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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Physical Chemical Studies of Soluble Antigen–Antibody Complexes. IV. The Effect of *p*H on the Reaction between Bovine Serum Albumin and its Rabbit Antibodies[1]

BY S. J. SINGER AND D. H. CAMPBELL

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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

(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. A preliminary account of some of the results of this investigation has appeared in S. J. Singer and D. H. Campbell, THIS JOURNAL, **76**, 4052 (1954).

(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).

changes of buffer, with magnetic stirring. In reporting electrophoretic mobilities, the conductivity of the buffer at 0° has been used throughout. Measurements of *p*H were made with a Beckmann model G instrument, at temperatures near 25°.

Experimental Results and Discussion

Ultracentrifugal Studies in the *p*H Range 7.5 to 5.0.—A series of ultracentrifuge experiments were performed in buffers of ionic strength $\Gamma/2$, 0.1 in the *p*H range 7.5 to 5.0. This is a particularly interesting *p*H range since at *p*H 7.5 the BSA and antibody γ-globulin molecules both bear a net negative charge, while at *p*H 5.0, the former molecule is negatively, the latter positively, charged. If the net charges on Ag and Ab play a significant role in the reaction, the equilibrium distribution of species in these solutions should change noticeably in this *p*H range. Data obtained with solutions made from preparation III are given in Table I, from which it is apparent that no marked change in the distribution of species occurs. Some representative

TABLE I

SOLUTION III FROM *p*H 7.5 TO 5.0

*p*H	Buffer	Apparent % free Ag		Apparent % a-complex	
		Elec-troph.	Ultra-cent.	Elec-troph.	Ultra-cent.
7.50	Phosphate	67	74	..	15
6.54	Cac–NaCl[a]	66	75	17	13
6.29	Phosphate	66	..	20	..
6.13	Cac–NaCl[a]	66	79	19	16
5.80	Cac–NaCl[a]	..	78	..	15
5.43	Acetate	69	75	18	16
5.00	Acetate	67	74	20	16

[a] 0.02 *M* Na cacodylate and 0.08 *M* NaCl.

sedimentation patterns in this *p*H range are reproduced in Fig. 1.

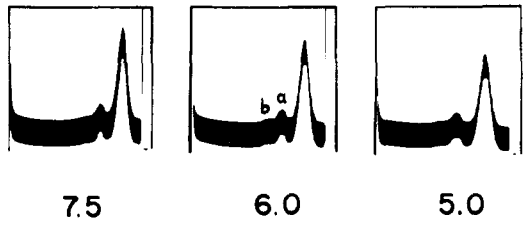

Fig. 1.—Ultracentrifuge diagrams of solution III (BSA–rabbit–anti–BSA) in the *p*H range 7.5 to 5.0. Sedimentation proceeds to the left.

With preparations III and V, but not with IV, a small amount of precipitation occurred in the *p*H range from about 6.0 to 4.5, with maximal precipitation (no more than 6% of the total protein) occurring at a *p*H about 5.4.

Electrophoresis in the *p*H Range 7.5 to 5.0.—Electrophoresis in buffers of ionic strength 0.1 was carried out with solution III (Fig. 2) and the apparent per cent. free Ag was determined[4] from the ascending pattern in each experiment (Table I). The area determinations were made somewhat difficult in the *p*H range from about 5.0 to 5.5 because some of the components are near their isoelectric points, and it was not possible to subtract the area of the stationary anomaly from that of the complexes. While the mobilities of the various species change with *p*H, of course, there seems to be no profound change in the distribution of species as a function of *p*H in this range,[7] which is in agreement with the ultracentrifugal results just discussed.

