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Physical-Chemical Studies of Soluble Antigen-Antibody Complexes. X. The Reaction of a Univalent Protein Antigen with Antibody^{1,2}

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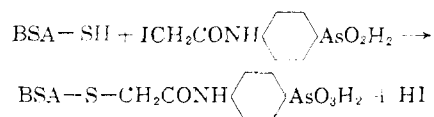
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A univalent protein antigen, BSA-S-R₁, was prepared by the complete reaction of the one SH group of bovine serum mercaptalbumin with N-(*p*-benzenearsonic acid) iodoacetamide. The reaction of BSA-S-R₁ with pure antibodies (Ab) to the benzenearsonic acid (R) group was studied quantitatively by electrophoresis, ultracentrifugation and light scattering. The results are completely in accord with the concepts of the framework theory. Equilibrium constants calculated, with the aid of stated assumptions, from the electrophoresis and light scattering data, are in good agreement. A comparison of these constants with those obtained with multivalent R antigen:anti-R ab systems shows that to a good approximation the intrinsic bond strength is independent of the size of the antigen-antibody aggregate, at least for aggregates which are soluble. The difference between ΔF° for hapten R:Ab and protein R:Ab reactions largely can be accounted for by a difference in the loss of translational entropy accompanying the two reactions.

Introduction

The reaction between protein antigens (Ag) and their specific antibodies (Ab) is difficult to study quantitatively because of its complexity. It is generally agreed that both Ag and Ab have more than one reactive site per molecule, and their reaction is therefore roughly analogous to a copolymerization of two multifunctional monomers, resulting in the formation of large networks containing the two molecules. For many purposes, it would be extremely useful to reduce this complexity by making available an antigen with all the properties of a typical protein Ag, with, however, only one reactive site per molecule: a univalent protein Ag. In combination with bivalent Ab, a univalent Ag should form only the aggregates AgAb and AgAbAg, and therefore only two reactions need be considered instead of the large multitude which are involved in multivalent Ag-Ab systems.

A naturally-occurring univalent protein Ag is not known. We have recently, however, described the preparation of a partly synthetic univalent protein Ag³ with properties suitable for physical chemical studies. The one sulfhydryl group of bovine serum mercaptalbumin⁴ (BSA-SH) may be completely and specifically combined with N-(*p*-benzenearsonic acid) iodoacetamide, as indicated



to give a derivative (BSA-S-R₁) with one benzenearsonic acid (R) residue covalently bound per BSA molecule. BSA-S-R₁ is a univalent Ag with respect to antibodies directed against the benzenearsonic acid group (anti-R Ab), which may be prepared and isolated in pure state.⁵

(1) This work was taken in part from the thesis submitted by F. A. Pepe as partial fulfillment of the requirements for the Ph.D. degree in Chemistry, Yale University, June, 1957. The research was supported by grant E-1204C from the National Institutes of Health, United States Public Health Service, and by a grant from the Rockefeller Foundation.

(2) The previous paper in this series is S. I. Epstein and S. J. Singer, *THIS JOURNAL*, **80**, 1274 (1958).

(3) F. A. Pepe and S. J. Singer, *ibid.*, **78**, 4583 (1956).

(4) W. L. Hughes, Jr., *Cold Spring Harbor Symposia Quant. Biol.*, **14**, 79 (1950).

(5) D. H. Campbell, R. H. Blaker and A. B. Pardee, *THIS JOURNAL*, **70**, 2496 (1948).

In our preliminary communication,³ we reported on the qualitative aspects of the reaction between BSA-S-R₁ and anti-R Ab. In this paper a quantitative physical chemical investigation of this system is presented, utilizing the techniques of ultracentrifugation, electrophoresis and light scattering.

