1
5′	0.5		0.036	−	+	−	0.288 lost	0.252	7.0
6′	0.5		0.040	−	−	−	0.291 0.292	0.251	6.3
7′	0.5		0.044	−	−	−	0.318 0.301	0.265	6.0
8′	0.5		0.048	+	+	+	0.325 0.319	0.274	5.7
9′	0.5		0.056	−	−	+	0.317 0.319	(0.262)	4.7
10′	0.5		0.080	−	+	−	0.262 0.233		
11′	0.5		0.160	−	−	+	0.110 0.107		

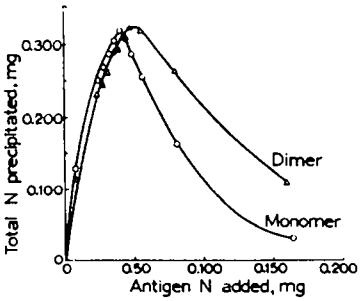

Fig. 1. Precipitin reaction of albumin dimer and monomer with rabbit anti-monomer.

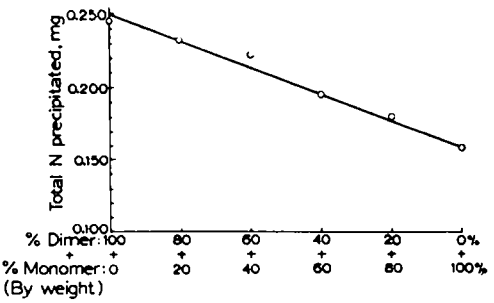

Fig. 2. Effect of varying proportions of dimer and monomer on N precipitated in antigen excess.

bilized by excess BSA, reacted with antibody to HSA. Since BSA did not cross-react with antibody to HSA, it was apparent that at least some HSA–S–Hg–S–BSA was present. The quantity of HSA–S–Hg–S–BSA in the mixture could not be determined, and the question as to whether the differences in antigen excess were due to more determinant groups per molecule or just to increase in molecular size could not be unequivocally answered.

The reaction of albumin monomer and dimer with antibody was not studied by complement fixation since the mercury dimer dissociates in dilute solution. The dimer described by MOORE AND WARD[8] prepared with N,N'-(1,3-phenylene)-bismaleimide appeared promising for complement fixation since it should not dissociate in dilute solution. In agreement with these authors, it was found that dimerization was never complete under any of the conditions used, although extent of dimerization was determined in the ultracentrifuge, varying pH, albumin concentration, reaction time, temperature, and mole ratio of reactants. Dimerization proceeded further, in general, with bovine serum albumin than with human serum albumin. The product was not examined immunologically.

Immunochemical estimation of dimer

The finding that dimer–antibody complexes are less soluble than monomer–antibody complexes in the region of antigen excess suggested study of the relationship between this observed difference and the percentage of dimer in an albumin solution. Therefore, precipitin reactions were set up with 93 μg of antigen N and 0.5 ml of antiserum. The total antigen was fixed and the proportions of dimer N and monomer N were varied (Fig. 2). The resulting linear relationship can be used to estimate the percentage of dimer in a given solution and is the basis for the study of the kinetics of monomerization of mercaptalbumin with mercury salts (Reaction III).

Kinetics of monomerization

Reaction III, HSA–S–Hg–S–HSA + $HgX_2 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}}$ 2HSA–SHgX, is very rapid being essentially complete by the time light-scattering measurements can be made[1]. The antigen–antibody reaction is also rapid and has been shown to be at least 90% complete in about 3 seconds[9]. If the dimer is unaffected by excess Hg^{++} when combined with antibody, that is, not susceptible to reaction III, the rate of monomerization can be measured by analysis of dimer present at any given time.

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The following experiments indicate that dimer complexed with antibody is not monomerized by mercury salts. A 20-fold mole excess of mercuric acetate added five seconds after the addition of dimer to antiserum caused no change in precipitable N compared to controls in reaction mixtures incubated one, two, or four days. Addition of mercuric acetate to the antibody before dimer was added caused no difference in precipitable N compared to controls without mercury.