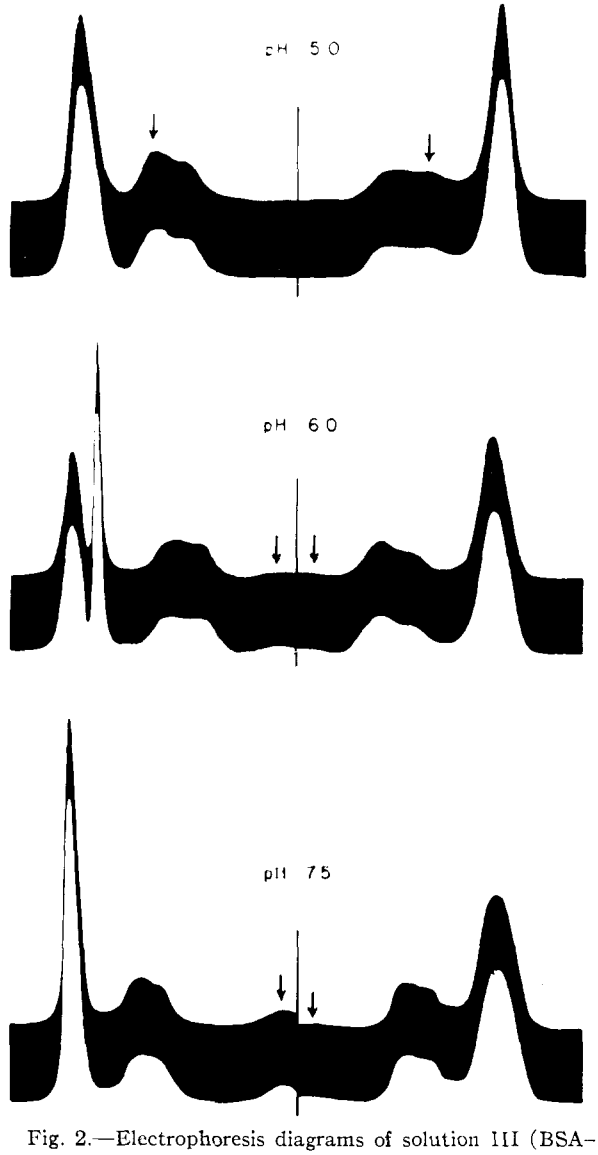

Fig. 2.—Electrophoresis diagrams of solution III (BSA–rabbit–anti–BSA) in the *p*H range 7.5 to 5.0. Starting positions are indicated by the arrows.

Between *p*H 7.5 and 5.0, both ascending and descending electrophoresis patterns (Fig. 2), for these solutions in fairly large Ag excess, showed three maxima.[7] According to our interpretation,[2] which is substantiated by the present results, the peak with the largest negative mobility is due to free Ag; the peak of intermediate mobility is due to the $(Ag)_2Ab$ complex; and the last peak is due to all the other complexes, which are poorer in Ag than

(7) In cacodylate–NaCl buffers in the *p*H range 6.5 to 5.5, the free BSA peak in the ascending limb, but not in the descending limb, was always sharply split into two parts. We have ascertained, in unpublished experiments, that this is an effect which has nothing to do with the interaction of BSA with its Ab, but occurs with BSA itself. The effect is completely reproducible, and is eliminated by substitution of I for Cl but its exact nature is not known.

the $(Ag)_2Ab$ complex, and are not much resolved from one another. The descending mobilities[8] are plotted in Fig. 3. If the Ag–Ab complexes were in very rapidly adjusted equilibrium, the fast peak in the descending limb would have a *constituent* mobility smaller than that of BSA.[10] The descending mobility of the free Ag peak in Ag–Ab solutions is the *same* as that of BSA itself, in accord with our conclusion that the rates of re-equilibration are relatively slow under the conditions of these experiments.

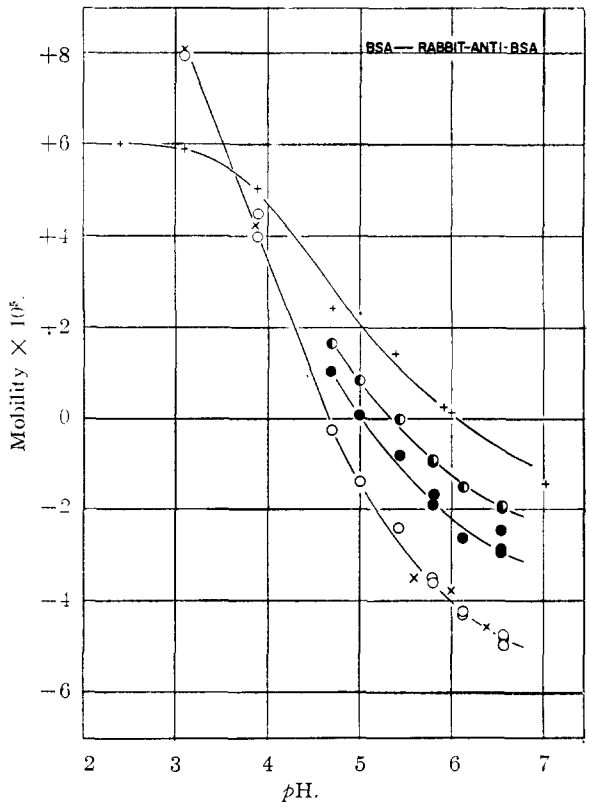

Fig. 3.—Electrophoretic mobility as a function of *p*H for: ×, BSA itself; O, free BSA in Ag–Ab solutions; ●, a-complex; ◐, other complexes; +, normal rabbit γ-globulin. All buffers are at 0.1 ionic strength.