There have been several useful results of this study. From a qualitative point of view, it has completely confirmed the predictions of the framework theory of Ag-Ab reactions,⁶ according to which, in particular, one would not expect any Ag-Ab precipitates to form in a mixture containing a univalent Ag and a bivalent Ab, whatever their proportions. From a quantitative point of view, it has provided strong evidence for the hypothesis that the intrinsic Ag-Ab bond strength is, to a good first approximation, independent of the size of the Ag-Ab aggregate containing the bond. This hypothesis is the crux of the Goldberg theory⁷ of the Ag-Ab reaction. This study has therefore further validated the estimates of free energy changes of protein Ag-Ab reactions which have been obtained with the use of the Goldberg theory.⁸⁻¹¹ In addition, the BSA-S-R₁:anti-R system serves as a relatively simple model system with which to investigate the effects which are produced when a mixture of species originally in equilibrium is subjected to electrophoretic or ultracentrifugal resolution. This problem is examined in the accompanying paper.¹²

Materials and Methods

Buffer Materials.—For light scattering experiments, the tris-(hydroxymethyl)-aminomethane obtained from Commercial Solvents Company was recrystallized from ethanol. Na *p*-arsanilate was prepared from Eastman *p*-arsanilic acid by adding to it enough C.P. NaOH to bring the pH to 7.0, and then precipitating the salt with an equal volume of ethanol, filtering and drying in air. All other buffer materials were C.P. grade or the equivalent.

Preparation of the Univalent Antigen.—The preparation of BSA-S-R₁ has been described in detail previously.³

Preparation of Anti-R Antibody.—Antisera were prepared by injecting rabbits with a highly coupled benzenearsonic acid-azo-bovine, γ -globulin (RBG). RBG was made by

(6) J. R. Marrack, "The Chemistry of Antigens and Antibodies," Med. Research Council Brit. Special Rept. Series, No. 230, 1938.

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

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

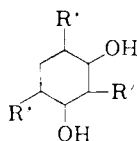
(9) S. J. Singer and D. H. Campbell, *ibid.*, **77**, 3199 (1955).

(10) S. J. Singer and D. H. Campbell, *ibid.*, **77**, 4851 (1955).

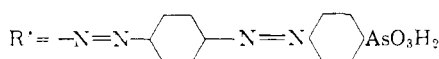
(11) M. C. Baker, D. H. Campbell, S. I. Epstein and S. J. Singer, *ibid.*, **78**, 312 (1956).

(12) S. J. Singer, F. Pepe and D. Ilten, *ibid.*, **81**, 3887 (1959).

coupling diazotized *p*-arsanilic acid to Armour bovine γ -globulin at 0° while maintaining the pH between 7 and 8. The product was exhaustively dialyzed against phosphate buffer, pH 7.5, $\Gamma/2$ 0.1 and then was lyophilized. Analysis for total N and total As established that there were about 180 benzenearsonic acid groups per RBG molecule. The RBG was administered subcutaneously by the Freund adjuvant technique, in which the RBG was emulsified with an oil containing killed and dried *Mycobacterium butyricum*.¹³ A second injection of 20 mg. of RBG was given three weeks after the first, and two weeks later ear bleedings were begun and carried on over an extended period, interspersed with several further subcutaneous injections to keep the serum titers at a sufficiently high level. The sera were titrated with RBG and with a synthetic trivalent hapten



where



This hapten is referred to as R'_3 -resorcinol and was obtained through the kindness of Drs. D. Pressman and D. H. Campbell. It was found that none of the antisera precipitated a measurable amount of bovine γ -globulin itself and that RBG precipitated about three times as much Ab as did R'_3 -resorcinol. The significance of this observation will be discussed later. Approximately one-third of the rabbits showed a maximum titer of from 0.6 to 1.0 mg. of anti-R per ml. of serum, using RBG as the precipitating Ag. Their sera only were pooled. The large quantity of Ab required for the studies reported in this paper placed a restriction on the number of experiments we were eventually able to perform.

