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Physical Chemical Studies of Soluble Antigen–Antibody Complexes. II. Equilibrium Properties[1]

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From a previous ultracentrifugal and electrophoretic study of the soluble complexes formed between lightly iodinated bovine serum albumin (antigen) and precipitating rabbit antibodies to bovine serum albumin, data are available concerning the amounts of free antigen present in equilibrium in solutions of known total antigen and antibody concentrations. These data are now used in conjunction with part of the theory recently developed by Goldberg for the antigen–antibody reaction, to test the theory, and to obtain an estimate of the free energy change in this reaction for the particular system studied.

A specific precipitate formed between an antigen (Ag) and its antibody (Ab) can be completely dissolved by adding to it a sufficient excess of the antigen in solution. In this process, the precipitate is converted into soluble antigen–antibody aggregates, or complexes, which are in equilibrium with one another in solution. In the first paper of this series,[2] an ultracentrifugal and electrophoretic study was presented of the soluble complexes formed between lightly iodinated bovine serum albumin (as the antigen) and rabbit antibodies made against uniodinated bovine serum albumin. (The antigen was lightly iodinated in order to facilitate analyses of total antigen and total antibody in solutions of their complexes. Precipitin tests showed that the iodination did not significantly alter the properties of the bovine serum albumin antigen in its reaction with antibodies made against the uniodinated bovine serum albumin.) Although the various molecular species are in equilibrium in these solutions, it is fortunately possible to resolve some of them ultracentrifugally and electrophoretically because the rates of re-equilibration among these species are sufficiently slow.

Several points of interest, which were developed in the previous study, may be summarized.

(1) The three slowest-sedimenting species in these solutions were investigated in the ultracentrifuge. In the order of increasing sedimentation constant they were: (i) the uncombined, or free, antigen; (ii) the a-complex, with $s^{w}_{20} = 8.7$ S; and (iii) the b-complex, with $s^{w}_{20} = 12.0$ S. Other faster-sedimenting complexes were discernible, but were poorly resolved and were not further examined.

(2) The a-complex, which predominates over all other complexes in great antigen excess, was shown to consist largely, if not completely, of $(Ag)_2Ab$ molecules. *The antibody in this system must, therefore, be bivalent.*

(3) The amounts of free antigen in equilibrium in solutions of known total antigen and total antibody contents were determined both in the ultracentrifuge and in electrophoresis, with essential agreement between the two methods.[3]

It is clear that from information concerning the concentrations of various species in equilibrium in these solutions, equilibrium constants and free energy changes characterizing antigen–antibody reactions may be obtained. The study of soluble antigen–antibody complexes by ultracentrifugation and electrophoresis represents a new approach to obtaining such thermodynamic data. The purpose of this paper is to explore some of the possibilities of this method with the data presented in the previous paper.

Not enough accurate information is available for this system, however, to enable us to evaluate any equilibrium constant directly. The concentrations of free antigen were determined accurately, and those of the $(Ag)_2Ab$ complex fairly accurately, but those of all other species were either not capable of being precisely measured or like those of free antibody and the AgAb complex were too small to be detected. It should be emphasized that the same system examined under different conditions, or a different system, might yield enough information to permit a direct determination of equilibrium constants by our methods. In the present case, nevertheless, we must proceed indirectly.

For this purpose we may try to use Goldberg's recent theory,[4] which gives the most probable distribution of antigen–antibody complexes subject to the following special assumptions.

(1) There are no cyclic complexes. In antigen excess, where most of the complexes are small, this assumption is surely valid.

(2) All antigen valences have equal affinity for antibody valences, and *vice versa*, regardless of the size or shape of the complex in which these valences occur.

For bivalent antibody, Goldberg's equation, which follows rigorously from his model, may be written as

$$m_{ik} = \frac{fG(fk-k)!}{(fk-2k+2-q)!k!q!}\left(\frac{fG}{2A}\right)^{k-1} p^{k+i-1} (1-p)^{fk-k-i+1}\left(1-p\,\frac{fG}{2A}\right)^{i-k+1} \quad (1)$$

where m_{ik} = number of complexes each containing i bivalent antibody molecules and k antigen molecules; f = valence of antigen, the number of effective reaction sites on each antigen molecule; G = total number of antigen molecules in the system; A = total number of antibody molecules in the system; p = fraction of antigen sites in the system which have reacted; q = number of free antibody sites on a complex $= i - k + 1$.

(1) This work was supported by grants from the Rockefeller Foundation and from the United States Public Health Service. This paper was presented before the meeting of the American Society of Immunologists at Chicago, Illinois, April, 1953.

