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Physical Chemical Studies of Soluble Antigen–Antibody Complexes. III. Thermodynamics of the Reaction between Bovine Serum Albumin and its Rabbit Antibodies[1]

BY S. J. SINGER AND DAN H. CAMPBELL

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Solutions of soluble complexes formed between bovine serum albumin (as antigen) and its rabbit antibodies have been subjected to electrophoresis and ultracentrifugation. From these experiments it has been possible to obtain the equilibrium concentration of uncombined antigen (Ag) in a given solution of known total antigen and total antibody (Ab) content. With the aid of the Goldberg theory, this information has been interpreted to give equilibrium constants for the reaction: $Ag + AgAb \rightleftarrows (Ag)_2Ab$; $K = (2.5 \pm 0.5) \times 10^4$, $\Delta F^0 = -5.5 \pm 0.2$ kcal., $\Delta H^0 = 0 \pm 2$ kcal., and $\Delta S^0 = +20 \pm 8$ e.u., for this reaction in this system in veronal buffer, *p*H 8.5, at 0°. The significance of these thermodynamic parameters is discussed.

Much of our information concerning the fundamental nature of the antigen–antibody reaction has been acquired through the study of artificial antigens, prepared by coupling simple chemical groups (haptens) to proteins.[2] The detailed molecular basis of the reactions between natural protein antigens and their antibodies, however, is still largely conjectural. This is because knowledge of the detailed structure of proteins as well as appropriate techniques of investigation have been lacking. Even the fundamental thermodynamic data for these reactions are not available, and suitable methods, other than calorimetry, for obtaining them have not been described. In our earlier studies[3,4] on the physical chemistry of soluble antigen–antibody complexes, we reported on a new method for obtaining such data. In this paper, the method is critically examined, and has been applied to the system containing bovine serum albumin (BSA) as the antigen and rabbit antibodies to BSA.

It is well known that a specific precipitate formed between an antigen (Ag) and its antibody (Ab) can generally be completely dissolved by adding to it a sufficient excess of the antigen in solution. In this process, the precipitate is converted into an equilibrium distribution of various soluble molecular aggregates, or complexes, of Ag and Ab. Our method relies on the fact that it is generally possible to effect at least a partial resolution of the various components in these solutions either by ultracentrifugation or electrophoresis, or by both independently, and, from these experiments, to determine the equilibrium concentrations of one or more species.

In the rabbit anti-BSA system,[3] at a *p*H near 7, it is not possible by these means to determine accurately enough the concentrations of more than two components and so equilibrium constants cannot be evaluated directly. (However, in the *p*H range from 4.5 to 3.0, in which the complexes are extensively dissociated, such direct constants can be obtained.[5]) If we assume that every antigen–antibody bond is intrinsically equivalent, regardless of the size of the complex in which the bond occurs, then a knowledge of the equilibrium concentration of any *one* species is sufficient to determine the equilibrium constants. The adequacy of this assumption, in view of current experimental errors, is explicitly demonstrated in the following paper.[5] At a *p*H near 7, the equilibrium concentration of uncombined, or free, Ag can be accurately determined by electrophoresis. This fixes the equilibrium constants and standard free energy changes. In addition, the temperature dependence of the ultracentrifugal behavior of these solutions can be studied to determine the variation of equilibrium constants with temperature, and enthalpy and entropy changes for the reactions.

Materials and Methods

Solutions of Soluble Complexes.—Four independent solutions of complexes were studied in the period July, 1952, to July, 1954. These are labeled III to VI, in chronological order, solutions I and II having been used in our previously reported work.[3] In III–VI, in contrast to I and II, the Ag was the unaltered crystalline BSA. In the earlier work, the BSA had been iodinated in order to facilitate analyses of total Ag and total Ab. Since then, we have developed an electrophoretic method for such analyses,[5] which makes the labelling unnecessary.