Purification of Anti-R Antibody.—The purification of Ab was carried out essentially by Epstein's method B.¹⁴ The Ab was precipitated from the γ -globulin fraction of the pooled antisera by the equivalent amount of R'_3 -resorcinol. After suitable washing, the precipitate was dissolved in sodium arsenilate solution (pH 8.3) and the R'_3 -resorcinol was removed as the bariun salt by centrifugation. Dialysis of the remaining solution free of the arsenilate yielded the pure Ab. The Ab was stored as a precipitate under 37% saturated ammonium sulfate at 4° until needed.

Two separate primary pools of Ab were used in this study. Following our previous nomenclature,¹¹ we refer to these as anti-R-III and anti-R-IV. The former was employed in the electrophoresis and ultracentrifuge studies at pH 8.6, and the latter in the light scattering experiments.

Electrophoresis Experiments.—A Perkin-Elmer Model 38 Tiselius apparatus was used, operating at 2°. For the main series of experiments solutions of BSA-S- R_1 and of anti-R Ab from pool III were separately dialyzed to equilibrium against barbital buffer, pH 8.70, $\Gamma/2$ 0.1. Their refractive increments were determined, and aliquots were then mixed by weight to provide solutions with a range of Ag-Ab ratios. These mixtures then were redialyzed against the barbital buffer before electrophoresis. Further conditions of the experiments are given in the legend of Fig. 5.

A few experiments were performed in other buffers as indicated.

Area measurements were made with a planimeter from enlarged tracings of the patterns. The areas were partitioned by drawing Gaussian curves where peaks overlapped slightly.

Ultracentrifugation.—These experiments were performed in a Spinco Model E Ultracentrifuge at 50,740 r.p.m. near 25°, with solutions which had been recovered from the electrophoresis experiments described above, of BSA-S- R_1 and anti-R Ab, in barbital buffer, pH 8.70, $\Gamma/2$ 0.1. Cells with 30 mm. centerpieces were used. Further conditions for the

experiments are given in the legend of Fig. 4. Area measurements¹² were made as previously described.¹⁵

Light Scattering Experiments.—The general aspects of our light scattering technique were similar to those used by Epstein, Doty and Boyd.¹⁴ A Brice-Phoenix photometer was employed at λ 4360 Å. Since the aggregates in this system were small in size and were present in only small quantities, no significant dissymmetry of light scattering was expected,¹⁴ and all measurements were made at 90°. Beckman spectrophotometer 1 cm. square silica cells were used, which were mounted reproducibly in the apparatus. Pipets were cleaned in a hot H_2SO_4 - HNO_3 bath at least overnight and rinsed thoroughly with tap and distilled water and finally with optically clean redistilled water. Cells were washed thoroughly with warm detergent solution using a jeweler's rouge cloth and then were rinsed as were the pipets. Protein solutions were centrifuged in a Spinco Model L Ultracentrifuge at 29,500 r.p.m. for 90 minutes, in polyethylene centrifuge tubes of a variety of capacities which were shaped to the holes in the #30 rotor of the centrifuge and fitted snugly with polyethylene caps. Solvents were clarified by filtration through ultrafine sintered glass filters until optically clean.

TABLE I

BUFFERS USED FOR LIGHT SCATTERING

	Enough solid Tris was added in each case to give pH 7.0.
I	0.05 M HCl-0.35 M NaCl; plus solid Tris, $\Gamma/2$ 0.40
II	.05 M HCl-0.85 M NaCl; plus solid Tris, $\Gamma/2$ 0.90
III	.05 M HCl-0.35 M NaCl-0.50 M Na <i>p</i> -arsanilate; plus solid Tris, $\Gamma/2$ 0.90
IV	.05 M HCl-0.45 M NaCl; plus solid Tris, $\Gamma/2$ 0.50
V	.05 M HCl-0.35 M NaCl-0.10 M Na <i>p</i> -arsanilate; plus solid Tris, $\Gamma/2$ 0.50