The intermediate peak could be precisely identified from its isoelectric point and from its mobility as a function of *p*H, if enough other information for the Ag and Ab were available, and if the theory of electrophoretic mobility were more nearly complete. At present, knowing that the mobility of a complex is likely to be determined more by its constitution than by its shape, we can make the rough assumption that it is given by $\mu = \Sigma\omega_i\mu_i$, where ω_i is the weight fraction and μ_i the mobility of the constituent i. Now, if the intermediate peak is due to $(Ag)_2Ab$, the other complex peak is due to species poorer in Ag, whose average composition might be represented as $(Ag)_n(Ab)_n$.[2] The sum $\Sigma\omega_i\mu_i$ has therefore been calculated (Table II) for $(Ag)_2Ab$ and $(Ag)_n(Ab)_n$ at *p*H 7.0, 6.0 and 5.0; the agreement with the observed mobilities is such as to lend at least qualitative support to these species assignments, and to confirm the conclusion that the antibody in these solutions is bivalent.[2,4]

TABLE II

MOBILITIES OF Ag–Ab COMPLEXES

*p*H	Obsd. mobilities[a] a-Complex	Obsd. mobilities[a] Other complexes	Calcd. mobilities[a] $(Ag)_2Ab$	Calcd. mobilities[a] $(Ag)_n(Ab)_n$
7.0	−3.3	−2.3	−2.9	−2.3
6.0	−2.2	−1.3	−1.9	−1.2
5.0	0	+0.85	+0.4	+1.0

[a] × 10^5 cm./sec./volt/cm.

For these and other purposes, electrophoresis experiments were performed in the same buffers with I SA itself and with a normal rabbit γ-globulin preparation, RGG-I.[4] The descending mobilities so obtained are included in Fig. 3.

Electrophoresis at *p*H 2.4.—In the *p*H range 4.6 to 3.0, the *p*H–mobility curves of BSA and rabbit γ-globulin cross (Fig. 3) and electrophoretic resolution of the species in these solutions is poor. The ultracentrifuge has therefore been used in this critical *p*H range. At *p*H 2.4, however, the electrophoretic mobilities are again quite different. Furthermore, the Ag–Ab bonds are completely dissociated, so that electrophoresis at this *p*H affords an analytical method for the determination of total Ag and total Ab in a given solution.

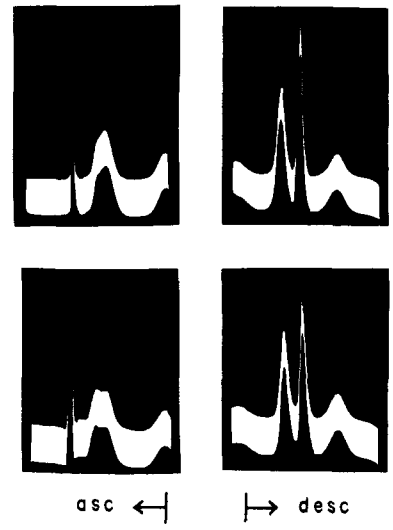

Fig. 4.—Electrophoresis diagrams in glycine–HCl buffer, *p*H 2.35, Γ/2 0.1 after 5700 sec. at 0.0080 amp.: (a) mixture of BSA and normal rabbit γ-globulin preparation RGG-II containing 49.12% BSA; (b) solution V-1.

In Fig. 4 are reproduced electrophoresis diagrams obtained in glycine–HCl buffer, *p*H 2.35, Γ/2 0.1, for (a) a mixture of BSA and *normal* rabbit γ-globulin, and (b) BSA and rabbit anti-BSA. The ascending and descending patterns are far from being mirror images of one another but the two descending patterns are practically indistinguishable. In these latter patterns there are three well-resolved peaks. The nature of the fastest peak is not

(8) The errors involved in the determination of the mean ordinates of the peaks due to the complexes are large enough so that we have neglected the fact that the conductivities of the solutions in the region of these boundaries are not the same as for the original solution.[9]

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

(10) L. G. Longsworth and D. A. MacInnes, *J. Gen. Physiol.*, **25**, 507 (1942).

clear,[11] but is not of importance for our purposes. The second and third peaks, however, are primarily due to BSA and γ-globulin, respectively. In order to eliminate uncertainties due to electrophoretic anomalies, the relative areas under these peaks were calibrated by experiments with accurately prepared mixtures of BSA and normal rabbit γ-globulin.[12] The rabbit γ-globulin preparation, RGG-II, was prepared by electrophoresis-convection and was 98% γ-globulin.[4] Differential refractometry was used to measure relative protein concentrations of the BSA and RGG-II solutions in phosphate buffer, *p*H 7.50, Γ/2 0.1, and mixtures of the two were prepared by weight.[4] The mixtures were then thoroughly dialyzed against the glycine–HCl buffer, and remained perfectly clear. At a total protein concentration of 17 mg./ml., electrophoresis experiments were performed in the glycine–HCl buffer, and the apparent relative areas under the second and third peaks in the descending patterns were determined as a function of the known composition of the solutions (Table III). As a check on the reliability of these results as applied to the Ag–Ab solutions, independent electrophoresis experiments under identical conditions were performed with all the solutions made from preparation V, at protein concentrations near 17 mg./ml. The per cent. total BSA in each solution was then obtained with the aid of the calibration data (Table III). On the other hand, if the master solution V is assigned the composition so determined the composition of each of the other solutions is known from the amounts of solution V and the standard BSA solution mixed in its preparation. The per cent. total BSA as determined by these two methods (Table IV) agree to within ±0.5 unit.