The preparation of solutions III–VI was in part similar to that previously described.[3] High titer hyper-immune rabbit sera, containing an average of about 3 mg./ml. of anti-BSA protein, were pooled, rapidly frozen, and stored at −40° for various periods of time. Immediately before use, the sera were carefully thawed, and the γ-globulin fraction was largely freed of other serum proteins by precipitation in 1/3 saturated $(NH_4)_2SO_4$ solution adjusted to *p*H 7.8. The precipitate was washed, and dissolved in saline at *p*H 7.5. BSA dissolved in saline at *p*H 7.5 was added to this γ-globulin solution in equivalent amount, as determined by preliminary titration under identical conditions. The mixture was kept at room temperature for 2 hours, then stored at 4° for 24 hours. The specific precipitate was then centrifuged and washed several times with cold saline. An amount of BSA in saline solution about twice the weight of Ab precipitated was then added, and the mixture rotated at 4° for about 24 hours. Solutions III, IV and V, at this stage, were then packed cold in insulated containers and shipped air-mail from Pasadena to New Haven. They were two days in transport, and were used immediately after arrival. Solution VI was investigated directly at Pasadena.

The solutions were centrifuged to remove small amounts of undissolved protein. V and VI were brought to 1/2 saturation with respect to $(NH_4)_2SO_4$ adjusted to *p*H 7.8.[3] The resultant precipitates were centrifuged, and completely redissolved in saline at *p*H 7.5. (This procedure results in a preparation in lower antigen excess than would otherwise be possible). Preparations III to VI were then dialyzed against appropriate buffers, and solutions in greater antigen

(1) This work was supported in part by grants from the Rockefeller Foundation and the United States Public Health Service. This paper was presented before the Meeting of the American Chemical Society at New York City, September, 1954.

(2) K. Landsteiner, "The Specificity of Serological Reactions," 2nd Edition. Harvard Univ. Press, Cambridge, Mass., 1945.

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

(4) S. J. Singer and D. H. Campbell, *ibid.*, **75**, 5577 (1953).

(5) S. J. Singer and D. H. Campbell, *ibid.*, **77**, 3504 (1955).

excess were accurately made from aliquots of the master solutions by the addition of known amounts of standardized BSA solutions (see section on Protein Analyses).

Normal Rabbit γ-Globulin Preparations.—Two preparations, RGG-I and RGG-II, were employed for various purposes in these studies. RGG-I was a crude preparation precipitated from pooled normal rabbit serum with 1/3 saturated $(NH_4)_2SO_4$ at *p*H 7.8. In electrophoresis, it analyzed for about 85% γ-globulin and 15% β-globulin. RGG-II was a refined preparation made as follows. Pooled normal rabbit serum was treated with 1/3 saturated $(NH_4)_2SO_4$ at *p*H 7.0. The precipitate was washed several times with cold 1/3 saturated $(NH_4)_2SO_4$, then redissolved in phosphate buffer, ionic strength 0.2 *p*H 6.02 (the mean isoelectric point of normal rabbit γ-globulin[5]). The solution was dialyzed against the same buffer, and fractionated by electrophoresis-convection,[6,7] at a field strength of 1.34 volts/cm. at 2°. After 72 hours, the top fraction was removed and used as RGG-II. It analyzed electrophoretically for 98% γ-globulin and 2% β-globulin.

Bovine Serum Albumin.—Several different batches of Armour's crystalline BSA were used, both for immunization and for the preparation of Ag–Ab solutions.

The normal bovine γ-globulin was an Armour Fraction II preparation.

Protein Analyses.—Nessler analyses were performed, using the factor 6.25 to convert from N to protein content. For the more quantitative experiments with solutions V and VI, refractive increments determined with a Brice-Phoenix differential refractometer were employed as follows. It was desired to make up a series of accurately known solutions from a master solution of complexes and a master solution of BSA. The two master solutions were thoroughly dialyzed against the same buffer, refractive increments against that buffer were measured, and aliquots of the two solutions were then weighed together. The *relative* compositions of the secondary solutions compared to the master solutions were thus known with a precision better than ±0.3%.

Ultracentifugation and Electrophoresis.—Ultracentrifuge experiments were performed in a Spinco Model E instrument, employing a Philpot-Svensson cylindrical lens-diagonal bar optical system. The temperature was near 25° unless otherwise indicated. Electrophoresis was carried out in a Perkin–Elmer Model 38 Tiselius apparatus, with a Longsworth scanning optical system. Area measurements were made as described previously,[3] on enlarged tracings made independently in triplicate and measured with a planimeter. The reproducibility of the apparent per cent. free Ag is about ±2 units for the ultracentrifuge experiments and 0.5 unit in electrophoresis.