Experiments were then set up to measure the k_3 of reaction III and the data analyzed assuming a bimolecular reaction described by the equation

$$k_3 = \frac{1}{T([Hg^{++}]_0 - [HSA\text{–}S\text{–}Hg\text{–}S\text{–}HSA]_0)} \ln \frac{[HSA\text{–}S\text{–}Hg\text{–}S\text{–}HSA]_0 ([Hg^{++}]_0 - X)}{[Hg^{++}]_0 ([HSA\text{–}S\text{–}Hg\text{–}S\text{–}HSA]_0 - X)}$$

where the subscript $_0$ refers to initial concentrations and X is the molar concentration of dimer that has reacted. In the experiments, 1.0 ml of dimer was pipetted into a series of acid-cleaned test tubes. One ml of mercuric acetate solution was added with a calibrated tuberculin syringe. After an interval 0.5 ml of antiserum was added by blowing the contents of the pipette into the reaction mixture and mixing at once. The elapsed time (T) was taken as the time in seconds between completion of the mercuric acetate addition and mixing the added antibody. In every experiment, the effectiveness of stopping monomerization by antibody was controlled by adding antibody to the dimer followed by mercuric acetate five and ten seconds later. The reaction mixtures were incubated two days at 0° C and the precipitates collected, washed, and analyzed for N.

TABLE II

DETERMINATION OF k_3

Time Hg^{++} added sec	*N precipitated mg*	*k_3 l/mole sec × 10^5*
—9.2	0.245	
—4.9	0.250	—
9.4	0.233	3.32
20.3	0.227	2.44
39.9	0.210	2.91
59.3	0.207	2.16
79.2	0.207	1.62
99.6	0.198	1.82
123.9	0.195	1.67
180.1	0.187	1.52
301.0	0.169	1.87
1209.0	0.155	—

The results of such an experiment, using 4.73 mμM of $HgAc_2$ per ml and 2.27 mμM of dimer per ml are shown in Table II. The data fit second-order kinetics reasonably well. In a similar manner, the rate constants were determined over a 10-fold change in Hg^{++} concentration (Table III). The assay procedure restricts the albumin concentration to rather narrow limits so that it was not possible to study the effect of different initial dimer concentrations.

Activation energy of the monomerization reaction

The effect of temperature on the rate of reaction was studied. The experimental procedure described above was repeated at 0° C, 9.8° C, and 20.1° C. About 15 minutes after completion of the experiment, the reaction mixtures were incubated at 0° C for two days and the precipitates collected, washed with chilled phosphate buffer, and analyzed for N. The data of these experiments are shown in Fig. 3. The rate of reaction is approximately doubled with each 10° rise in temperature. The energy of activation of the reaction is 5,700 calories per mole (Fig. 3). No correction was made for the effect of temperature on the activity of the mercury salts.

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TABLE III

EFFECT OF MERCURIC ACETATE CONCENTRATION ON k_3

Mercuric acetate concentration mμM/ml	*k_3 (average) l/mole sec × 10^4*
11.9	1.95
4.73	2.16
4.43	2.69
1.19	1.19

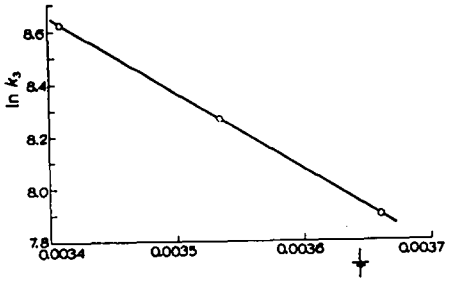

Fig. 3. Calculation of energy of activation.