The special problems faced in this study stemmed from the very small increments in light scattering produced by Ag-Ab reactions in this system, and these only at relatively high total protein concentrations. Preliminary experiments indicated that the most careful controls were required to determine these increments with any satisfactory accuracy. To this end, for detailed reasons which will become apparent later, four types of solutions and their dilutions were examined: (L1) a solution of BSA-S- R_1 and anti-R at a weight ratio of the two calculated to give maximal aggregation; (L2) solution L1 containing in addition a heavy molar excess of Na *p*-arsanilate (0.1 M) over BSA-S- R_1 ; (L3) a solution containing BSA-SH (the preparation from which BSA-S- R_1 was synthesized) and an aliquot of the same anti-R Ab, at the same ratio of components, as solution L1; and (L4) solution L3, containing in addition the same amount of excess Na *p*-arsanilate as solution L2. The anti-R Ab used in all the light scattering experiments was from pool IV. In addition, a series of optically clean buffer solutions, which are listed in Table I, was prepared so that the final buffer ion concentrations of a series of dilutions of any one protein solution could be made identical. Each solution examined by light scattering was at pH 7.0 and $\Gamma/2$ 0.50. All those which contained Na *p*-arsanilate consisted of 0.05 M Tris Cl (tris-hydroxymethylaminomethane), 0.35 M NaCl, and 0.1 M Na arsenilate, while all those which had no arsenilate, consisted of 0.05 M Tris Cl and 0.45 M NaCl. The pH and $\Gamma/2$ were chosen so as to minimize the non-ideal behavior of these multi-component solutions.

The protein solutions L1 and L2 and their dilutions, for example, were prepared and examined as follows. A solution of weight ratio BSA-S- R_1 :anti-R Ab = 0.80 was prepared from solutions of the two components separately dialyzed against buffer I, which were mixed by weight after their refractive index increments were determined. This solution was centrifuged in two polyethylene tubes each of 12-ml. total capacity. The top 9.2 ml. of clean solution was removed carefully from each tube, with the aid of a rack-and-pinion device and gentle suction, and transferred to a single clean vial containing a glass-covered magnetic stirring rod. The vial and stirrer were previously brought through

(13) Ready prepared adjuvants may be obtained from Difco Laboratories, Detroit 1, Mich.

(14) S. I. Epstein, P. Doty and W. C. Boyd, *THIS JOURNAL*, **78**, 3306 (1956).

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

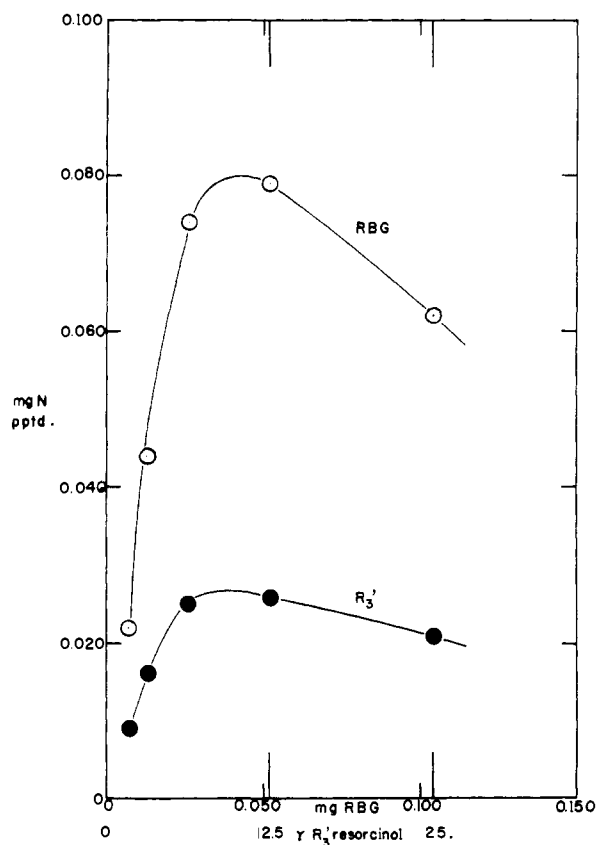


Fig. 1.—Ag-Ab precipitin titration of pooled high titer anti-R sera with the two Ag's, RBG and R_3' -resorcinol. The serum was diluted to $\frac{1}{3}$ its original concentration with borate-NaCl buffer, pH 8.0, $\Gamma/2$ 0.16, and 1.0 ml. was added to 1.0 ml. of serial dilutions of Ag in the same buffer.