Results

Effects of Re-equilibration during Electrophoresis.—The various species in these Ag–Ab solutions are initially in equilibrium, but as the boundaries resolve, the distribution of species readjusts toward new equilibrium conditions. It is necessary, therefore, to justify the quantitative use of the schlieren diagrams obtained. In a previous paper,[3] it was demonstrated that for the BSA system at a *p*H near 7, the effects of re-equilibration are minimized in the *ascending* limb in electrophoresis. If the schlieren diagram returns to the baseline in the region between the peaks due to free Ag and to the complexes, then, unambiguously, the area under the free Ag peak is proportional to the equilibrium concentration of free Ag in the original solution (subject to corrections for anomalies occurring in conventional electrophoresis, see below). There may be systems, however, for which the schlieren diagram is elevated from the baseline in the region between the two peaks, due solely to the limited electrophoretic resolution of the various species, and not necessarily to the effects of re-equilibration. An independent experimental test of the importance of the latter effects has therefore been developed. Several experiments with aliquots of the same Ag–Ab solution in the same buffer are performed, in which the number of coulombs passed through the cell, and hence the distances moved by the various boundaries, are the same, but the current and duration of the experiment are varied inversely. If the apparent area under the free Ag peak does not vary with the duration of the experiment, then the effects of re-equilibration are negligible. (If the apparent area does vary, an extrapolation to zero time may be possible.)

A number of such series of experiments have been carried out with the rabbit anti BSA system. All of them have shown no variation of the area, within experimental errors, under the free Ag peak over an eightfold variation in the duration of an experiment. A typical example is given in Fig. 1, with solution V-2 in phosphate buffer, *p*H 7.50, Γ/2 0.1. We conclude that, in this system, the interpretation of the area under the free Ag peak as proportional to the *equilibrium* concentration of free Ag in the original solution, is justified.

Electrophoresis Results.—A series of solutions of soluble complexes derived from preparation V were examined electrophoretically at a total protein concentration of 17 mg./ml. in phosphate buffer, *p*H 7.50, Γ/2 0.1. All experiments were run for 10,400 sec. at a field strength of 4.16 volts/cm. The *apparent* relative concentration of free Ag was determined from the ascending pattern in an experiment (Table I). The per cent. total Ag was obtained by electrophoresis at *p*H 2.4[5] (Table I). Solutions derived from preparation VI were studied electro-

TABLE I

SOLUTIONS OF PREPARATION V IN PHOSPHATE BUFFER, *p*H 7.50, Γ/2 0.1

Solution	Total Ag,[a] %	Apparent relative areas: Free Ag (electroph.)	Free Ag (ultracent.)	a-complex (ultracent.)
V	39.3	20.6	19	8
V-1	48.1	31.4	28	13
V-2	56.9	43.4	41	15
V-3	66.8	56.0	52	14
V-4	71.8	63.5	61	13
V-5	78.7	72.6	69	11
V-6	89.4	86.4	84	6

[a] As determined by electrophoresis in glycine–HCl buffer *p*H 2.4.[5]

TABLE II

ELECTROPHORESIS OF PREPARATION VI SOLUTIONS IN VERONAL–NaCl BUFFER, *p*H 8.43, Γ/2 0.3

Solution	Total Ag,[a] %	Apparent % free Ag	$K \times 10^{-4}$ [b]
VI	48.0	17.2	140[c]
VI-1	55.7	30.2	2.4
VI-2	62.4	37.8	4.2
VI-3	69.1	48.0	3.3
VI-4	76.3	59.6	2.6
VI-5	83.3	70.7	3.0

[a] As determined by electrophoresis in glycine–HCl buffer *p*H 2.4[5]. [b] For the reaction Ag + AgAb ⇄ $(Ag)_2Ab$, see text. [c] Large value caused by too small value for the % free Ag, reason for which is not known.

(6) J. R. Cann, J. G. Kirkwood, R. A. Brown and O. J. Plescia, THIS JOURNAL, **71**, 1603 (1949).

(7) The coöperation of Dr. S. N. Timasheff in performing the electrophoresis convection experiments is gratefully acknowledged.