TABLE III

ELECTROPHORETIC ANALYSES WITH MIXTURES OF BSA AND NORMAL γ-GLOBULIN IN GLYCINE–HCl, *p*H 2.4

% BSA, analyt.[a]	Apparent % BSA[b]	% BSA, analyt.[a]	Apparent % BSA[b]
19.8_3	15.5	67.8	62.4
29.5_6	24.1	78.5	73.8
39.1_5	32.9	87.8	84.0
49.1	42.0	51.0[c]	44.8[c]
57.8	51.3	59.7[c]	53.8[c]

[a] From preparation of known mixtures, see text. [b] Ratio of area under second peak/sum of areas under second and third peaks, in decending electrophoresis patterns, see text. [c] Mixture with bovine (instead of rabbit) γ-globulin.

TABLE IV

ELECTROPHORETIC ANALYSIS IN GLYCINE–HCl, *p*H 2.4

Soln.	BSA, % Electroph.	BSA, % Analyt.	Soln.	BSA, % Electroph.	BSA, % Analyt.
V	39.3	..	V-4	71.8	72.7
V-1	48.1	47.8	V-5	78.7	78.6
V-2	56.9	56.9	V-6	89.4	88.9
V-3	66.8	66.5			

Independent experiments showed that β-globulin migrates under the γ-globulin peak under these conditions, so that any residue of β-globulin present in the γ-globulin preparation used to obtain the calibration data, does not interfere. Furthermore, experiments with mixtures of BSA and *bovine* γ-globulin have given the same results, within experimental error, as mixtures with *rabbit* γ-globulin (Table III).

This electrophoretic analysis has been employed to determine the composition of preparations IV, V and VI used in this and an earlier study,[4] and has obviated the labeling of BSA which was previously used.[2]

In the ultracentrifuge, BSA–anti BSA solutions also exhibit behavior identical with mixtures of BSA and normal rabbit γ-globulin in this glycine–HCl buffer. For various reasons, however, including the Johnston–Ogston anomaly to be discussed below and the relatively less accurate area determinations obtained from sedimentation patterns, the electrophoretic method is much more suited to accurate analyses. It should also be remarked here that there has never been observed at *p*H 2.4 any evidence, either in electrophoresis or sedimentation, of any components other than BSA and Ab in these Ag–Ab solutions.

Similar electrophoretic techniques should be applicable to the analysis of total Ag and Ab in other systems as well.

Ultracentrifugation in the *p*H Range 5.0 to 2.4.—As the *p*H is made more acid than about 4.5, the ultracentrifuge diagrams of solutions of Ag–Ab complexes change profoundly. From *p*H 7.5 to 4.6, there is an almost imperceptible amount of a component with the sedimentation rate of γ-globulin, between the peaks due to the free Ag and the slowest-sedimenting complex (the a-complex).[2] With further decrease in *p*H, however, such a γ-globulin peak becomes progressively larger while the amounts of the complexes diminish. It is clear that this γ-globulin component is *free* Ab, and that extensive dissociation of the complexes has occurred.[13]

These conclusions are evident from the results obtained with solution IV (containing 63.0% total Ag and 37.0% total Ab) at a concentration of 21 mg. total protein/ml. in buffers of 0.1 ionic strength (Fig. 5). At *p*H 2.4, the sedimentation diagram was that of a corresponding mixture of BSA and normal γ-globulin. A solution at *p*H 3.1 dialyzed back to *p*H 7.5 exhibited a diagram indistinguishable from that of a solution kept at *p*H 7.5, indicating that the acid dissociation of the complexes was completely reversible under these conditions. The apparent relative areas under the free Ag, free Ab and a-complex peaks only, for solution IV

(11) We have found a peak of the same mobility and similar area present in the descending patterns in experiments with the individual proteins BSA, γ-globulin, β-lactoglobulin and ovalbumin. Its universal presence suggests that it may be due to the buffer rather than the protein; perhaps the hydrogen ion constituent attains an electrophoretically significant concentration in this buffer.

(12) The glycine–HCl buffer, *p*H 2.35, Γ/2 0.1 was prepared from 12.70 g. of Mallinckrodt NF VIII glycine and 8.25 cc. of C.P. concd. HCl per liter of solution. The experiments were run for 5700 sec., at 8.0 ma. current. The second and third peaks in the descending patterns are practically completely resolved under these conditions, and were partitioned by dropping a perpendicular from the minimum between the peaks to the baseline.

(13) The sedimentation constants of all the species in these Ag–Ab solutions decrease as the *p*H becomes more acid than 4.0, in buffers of Γ/2 0.1. From *p*H 4.0 to 3.0, in solution IV, the *S* values for the free BSA peak decreased from 4.1 to 2.7 *S*; for the free Ab peak, from 6.2 to 4.4 *S*; and for the a-complex peak, from 8.3 to 5.7 *S*. Independent experiments with mixtures of BSA and normal γ-globulin confirmed these trends.