(2) S. J. Singer and Dan H. Campbell, THIS JOURNAL, **74**, 1794 (1952).

(3) The ultracentrifuge experiments were performed near 25° in phosphate buffer, pH 7.60, μ 0.1, while the electrophoresis measurements were carried out at 0°, in veronal buffer, pH 8.50, μ 0.1.

(4) R. J. Goldberg, THIS JOURNAL, **74**, 5715 (1952).

From this general formula, expressions may be obtained for the concentrations in grams/liter of the free antigen, C_{01}, of the AgAb complex, C_{11}, of the $(Ag)_2Ab$ complex, C_{12}, etc.

$$C_{01} = C_G(1 - p)^f \quad (2)$$

$$C_{11} = fC_G\left(\frac{M_A + M_G}{M_G}\right)p(1 - p)^{f-1}\left(1 - p\frac{fC_GM_A}{2C_AM_G}\right) \quad (3)$$

$$C_{12} = \frac{f^2}{4}\frac{C_G^2}{C_A}\frac{M_A(M_A + 2M_G)}{M_G^2}p^2(1 - p)^{2f-2}, \text{ etc.} \quad (4)$$

Here C_G and C_A are the total concentrations in grams/liter, and M_G and M_A are the molecular weights, of antigen and antibody, respectively. In this paper, M_G is taken as 70,000, M_A as 160,000. If we now consider a particular reaction, for example, that between free antigen (Ag) and the complex AgAb to form the complex $(Ag)_2Ab$

$$Ag + AgAb \rightleftarrows (Ag)_2Ab \quad (5)$$

we may write the equilibrium constant for this reaction in terms of concentrations in moles/liter as

$$K = \frac{fM_Ap}{4C_A(1 - p)\left(1 - p\frac{fC_GM_A}{2C_AM_G}\right)} \quad (6)$$

In this equation, concentrations are used instead of the activities of the components.

The results of calculations using equations 2–6 are summarized in Table I, for five solutions of various antigen–antibody ratios. Taking the valence of antigen, f, equal to 6, a value of p for each solution is determined from equation 2. (For $f >$ 4, the K values are essentially independent of f.) There is good agreement between the concentration, C_{12}, of the a-complex as determined experimentally and as calculated by equation 4. The equilibrium constants calculated by equation 6, however, decrease as the antigen excess is increased. In part, this may be due to experimental error; it appears that the value of C_{01} in solution I is too small and that K for this solution is therefore too large. Possibly some systematic error is responsible, although aside from chemical determinations of total antigen and antibody, the free antigen concentrations are the only experimental quantities utilized in this analysis. It is therefore unlikely that possible re-equilibration of the complexes during the experiments is involved.[2] On the other hand, if assumption (2) of the theory, that all antigen and all antibody valences are equally effective, is at fault, the suggestion would be that the antigen–antibody bond is stronger in larger complexes than in smaller ones in these particular solutions.

TABLE I

Soln.[a]	C_{01},[b] g./l.	C_G, g./l.	C_A, g./l.	p (f = 6)	C_{12}, g./l. Obsd.[c]	Calcd.	K
I	1.55	6.25	11.75	0.207	0.9	1.2	20000
II	2.23	6.44	11.56	.162	1.1	1.4	10400
I-1	4.41	7.85	7.85	.092	1.6	2.2	8100
I-2	8.82	12.88	7.82	.061	3.3	3.7	6300
II-2	10.83	14.50	6.80	.047	2.9	3.7	5300
						Av. =	10000
						Av. dev. =	4000

[a] Designations from ref. 2. [b] From ascending electrophoresis patterns, ref. 2. [c] From ultracentrifuge data, ref. 2.

In view of the variation in K, its significance is uncertain. However, it may be noted that a K value of 10,000 ± 4000 corresponds to a standard free energy change, $\Delta F^\circ = -RT \ln K$, of −5400 ± 400 cal. per mole, for the reaction given in equation 5 (in veronal buffer, pH 8.50, μ 0.10, at 0°, the conditions used in the electrophoresis experiments from which C_{01} values were determined).

Much more work, with a variety of systems, is needed to explore the applicability of the Goldberg theory to solutions of antigen–antibody complexes, as well as to find experimental conditions in which direct determinations of equilibrium constants are feasible. In acid solution, for example, in which the antigen–antibody bond should weaken considerably, the concentration of free antibody might become large enough to measure accurately, along with that of the free antigen and a-complex, so that the equilibrium constant for the formation of a could be evaluated directly.

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