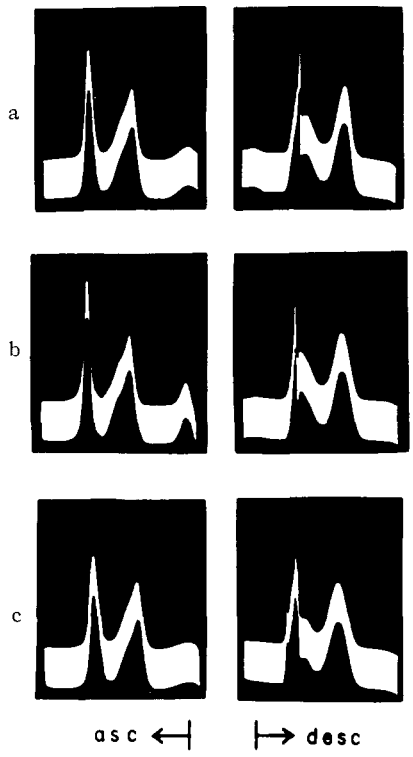

Fig. 1.—Electrophoresis patterns of solution V-2 in phosphate buffer, *p*H 7.50, Γ/2 0.1: (a) 2950 sec. at 0.0120 amp.; (b) 10,400 sec. at 0.0034 amp.; (c) 20,800 sec. at 0.0017 amp. Starting positions are indicated by the arrows.

phoretically at a total protein concentration of 15 mg./ml. in a veronal–NaCl buffer, *p*H 8.43, Γ/2 0.3, containing 0.2 *M* NaCl. The experiments were run for 13,500 sec. at a field strength of 4.16 volts/cm. The results of analyses of apparent free Ag and of total Ag are given in Table II.

In all our electrophoretic and ultracentrifugal studies, there was no indication of the presence in these solutions of any components other than Ag, Ab, and aggregates of the two.

Ultracentrifuge Results.—The solutions derived from preparation V were examined in the ultracentrifuge as well as in electrophoresis in the same phosphate buffer. The sedimentation patterns were in all respects similar to those of Fig. 2 of reference 3. The apparent relative areas under the free Ag peak and the a-complex peak (the a-complex peak at this *p*H being due to the $(Ag)_2Ab$ aggregate[3,5]) were measured (Table I). These relative areas as a function of the ratio of total Ag to total Ab are, within experimental error, the same as previously reported,[3] indicating that the results obtained are entirely reproducible.

Ultracentrifuge experiments with some of these solutions were also performed at different temperatures. The ultracentrifuge rotor and the cell filled with the solution to be studied were incubated at the appropriate temperature for about 2 hours before the experiment, and were then immediately accelerated. Solutions in low Ag excess were less stable at higher temperatures than those in greater excess. Thus solution V at 47° for 2 hours had

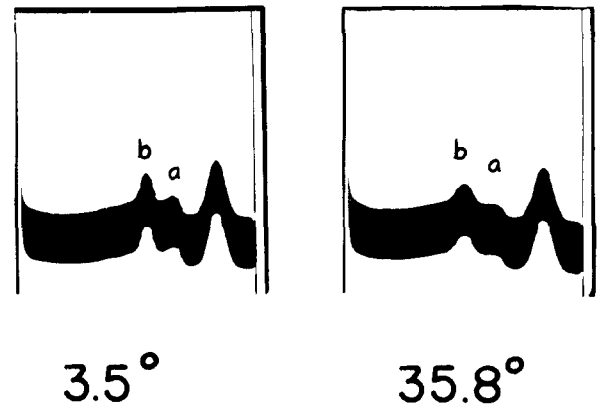

Fig. 2.—The effect of temperature on ultracentrifuge patterns of solution V (BSA-rabbit-anti-BSA). Sedimentation proceeds to the left.

heavily precipitated, but was perfectly clear at 37° after 2 hours. Solution V-3, however, remained clear at 47° for 2 hours, and was examined at that temperature. The low temperature runs were performed at a mean temperature of about 4°, with refrigeration of the ultracentrifuge rotor chamber. The mean temperature in a given run was taken as the average of the rotor temperatures immediately before and after the experiment.

In Fig. 2 are reproduced examples of the effect of temperature upon the sedimentation patterns. The sedimentation rates of the various species, of course, increase with increasing temperature, due primarily to a decrease in the solvent viscosity, so that patterns at two temperatures are compared in which a given peak has moved comparable distances from the meniscus. The patterns of Fig. 2 are very similar, but closer inspection of the region of the diagrams between the a- and b-complex peaks reveals an interesting difference. At the lower temperature, in spite of a longer time of sedimentation, the two peaks are better resolved than at the higher temperature. This is not due to diffusion, but is most likely due to slower rates of re-equilibration at the lower temperature.