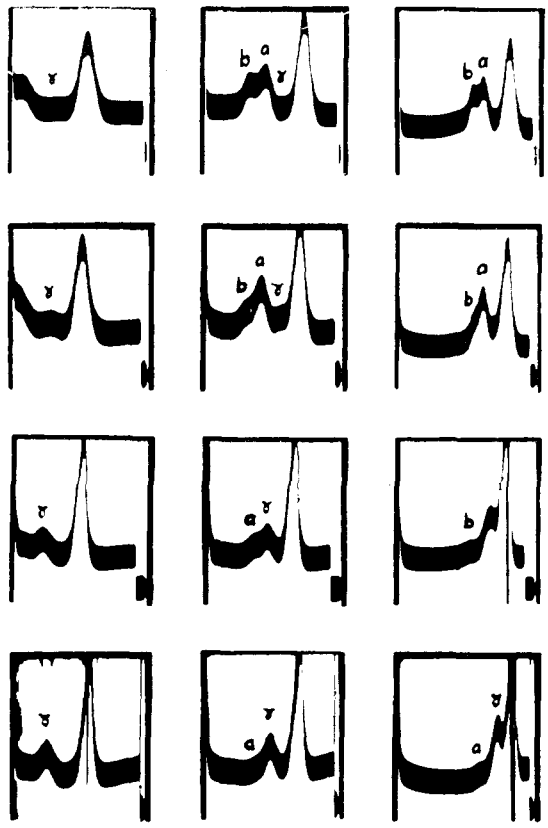

Fig. 5. — Ultracentrifuge diagrams of solution IV in buffers of different *p*H and Γ/2 0.1. Three patterns at different times in each experiment are illustrated, from right to left chronologically. Sedimentation proceeds to the left. The γ-peak represents free Ab, a and b, the *a*- and *b*-complexes.

in this series of buffers are given in Table V. It is to be noted that the dissociation appears to be quantitatively independent of the chemical nature of the buffer.

TABLE V

EFFECT OF *p*H ON Ag-Ab EQUILIBRIA

		Relative areas[a]								
		Free Ag		Free Ab		a-complex[b]		Ag-Ab[c]	K^d	
*p*H	Buffer	App.	Cor.	App.	Cor.	App.	Cor.		× 10^{-3}	log *K*
4.22	Lactate	50	42	5.3	5.3	26	30	17	18	4.25
3.90	Lactate	57	49	9	10	31	35	23	11	4.04
3.88	Acetate	57	49	9	10	25	30	20	9.4	3.97
3.60	Lactate	66	57	13	16	17	22	18	4.6	3.66
3.42	Lactate	68	59	19	22	12	17	15	2.8	3.45
3.31	Lactate	71	62	23	27	10	14	13	1.9	3.28
3.12	Glycine-HCl	68	59	29	34	5	10	10	1.2	3.08
2.49	Glycine-HCl	73	63[f]	27	37[f]	..	..	..	..	..

[a] Given as per cent. of total area expected for total protein content of solution, 21 mg./ml. [b] Taken as constituted of AgAb and $(Ag)_2Ab$. [c] Calculated as described in text. [d] For Ag + Ab ⇄ AgAb, in liter mole^{-1}. [e] As determined by electrophoresis in glycine-HCl buffer, *p*H 2.35, Γ/2 0.1.

Other than the free Ab peak, however, no new peak appears in the sedimentation diagrams in this *p*H range. On the other hand, in solutions containing appreciable amounts of free Ab, significant amounts of the AgAb complex should also be present. We infer that the AgAb complex sediments at a rate similar enough to that of the $(Ag)_2$-Ab complex in these solutions, that the two species are not resolved. Therefore, while at *p*H 7.5, the a-complex peak was shown[2] to be due largely to the $(Ag)_2Ab$ species, we conclude that at a *p*H more acid than 4.5 the area under the a-complex peak is attributable to the *sum* of the concentrations of the AgAb and $(Ag)_2Ab$ complexes.

At *p*H 7.5, one cannot determine the equilibrium concentration of enough species for a direct evaluation of equilibrium constants.[2,4] Instead, recourse is had to the Goldberg theory for Ag–Ab reactions,[6] the model for which assumes that all Ag–Ab bonds are intrinsically equivalent. In the *p*H range 4.5 to 3.0, however, almost entirely direct equilibrium constants may be obtained, as follows.[1] First, the apparent relative areas under the free Ag, free Ab, and a-complex peaks listed in Table V are corrected for the Johnston-Ogston anomaly.[14] The equilibrium concentrations of the free Ag and Ab at a given *p*H are thus directly determined from the corrected areas under their respective peaks. In addition, the corrected relative area under the a-complex peak is taken as the sum of the relative concentrations of the AgAb and $(Ag)_2Ab$ species. The *fraction* of the a-complex area attributable to the AgAb is evaluated, as a first approximation, on the assumption of the intrinsic equivalence of the bonds in the AgAb and $(Ag)_2Ab$ complexes. This assumption requires[6,15] that the concentrations of the two complexes in a given solution be related as