the acid bath, rinses and drying as a unit. At this point, after stirring, the solution was examined at low angles in a strong light beam in the photometer, to detect dust particles. If dust was present, the solution was recentrifuged and placed into another clean vial and re-examined. From a clean solution two 8-ml. aliquots were withdrawn with optically clean graduated pipets, using the same mechanism as that used for withdrawing solution from the centrifuge tubes. The aliquots were placed in weighed clean small vials each containing a small glass covered magnetic stirring rod, and the solutions were again examined in the light beam for cleanliness. To one of the vials was added 2.0 ml. of filtered buffer II and to the other 2.0 ml. of buffer III, to give solutions L1 and L2, respectively. The vials were reweighed, the solutions were stirred on a magnetic stirrer and then examined for cleanliness. For light scattering measurements of L1 at various concentrations, for example, the solution was drawn into a calibrated pipet and 3.0, 2.4, 1.8, 1.2 and 0.6 ml. were transferred to five clean weighed silica cells. The cells were reweighed. Then filtered buffer IV was added to give a total volume of 3.0 ml. in each cell, and the cells were weighed again. Clean aluminum foil cell covers then were pressed firmly to the tops and the cells were tipped gently five times to mix the solutions. Light scattering readings were taken immediately. The concentrations of the protein solutions were high (0.5–2.0%), and any small amount of dust introduced by the mixing procedure did not significantly change the total light scattered by the solution at 90° . This was demonstrated by the finding that further brief mixing of the solutions caused no significant changes in the light scattered. The concentrations of the solutions were calculated from the measured densities of the buffers and the added weights of solutions, taking \bar{V} for the proteins as 0.75 ml./g. Dilutions of L2 were prepared and examined in a similar manner, thereby ensuring as close as possible a parallelism between the two sets of results. In

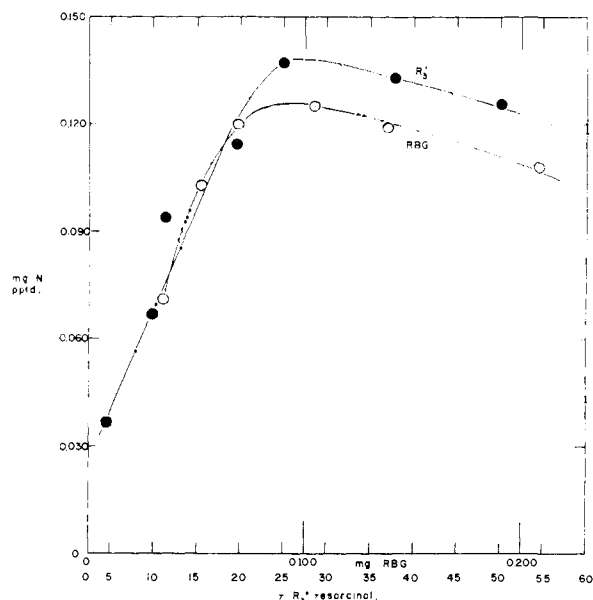


Fig. 2.—Ag-Ab precipitin titration of purified anti-R IV Ab with the two Ag's, RBG and R_3' -resorcinol. To 1.0 ml. of serial dilutions of Ag in borate-NaCl buffer, pH 8.0, $\Gamma/2$ 0.16, was added 0.182 mg. of Ab N in 1.0 ml. of the same buffer.

an exactly analogous fashion, solutions L3 and L4 were prepared from a common centrifuged solution containing a weight ratio of BSA-SH to anti-R Ab of 0.81.