In other respects, however, the diagrams are indistinguishable. In order to assess the effect of temperature accurate measurements of the free Ag area were made. Comparable pairs of patterns from runs at two different temperatures were enlarged and traced superimposed. This minimized uncertainties in the baselines, particularly. Within experimental error (about ±3%) no change in the free Ag area was observed in the temperature range 3–36° with solutions V and V-2, or in the range 3–47° with V-3.

Discussion

The Free Ag Concentration.—In a system of non-reacting components in electrophoresis, the relative areas under the peaks in a given diagram are subject to correction because of anomalies. These anomalies decrease with decreasing total protein concentration and with increasing ionic strength of the buffer; they make too large the apparent relative areas of the free Ag peak in our experiments; and they are larger in the ascending than in the descending patterns. For the purposes

of our earlier experiments,[3] these corrections were negligible in the veronal buffer, *p*H 8.50, Γ/2 0.1. For calculations of equilibrium constants, however, these small corrections are significant. Their magnitude for strong electrolyte systems is given by the Dole theory[8] as has been experimentally confirmed.[9] Since proteins are weak electrolytes, it is not surprising that the Dole theory accounts only qualitatively for the anomalies that occur[10] in protein mixtures. The observed anomalies are about twice the calculated ones. The apparent relative free Ag concentrations from the ascending patterns in our earlier paper have therefore been corrected by doubling the calculated Dole corrections in the veronal buffer[11] (Table III). These corrections are small enough to have no effect on the conclusions reached in reference 3.

TABLE III

EQUILIBRIUM CONSTANTS FOR SOLUTIONS I AND II[3,4]

Solution	Total Ag, %	Free Ag, % App.	Free Ag, % Cor.	$K \times 10^{-12}$ App.	$K \times 10^{-12}$ Cor.
E-I	34.7	8.6	6.6	2.0	3.7
E-II	35.8	12.4	10	1.0	1.8
E-I-1	50.0	28.1	25	0.81	1.5
E-I-2	62.2	42.6	39	0.63	1.6
E-II-2	68.1	50.9	47	0.53	2.1

[a] For the reaction Ag + AgAb ⇄ $(Ag)_2Ab$, see text.

In phosphate buffers, electrophoretic anomalies are much larger than those in veronal buffers of the same ionic strength.[10,12] This is evident in our own studies with the solutions from preparation V in phosphate buffer. The apparent per cent. free Ag determined by electrophoresis in a given solution is always larger than that obtained by ultracentrifugation (Table I). On the other hand, the latter values are themselves too large because of an area anomaly occurring in ultracentrifugation.[13,14] Furthermore, we have performed a few mixture experiments with BSA and a normal rabbit β-globulin fraction[15] prepared by electrophoresis–convection (β-globulin having a relative mobility comparable to that of the complexes). These experiments confirm the magnitudes of the electrophoresis area anomalies in phosphate buffer which are required for the data in Table I to conform with those obtained with preparation VI. Rather than make such fairly large corrections, however, we can turn to the independent data obtained with preparation VI. Solutions made from the latter were examined electrophoretically in a veronal–NaCl buffer at ionic strength 0.3[10] in order to reduce the magnitude of the anomalies below the experimental errors in area measurements. That the resultant anomalies are so reduced in this buffer has been confirmed.[15] The apparent relative free Ag concentrations determined with solutions of preparation VI are therefore taken as the true values without further correction.

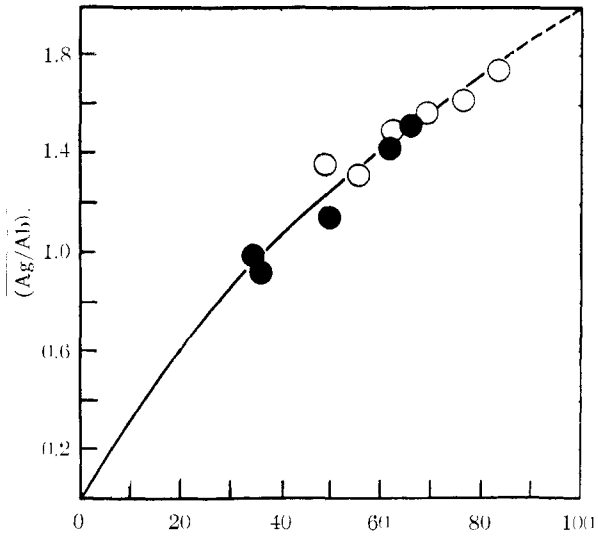

Fig. 3.—The binding of BSA by antibody: open circles for data of preparation VI, filled circles for preparations I and II (reference 3).