$$C_{(Ag)_2Ab} = C^2_{AgAb}/4C_{Ab}$$

where *C* represents molar concentration. This permits evaluation of quasi-experimental AgAb concentrations, and equilibrium constants, *K*, for the reaction Ag + Ab ⇄ AgAb, as a function of *p*H (Table V). In view of the approximations made in the calculations of the concentrations of the various components, the *K* values are uncertain to ±25%, but this introduces an uncertainty of only ±0.1 unit in log *K*.

The data obtained with solution IV also permit a direct test of the applicability of the Goldberg theory[6,4] to these Ag–Ab solutions. Two independ-

(14) J. P. Johnston and A. G. Ogston, *Trans. Faraday Soc.*, **42**, 789 (1946). An anomaly occurs with mixtures in the ultracentrifuge, such that the apparent areas of slower components are increased somewhat at the expense of the apparent areas of faster ones. Exact calculation of the anomalies in our system is at present impossible. Instead, empirical corrections have been applied, anchored by the following observed corrections at *p*H 7.5 and *p*H 2.4. At the former *p*H, due to this anomaly, the apparent per cent. free Ag determined ultracentrifugally is about 8 units higher than the true value,[4] at a total protein concentration of the order of 20 mg./ml. At the latter *p*H, the data in Table V indicate that the apparent per cent. free Ag is about 10 units higher than the true value. The corrections to the apparent per cent. free Ag at *p*H values between these two have therefore been interpolated between 8 and 10 units. The corrections to the apparent per cent. free Ab have been made as follows. At *p*H 2.4, the observed correction is + 10 units (Table V). It is assumed that that at *p*H 4.0, the correction is negligible, because of the presence of faster as well as slower components in the solution, and at *p*H values in between, the correction is obtained by interpolation. The corrections to the apparent per cent. of the a-complex are made similarly, with the restriction that in a given solution the algebraic sum of the corrections for the free Ag, free Ab, a-complex, and b-complex (the last, not included in Table V) be zero. The average correction to any apparent relative area is less than 20% of that area, and any residual uncertainties are not sufficient to alter the conclusions derived from these data.

(15) L. Pauling, D. Pressman, D. H. Campbell and C. Ikeda, THIS JOURNAL, **64**, 3003 (1942).

ent theoretical K values (Table VI) may be calculated for each solution, using either the free Ag concentration, or the free Ab, to evaluate the parameter p and hence K.[4,2,16] In view of the experimental uncertainties and the marked sensitivity of the theoretical K values to the free Ag or free Ab concentrations,[4] the agreement among the three sets of K values is satisfactory.

TABLE VI

TEST OF GOLDBERG EQUATIONS

pH	K(expt.) $\times 10^{-3}$	K(calcd.) $\times 10^{-3}$ From % free Ag	From % free Ab
4.22	18	34	18
3.90	11	10	10
3.88	9.4	11	10
3.60	4.6	2.4	6
3.42	2.8	1.4	3
3.31	1.9	0.5	1.9
3.12	1.2	1.5	0.5

In order to explain the effect of acid pH on Ag–Ab equilibria, let us consider a model system with the following properties: (a) in each of the two reactive sites of the Ab molecule there is one negatively charged group, and in all f sites of the Ag molecule one positively charged group, which must be ionized for reaction to occur. (Interchanging positive and negative charges would have no effect on the argument.); (b) the acid dissociation of the positive group occurs in the alkaline pH range; (c) the acid dissociation of the negative groups can be described by a single intrinsic equilibrium constant; (d) the effect on the reaction of the net charges on the entire Ag and Ab molecules is negligible. The equations governing the key equilibria in acid and neutral solutions may then be written

$$Ag^{+f} + Ab^{-2} \rightleftharpoons Ag^{+(f-1)}Ab^{-1};\ K_0 \qquad (1)$$

$$Ab^{-2} + H^+ \rightleftharpoons HAb^{-1};\ 2K_H \qquad (2)$$

$$HAb^{-1} + H^+ \rightleftharpoons H_2Ab;\ K_H/2 \qquad (3)$$

$$Ag^{+(f-1)}Ab^{-1} + H^+ \rightleftharpoons Ag^{+(f-1)}HAb;\ K_H \qquad (4)$$

The symbol for the equilibrium constant characterizing each reaction is written at the right. The apparent equilibrium constant, K, determined experimentally from the sedimentation patterns is given by

$$K = \frac{[AgAb]_{total}}{[Ag^{+f}][Ab]_{total}} \qquad (5)$$

where [S] represents the molar concentration of species S, $[Ab]_{total} = [Ab^{-2}] + [HAb^{-1}] + [H_2Ab]$, and $[AgAb]_{total} = [Ag^{+(f-1)}Ab^{-1}] + [Ag^{+(f-1)}HAb]$. From these relations it follows that

$$\log\left(\frac{1}{K} - \frac{1}{K_0}\right) = \log\frac{K_H}{K_0} - pH \qquad (6)$$

If *two* negative and *two* positive charges are involved in each bond, a similar but more complicated relation is obtained which, for pH < pK, reduces to the form of equation 6 with, however, a coefficient of *two* for the pH term.