In addition to these experiments, solutions of BSA-SH in the same Tris-NaCl buffers with and without 0.1 *M* arsenilate were studied as checks on our entire technique. Unfortunately, not enough purified Ab was available for other light scattering experiments than the ones described above.

Specific refractive increments for BSA-S-R₁ were taken as equal to those of BSA and were interpolated in the data of Perlmann and Longworth.¹⁶ Rabbit Ab γ -globulin was taken to be the same refractometrically as human γ -globulin, for which data are available.¹⁷ At 25°, in 0.5 *M* NaCl, at λ 4360 Å. we used $k_{BSA} = 2004 \times 10^{-6}$ and $k_{Ab} = 1960 \times 10^{-6}$.

Auxiliary Measurements.—Refractive increment measurements for the determination of protein concentrations were made in a Brice-Phoenix differential refractometer calibrated with KCl solutions at λ 5780 Å. A Beckman Model G pH meter was used for all pH determinations at 25° unless otherwise indicated, and a Beckmann DU Spectrophotometer for light absorption measurements.

Results and Discussion

Properties of Anti-R Ab Preparations.—The original antisera elicited by the Ab RBG contained different kinds of anti-R Ab, and precipitation with R_3' -resorcinol produced a fractionation of the Ab. This is demonstrated by the Ag-Ab precipitation data of Figs. 1 and 2, in which the original antisera and the purified Ab anti-R IV were titrated with RBG and R_3' -resorcinol. No precipitation of Ab was obtained with bovine γ -globulin itself. The purified Ab was precipitated more completely by R_3' -resorcinol than by RBG, whereas the latter Ag precipitated three times as much Ab from the original antiserum. No attempt was made to investigate experimentally the molecular basis for this Ab heterogeneity, but it is apparent that RBG contained

(16) C. E. Perlmann and L. G. Longworth, *THIS JOURNAL*, **70**, 2719 (1948).

(17) H. Neurath and K. Bailey, "The Proteins," Vol. 1A, Academic Press, Inc., New York, N. Y., 1954, p. 404.

antigenic sites which were more complicated than the simple *p*-azobenzene-arsonic acid group. This is probably related to similar phenomena observed by Haurowitz and Schwerin.¹⁸ The purified Ab, isolated after precipitation by R_3' -resorcinol, is likely to have contained a more homogeneous Ab population than the original whole antisera, but there are indications of some residual heterogeneity in binding capacity which will be discussed later.

The purity of the anti-R Ab preparations was investigated in several ways. The two most important impurities which might possibly have been present in these preparations were some R_3' -resorcinol not completely removed in the purification procedure, and inert γ -globulin. If the former were present in significant amounts, rapidly sedimenting peaks should appear in the ultracentrifuge patterns of the Ab preparations due to two or more Ab molecules attached to one R_3' -resorcinol molecule. Such peaks were not observed (Fig. 3a). Furthermore,

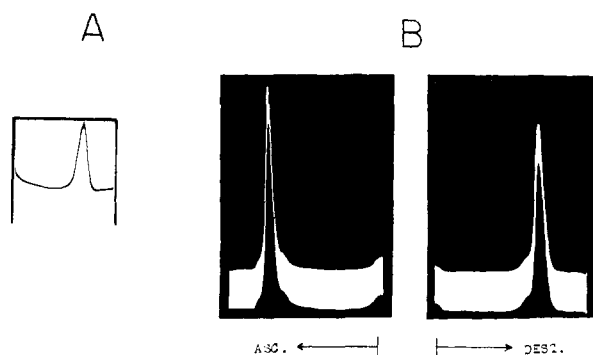


Fig. 3A.—An ultracentrifuge pattern of anti-R IV purified Ab at 4.0 mg./ml. concentration in borate-NaCl buffer, pH 8.0, $\Gamma/2$ 0.16, after 3360 sec. at 50,740 r.p.m. 3B.—Electrophoresis patterns of BSA-S-R₁, 10.9 mg./ml. in barbital buffer, pH 8.70, $\Gamma/2$ 0.1, after 3600 seconds at 12.9 v./cm. The starting positions and directions of migration are indicated by the arrows.