DISCUSSION

COHN, WETTER AND DEUTSCH[10] use the shape of the precipitin curve in the region of excess antigen as a criterion of immunochemical homogeneity. It is also stated that two to three times the amount of protein antigen required to reach the equivalence point should completely solubilize the antigen–antibody complex[11]. Failure to effect complete solubilization with this quantity of antigen is said to indicate that more than one antigen–antibody system is present. VAUGHAN AND KABAT[12] state that trailing off of the precipitin curve is insufficient evidence of nonhomogeneity unless the curve in this region of antigen excess rises from previously lower values.

Although factors affecting the precipitin curve in the region of antigen excess have not been extensively investigated, MACPHERSON AND HEIDELBERGER[13] have shown that aggregation of the antigen decreases the solubility of antigen–antibody complexes in antigen excess. COHN[11], however, has said that this decrease in solubilization is due to denaturation and results in a more insoluble antigen.

The data in the present report confirm the finding that aggregation does decrease the solubility of antigen–antibody complexes. This is evident from the data in Fig. 1. It is probable that antigens aggregated more than mere dimerization would show an even greater difference. The effect of aggregation on the precipitin curves also supports the concept[12] that too little is known about the region of excess antigen to accept the trailing off of the precipitin curve as a criterion of heterogeneity.

The similarity of the precipitin curves with decanol-crystallized albumin and mercaptalbumin indicates that the SH group of albumin is not involved in an antigenic site with the antisera used. If two like molecules are joined through an identical portion of each, any site occluded on one will also be obstructed on the other. In dimer, any group masked on one monomer molecule will be unavailable on the other. The valences of a given molecule of antibody may be directed towards the same site[14]. If this is so, the identical quantities of antibody N precipitated by either monomer or dimer indicate that no immunologically reactive site is occluded during dimerization.

The rate constants given are based on mercuric acetate concentration rather than on mercuric ion activity. This is equivalent to assuming that $Hg(OH)_2$ and HSA–S–Hg–S–HSA are the reactants. The Hg^{++} ion should be a fairly constant fraction of the nonprotein mercuric compounds in the system, so that the rate constants, in terms of mercuric ion activity, might be approximated using the data of BJERRUM[15],

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by multiplying k_3 by $10^{-1.4}$, $10^{-8.8}$, and/or $10^{-13.5}$ if $HgOH^+$, Hg^{++} and/or HSA–S are the reactive species[16].

Since it is possible to determine the rate constant for monomerization, it might be possible to determine the rate constant of the antigen–antibody reaction at various points in the antigen-excess region of precipitation by analyzing competitive reactions between substances causing monomerization and antibody for dimer*. Preliminary experiments showed that concentrations of Hg^{++} up to the point of protein precipitation did not compete effectively with antibody for the dimer. Methyl mercuric nitrate, which has a higher activity coefficient, not only competed with the antibody but also monomerized the dimer after antigen–antibody complexes were formed.

Although a rate constant has not been determined for the antigen–antibody reaction, it is possible to calculate an *upper limit* for completion under the experimental conditions. At a particular point in the region of antigen excess, no detectable monomerization occurred in a system containing 134.4 mμM/ml of mercuric acetate and 2.44 mμM/ml of dimer. Ten-per-cent monomer, an amount easily detected, should be formed in 0.37 of a second. The antigen–antibody reaction must be complete in a comparable time or less under these conditions.

SUMMARY

Quantitative precipitin studies with human serum albumin monomer and mercury dimer and rabbit antibody showed more precipitin nitrogen in the antigen-excess zone with dimer. This difference was used to determine the proportions of monomer and dimer in mixtures. A velocity constant of 20,000 l/mole sec at 0° C and an energy of activation of 5,700 cal/mole were found by immunologic studies of the kinetics of monomerization. Competition between the monomerization reaction in large excess of mercuric acetate and the antigen–antibody reaction indicates that the antigen–antibody reaction was complete in 0.37 sec or less under the experimental conditions.

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* Dr. R. B. SIMPSON found an approximate solution to the differential equations for such a system.