The Valence of Antibody.—In a previous paper[3] we have demonstrated that precipitating rabbit antibodies to BSA are largely, if not all, bivalent. In the present studies, this has been confirmed with additional data. As discussed in the last section, the amount of free Ag in solutions of known total Ag and total Ab has been determined. The amount of Ag bound in all the complexes in a solution is then given by the difference between the total Ag and the free Ag. If all the Ab in these solutions in Ag excess is considered bound in complexes, then the ratio (bound Ag)/(bound Ab) multiplied by the molecular weight factor 160,000/70,000 gives $\overline{(Ag/Ab)}_{B,N}$, *the average number of Ag molecules bound per Ab molecule in all the complexes in a solution.* The limit approached by this number as the antigen excess becomes large should be the antibody valence, the limiting number of Ag molecules that can be attached to an Ab molecule. In Fig. 3 is plotted such data for the solutions I, II and VI. Extrapolation leads to a value of two for the antibody valence. It has been reported[16] that such a plot obtained from electrophoretic data for the same system in very large antigen excess suggests an Ab valence larger than two. However, in very large antigen excess, the relative area under the free Ag peak is so large that the usual uncertainties in area measurement can now result in very large errors in

(8) V. P. Dole, THIS JOURNAL, **67**, 1119 (1945).

(9) L. G. Longsworth, *ibid.*, **67**, 1109 (1945).

(10) S. H. Armstrong, M. J. E. Budka and K. C. Morrison, *ibid.*, **69**, 416 (1947).

(11) For these calculations, it is assumed that the Ag–Ab solution is a two-protein system composed of free Ag and a complex species. The latter is assigned a relative mobility of −3.5, and the relative mobilities assigned to the other ions are: Na, +22.7; veronal ion, −10.3; and BSA, −6.0.[10] The average protein concentration is taken as 17 mg./ml.

(12) L. G. Longsworth, *J. Phys. Colloid Chem.*, **51**, 171 (1947).

(13) J. P. Johnston and A. G. Ogston, *Trans. Faraday Soc.*, **42**, 789 (1946).

(14) In reference 3, it was observed that the apparent per cent. free Ag obtained in phosphate buffer in the ultracentrifuge was consistently larger than that evaluated by electrophoresis in veronal buffer. We now recognize that this is due to the Johnston–Ogston anomaly.[13] Our previous inability to find the anomaly directly by performing experiments with the same solution at several total protein concentrations, and looking for a decrease in the apparent per cent. free Ag with decreasing concentration, was due to the large errors in area determinations at low concentrations as well as to the small magnitude of the anomalies.

(15) S. J. Singer and S. N. Timasheff, unpublished experiments.

(16) O. J. Plescia, E. L. Becker and J. W. Williams, THIS JOURNAL, **74**, 1362 (1952).

the ordinate of such a plot. In all the results obtained in this and the following paper,[5] there has been no observation inconsistent with the bivalence of antibody.

Equilibrium Constants and Thermodynamic Parameters.—With the assumptions (a) that 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, and (b) that no cyclic complexes can form, Goldberg[17] has developed a theory which gives the complete distribution of complexes in a solution in homogeneous equilibrium in terms of two parameters: f, the antigen valence, and p, the fraction of antigen sites in the solution which have reacted. In the case that all the antibody is bivalent, simple equations for the concentrations of any species in homogeneous equilibrium can be derived, and hence equilibrium constants for any Ag–Ab reactions, in terms of f and p. For example, for the reaction Ag + AgAb $\rightleftarrows$ $(Ag)_2Ab$, the equilibrium constant K, in terms of concentrations in moles/liter, is given by[4]

$$K = \frac{fM_A p}{4C_A(1-p)\left(1-p\frac{fC_G M_A}{2C_A M_G}\right)} \qquad (1)$$

in which C_A and C_G are the concentrations of total Ab and Ag, respectively, and M_A and M_G are the molecular weights of Ab and Ag, taken for the BSA system as 160,000 and 70,000, respectively. The parameter p for a given solution may be determined from the relation

$$C_{0l} = C_G(1-p)^f \qquad (2)$$

for which C_{0l}, the concentration of free Ag, and C_G are experimentally known, and the value $f = 6$ is chosen for BSA (for $f > 4$, the K values are essentially independent of f).