To test the applicability of this relationship to the data of Table V, let us first note that where appreciable dissociation of the complexes exists, $1/K >> 1/K_0$, and $\log(1/K - 1/K_0) \cong -\log K$. In such circumstances, $\log K$ is a linear function of pH with slope unity. It is evident that the data of the first and last columns of Table V conform to this behavior. For a more quantitative test, the value of K_0 must be determined. If at pH $\sim$ 8, the positively and negatively charged groups in the reactive sites of the Ag and Ab molecules are fully ionized, then $K = K_0$ at that pH. In a previous paper,[4,16] for the reaction Ag + Ab $\rightleftarrows$ AgAb at pH 8.6, K was determined to be 1.0×10^5. Using this value for K_0, and the K values of Table V, we obtain the data plotted in Fig. 6. The relation between $\log(1/K - 1/K_0)$ and pH is indeed linear with slope −1.2. The difference from the theoretical slope −1.0 may be due to systematic errors in K, or to other effects discussed below. Furthermore, these data indicate that $\log(K_H/K_0) \sim 0$, and $K_H \cong K_0$, from which it follows that the pK for the dissociation of the group in the reactive site is about 5. This value is, within experimental error, equal to the pK of an isolated carboxyl group.

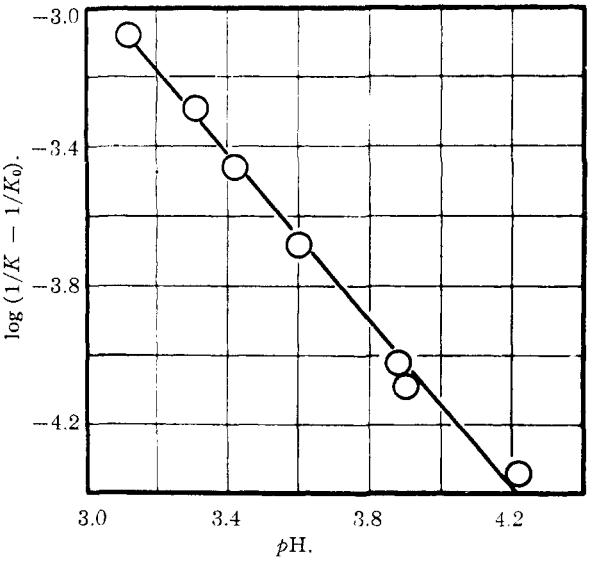

Fig. 6.—The variation of the apparent equilibrium constant with pH for solution IV.

Our results are therefore consistent with the presence of *one carboxyl* group in each Ag–Ab bond which must be ionized in order for the bond to form. Whether this carboxyl group is exactly the same from one site to the next, and whether it occurs in every Ag site or every Ab site, it is not possible to judge from the present results.

Several alternative explanations of the influence of pH on this system might be advanced. The two most important ones are: (a) as the Ag–Ab solutions become more acid and the Ag and Ab molecules acquire large net positive charges, non-specific *intermolecular* electrostatic repulsion of the Ag and Ab molecules might cause dissociation of the bonds between the two[17]; and (b) as the molecules be-

(16) K for the reaction Ag + AgAb $\rightleftarrows$ $(Ag)_2Ab$ is 1/4 of K for the reaction Ag + Ab $\rightleftarrows$ AgAb.[6,16]

(17) An approximate theoretical calculation of the magnitude of this effect may be made by means of the theory of Verwey and Overbeek,[18] but the validity of the calculation is uncertain. The Ag and Ab molecules are taken as spheres with a uniform surface charge density surrounded by ion atmospheres, the spheres being in contact with each other. With this model, a positive electrostatic free energy is pre-

come highly positively charged, *intramolecular* repulsive forces may cause reversible configurational changes in either or both the Ag and Ab molecules, such that the complementariness of the reactive sites is diminished and dissociation occurs.[19]

That non-specific intermolecular electrostatic forces are not of primary significance under the conditions of our experiments is indicated by several facts. (a) There is no gross effect on the Ag–Ab reaction as the *p*H is lowered from 8.6 to values between the isoelectric points of Ag and Ab, where the molecules are now oppositely charged (Table I). (b) We expect the electrostatic forces to be proportional to the product of the surface charge densities of the Ag and Ab molecules, and in turn to the product of their electrophoretic mobilities under given conditions. Yet this mobility product has about the same value in this system at *p*H 4.0 or *p*H 8.0 (Fig. 3). Therefore if the extensive dissociation at *p*H 4.0 were due to non-specific intermolecular electrostatic forces, similar dissociation should exist at *p*H 8.0, which is contradicted by our results.