R_3' -resorcinol absorbs strongly at 430 $m\mu$ and spectrophotometric measurements on a solution containing 24.3 mg./ml. anti-R IV showed that fewer than 2 moles of R_3' -resorcinol were present for every 100 moles of Ab. This amount of hapten could not significantly affect our results. This spectrophotometric test is an important one, since we have observed that it is difficult to remove the last traces of the dye from the purified Ab. These traces impart a distinct pink coloration to an Ab precipitate under 1/3 saturated $(NH_4)_2SO_4$; the precipitate of the Ab anti-R IV was colorless visually.

The fact that R_3' resorcinol precipitated a maximum of only about 75% of the protein in a purified anti-R Ab preparation is not an indication of the presence of 25% inert protein but rather is due to the equilibrium attained in the reaction of a trivalent hapten with bivalent Ab. That all the protein in the Ab preparation is active may best be demonstrated electrophoretically with a mixture of the Ab and a multivalent Ag in Ag excess.¹¹ In such a mixture, the Ab is bound in rapidly-migrating Ag-Ab aggregates, and the absence of a signifi-

(18) F. Haurowitz and P. Schwerin, *Brit. J. Exptl. Path.*, **23**, 146 (1942).

cant peak with the mobility of inert γ -globulin demonstrates the absence of inactive protein. Such experiments with the Ab of pool IV are shown in Fig. 10 and are discussed in a later section. The Ab protein by this criterion was essentially completely active.

The Framework Theory of Ag-Ab Precipitation.

—The behavior of the BSA-S-R₁:anti-R system is qualitatively entirely in accord with the predictions of the framework theory of Ag-Ab precipitation.⁶ This theory accounts for precipitate formation by postulating that natural Ag and Ab are both multivalent, that is, each has more than one reactive site per molecule, so that large three-dimensional frameworks of alternating Ag and Ab molecules may form which are insoluble. It has been demonstrated that rabbit anti-R Ab is bivalent.¹⁹ The theory therefore predicts that no precipitation should occur in mixtures of BSA-S-R₁ and anti-R, since only the aggregates Ag-Ab and Ag-Ab-Ag would be expected to form. Experimentally, this prediction is verified. Over a wide range of ratios of the two components at concentrations as high as 2% total protein at pH 8.7, no specific precipitation ever was observed.

The ultracentrifuge diagrams of BSA-S-R₁:anti-R mixtures (Fig. 4) similarly conform to the ex-

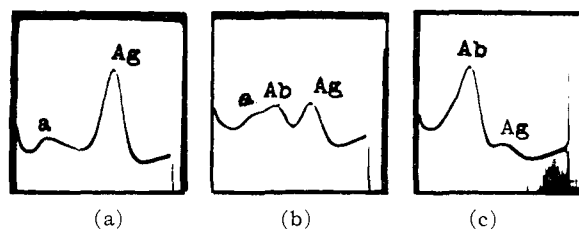


Fig. 4.—Ultracentrifuge patterns of BSA-S-R₁: anti-R mixtures. Sedimentation proceeds to the left: (a) solution L (Table II, column 1) at 8.3 mg. protein/ml. after 6627 sec. at 50,740 r.p.m.; (b) solution H at 7.6 mg./ml. after 5790 sec.; (c) solution E at 10.0 mg./ml. after 7211 sec. Peaks labelled Ag, Ab and a correspond to free BSA-S-R₁, free Ab and aggregates, respectively.

pected behavior of this system.³ No aggregates were present which sedimented more rapidly than expected for the $(Ag)_2Ab$ species, in marked contrast to the BSA:anti-BSA system,¹⁵ which contains a multivalent Ag, and hence forms large Ag-Ab aggregates.