This theory was previously used[4] with apparent free Ag concentrations without correction for the electrophoretic anomalies. The use of *corrected* free Ag concentrations (Table III) eliminates the previously noted trend in K values.

K values similarly obtained from the data for preparation VI are listed in Table II. These constants appear to be somewhat higher than those obtained with solutions I and II, but the difference is actually within experimental error. If we assign precision measures of ± 0.7 unit to the values of per cent. total and free Ag, the uncertainty in K is about ± 5000. The average value of K obtained from the corrected data with preparations I, II and VI is therefore $(2.5 \pm 0.5) \times 10^4$. From this the standard free energy change, $\Delta F^0 = -RT \ln K$, is $-(5.5 \pm 0.2)$ kcal. per mole for the reaction Ag + AgAb $\rightleftarrows$ $(Ag)_2Ab$ at 0° in veronal buffer at pH 8.5.

Within experimental errors, there is no effect of temperature on the equilibria in this system. Working back from the relation $d \ln K/dT = \Delta H/RT^2$, one can calculate that an uncertainty of $\pm 3\%$ in detecting a change in the free Ag area corresponds to an uncertainty of ± 2 kcal. in the enthalpy change in our experiments. Therefore, ΔH for this reaction is (0 ± 2) kcal. per mole. If the heats of dilution in this system are negligible, this may be taken as the standard enthalpy change per mole. These results lead, from the relation $\Delta F^0 = \Delta H^0 - T\Delta S^0$, to a standard entropy change of $+(20 \pm 8)$ e.u.

The reliability of these thermodynamic parameters depends on the adequacy of the assumptions made in the development of the Goldberg theory. In the following paper,[5] the theory is subjected to direct test, and is found to apply in this system within current experimental uncertainties. However, with regard to the assumption of equivalent sites, there is evidence of antibody heterogeneity[18] in some systems. When more accurate measurements become possible, therefore, the equilibrium constants may prove to depend somewhat on the ratio of total Ag to total Ab. At present, the results for K, ΔF^0 and ΔS^0 should be considered as effective average values.

There are few dependable thermodynamic data with which the results herein reported may be compared. For the reaction between red blood cells and their iso-agglutinins,[19] the magnitudes of the thermodynamic parameters are not very different from those reported here. A light scattering investigation[20] of the reaction between simple compounds containing two benzene-arsonic acid haptenic sites and purified antibodies to benzene-arsonic acid has been reported. For the reaction Ag + AgAb $\rightleftarrows$ $(Ag)_2Ab$, the results are $\Delta F^0 = -7.5$ kcal., $\Delta H^0 = -2.4$ kcal., and $\Delta S^0 = +17$ e.u. in borate buffer, pH 8.0, $\Gamma/2$ 0.15 at 25°. The striking similarity of these values, for systems as disparate as these, is strongly suggestive of a general pattern of behavior for all Ag–Ab reactions. The weakness of the binding, in view of the high order of specificity involved, is remarkable. The positive entropy change is also interesting, and is presumably attributable to the release of bound, or polarized, water from reactive sites upon reaction. In the benzene-arsonic acid system, the antigenic site involves a single negative charge, and there is probably a single positive charge in the antibody site, both of which can polarize a small number of water molecules. When the two sites combine, this water is released. In the BSA system, we have shown that a similar salt linkage is involved in the Ag–Ab bond.[5] Therefore, the above explanation of the positive entropy change is consistent for the two systems. On the other hand, since the benzene-arsonic acid antigen is so much smaller than the BSA molecule, it is not likely that the sizes of the reacting molecules are primary determinants in this positive entropy change. It is also significant in this connection that in the binding of small anions to bovine serum albumin, positive entropy changes of the same magnitude are encountered.[21]

The methods outlined in this paper should be applicable to a variety of other Ag–Ab systems. With present techniques, it is possible to obtain more accurate information from electrophoresis than ultra-

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

(18) E. A. Kabat and M. M. Mayer, "Experimental Immunochemistry," C. C Thomas, Springfield, Ill., 1948, p. 27.

(19) S. Filitti-Wurmser, Y. Jacquot-Armand and R. Wurmser, *J. chim. phys.*, **49**, 550 (1952).

(20) P. Doty and S. I. Epstein, *Nature*, **174**, 89 (1954).