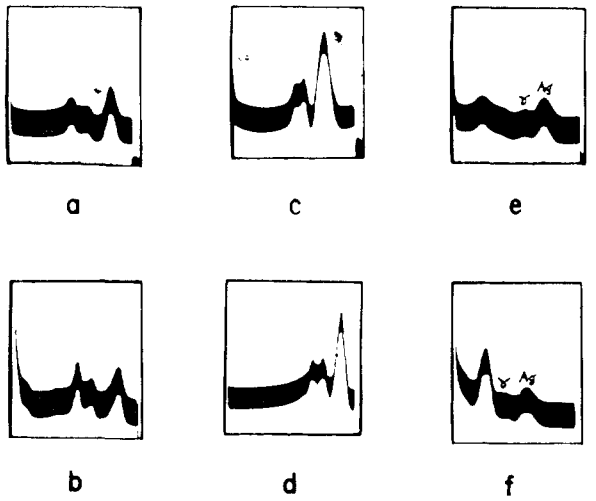

Fig. 7.—The effect of ionic strength on the ultracentrifuge patterns of Ag–Ab solutions; solution V in phosphate buffers, (a) *p*H 7.50, Γ/2 0.10; (b) *p*H 7.50, Γ/2 0.001; solution IV in acetate buffers, (c) *p*H 5.38, Γ/2 1.0 (containing 0.9 *M* NaCl); (d) *p*H 5.46, Γ/2 0.01; solution V in lactate buffers; (e) *p*H 4.02, Γ/2 0.10; (f) *p*H 4.03; Γ/2 0.001. Sedimentation proceeds to the left in all patterns.

This view is further substantiated by some ultracentrifuge experiments which were performed with Ag–Ab solutions at various ionic strengths. Electrophoresis and ultracentrifuge experiments over a wide range of ionic strengths are not easily interpreted quantitatively. In the former case, area anomalies greatly increase as the ionic strength is decreased, while in the latter, marked changes in the resolution of the species in a mixture may be observed with variations in ionic strength. The results of our experiments in this connection are therefore only semi-quantitative. In Fig. 7 are illustrated some pairs of ultracentrifuge patterns obtained with a given Ag–Ab solution at a particular *p*H and two ionic strengths differing by a factor of 100. There is no pronounced effect of ionic strength on the amounts of free Ag or free Ab in these solutions.

Evidence has accumulated that some kind of structural change occurs in the BSA molecule in acid solution, presumably due to intramolecular electrostatic repulsive forces.[20,21] Part of the reduction in sedimentation constants of the components of these Ag–Ab solutions[13] may be due to this effect. However, it is not likely that this is a major factor in the acid dissociation of BSA–anti BSA bonds since the *p*H regions in which the two phenomena occur do not coincide. At *p*H 4.0, the *K* value (Table V) for the Ag–Ab reaction is only $^1/_{10}$ its value at *p*H 8.6, while the effects of acid on the structure of the BSA molecule only begin to be noticeable at *p*H 4.0.[21]

We conclude, therefore, that a single ionized carboxyl group is an essential part of the Ag–Ab bond in this system. The inference is made that a positively charged group is present in each complementary site. This is consistent with the observation that similar antigen–antibody precipitates may be dissociated in sufficiently alkaline as well as acid solutions.[22] It is interesting that the involvement of a pair of oppositely charged groups in the bond provides a reasonable explanation of the positive standard entropy change accompanying the reaction.[4] Furthermore, that only one charged group is specifically involved is a reflection of the relatively small size of a reactive site on these large molecules.

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dicted which is large enough to cause dissociation of the Ag–Ab bonds in the acid *p*H range. This cannot be correct, however, since the theory would likewise predict dissociation at *p*H 8.0 comparable to that at *p*H 4.0 in this system, which is not observed experimentally.

(18) E. J. W. Verwey and J. T. G. Overbeek, "Theory of the Stability of Lyophobic Colloids," Elsevier Publishing Co., New York, N. Y., 1948, p. 152.

(19) If either one or both of these factors is significant, then the ionization of a specific group in the reactive site is probably not involved at all. For, if all of these factors were important, subtracting the effects of either intermolecular or intramolecular electrostatic repulsion, or both, would decrease the slope of the experimental plot in Fig. 6. A slope of much less than unity would imply that only some fraction of reactive sites contained an ionizable group. Such large differences among the sites seem improbable.

(20) C. Tanford, *Proc. Iowa Acad. Sci.*, **59**, 206 (1952).

(21) J. T. Yang and J. F. Foster, THIS JOURNAL, **76**, 1588 (1954).

(22) E. A. Kabat and M. M. Mayer, "Experimental Immunochemistry," C. C Thomas, Springfield, Ill., 1948, p. 480.