(21) I. M. Klotz, *Cold Spring Harbor Symp. Quant. Biol.*, **14**, 97 (1950).

centrifugation and it is therefore desirable to study antigens which are readily resolvable from antibody γ-globulin by electrophoresis. It is necessary for antigens to be well-defined homogeneous species of definite molecular weight in solution. On the other hand, since a specific Ag–Ab precipitate is dissolved in Ag excess, it is not necessary to isolate pure antibodies, which is a considerable advantage. (The enthalpy changes accompanying the reaction might be determined with greater precision by calorimetry, but the isolation of purified antibodies would be required.) Finally, it might be noted that although the precision attainable by these methods leaves much to be desired when compared with that attainable for small molecule reactions, the state of purity of most natural antigens is probably a limiting factor at the present time.

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Solutions of soluble complexes formed between bovine serum albumin (as antigen, Ag) and its rabbit antibodies (Ab) have been subjected to electrophoresis and ultracentrifugation over a range of *p*H. While the distribution of species in these solutions is apparently not grossly altered between *p*H 7.5 and 4.5, between *p*H 4.5 and 3.0 very extensive dissociation of the complexes occurs. Equilibrium constants, K, for the reaction Ag + Ab $\rightleftarrows$ AgAb can be calculated as a function of *p*H. The variation of K with *p*H provides strong evidence that a single carboxyl group is involved in every Ag–Ab bond in this system.

Little is known about the molecular details of the bonding between natural protein antigens (Ag) and their antibodies (Ab). The sizes of the reactive sites involved are thought to be considerably smaller than the entire Ag or Ab molecules but their structure has not yet been elucidated. If a small number of ionizable groups were present in the reactive sites and were intimately involved in the reaction, then the strength of the Ag–Ab bonds should depend on the state of ionization of these groups, and hence upon the *p*H of the solution containing the Ag and Ab. With appropriate methods for measuring the Ag–Ab bond strength, and with a careful appraisal of auxiliary effects which changes in *p*H might have upon such a system, the presence of such ionizable groups might thus be detected, and their number determined.

In previous papers of this series,[2-4] electrophoretic and ultracentrifugal studies have been carried out near neutral *p*H with the soluble Ag–Ab complexes formed by dissolving an Ag–Ab precipitate in an excess of Ag, in the system containing bovine serum albumin (BSA) as Ag and rabbit antibodies to BSA. These methods are well-suited to the determination of the influence of *p*H on Ag–Ab equilibria, and this application to the rabbit anti-BSA system is the major subject of this paper. Strong evidence has been obtained in this manner that *one ionized carboxyl group* is involved in every Ag–Ab bond in this system.

Other matters of interest also have been investigated and are reported. Additional evidence has been obtained confirming our earlier conclusions[2] concerning the rates of re-equilibration reactions, and the identities of the components appearing in the ultracentrifuge and electrophoresis diagrams of solutions of Ag–Ab complexes. In addition, we have developed an electrophoretic method for the determination of total BSA and total Ab in a solution, based on the observations that at *p*H 2.4 the Ag–Ab bonds are essentially completely dissociated, and that BSA and Ab γ-globulin are electrophoretically resolvable under these conditions. (That the Ag and Ab in this system can be dissociated in sufficiently acid solution has been observed previously.[5]) This now enables us to use unaltered BSA whereas previously it was found desirable to label the BSA by iodination.[2]

It also has been possible in this study to make a direct test of that part of the Goldberg theory for Ag–Ab reactions[6] which deals with systems in homogeneous equilibrium. This theory has been used[4] in the evaluation of thermodynamic parameters for the reactions in the rabbit anti-BSA system, and its validity has not previously been confirmed.

Materials and Methods

The Ag–Ab preparations, III, IV, V and VI, and the methods employed to study them have been described in detail in a previous paper.[4] Solutions to be examined in different buffers were dialyzed 48 hours at 4° against several

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(2) S. J. Singer and D. H. Campbell, *ibid.*, **74**, 1794 (1952).

(3) S. J. Singer and D. H. Campbell, *ibid.*, **75**, 5577 (1953).

(4) S. J. Singer and D. H. Campbell, *ibid.*, **77**, 3499 (1955).

(5) D. H. Campbell, E. Luescher and L. S. Lerman, *Proc. Nat. Acad. Sci.*, **37**, 575 (1951).

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