Electrophoresis at pH 8.7.—A series of electrophoresis experiments were performed covering a wide range of ratios of BSA-S-R₁ to anti-R (Fig. 5). In each limb there generally appear more than two peaks, the peaks of intermediate mobility clearly resulting from the formation of aggregates of BSA-S-R₁ and anti-R Ab.³ It is evident, however, that the two patterns of a given experiment are far from being mirror images, which is most probably due to re-equilibration reactions occurring during electrophoretic resolution of the mixture. The apparent mobilities of some of the prominent peaks (calculated using throughout the original buffer conductivity) are given in Table II; the peak designations are those indicated in Fig. 5.

(19) H. N. Eisen and F. Karush, *THIS JOURNAL*, **71**, 363 (1949).

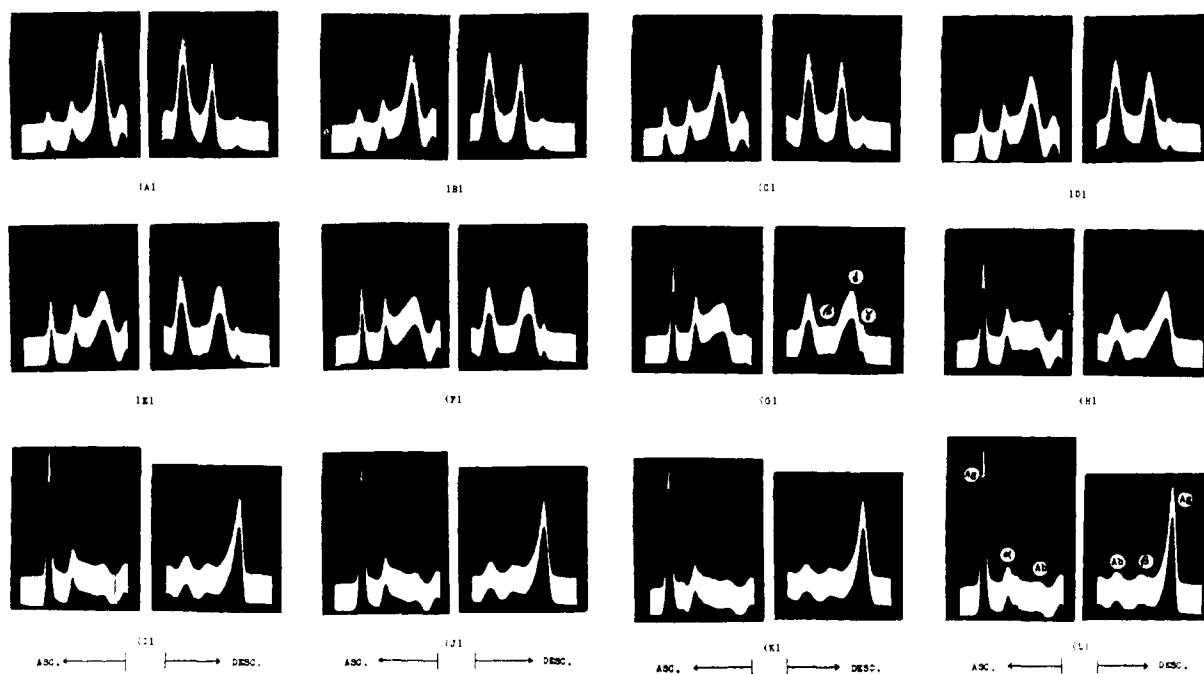


Fig. 5.—Electrophoresis patterns of BSA-S-R₁: anti-R mixtures. The letters under each pair of patterns designate the solution (Table II, column 1). All experiments in barbital buffer, *p*H 8.70, $\Gamma/2$ 0.1 after 4500 sec. at 20.2 v./cm. The peaks are labelled as in Table II. The arrows indicate the direction of migration in the ascending and descending limbs.

A more detailed discussion of the nature of these patterns is given in the accompanying paper.¹² For the present purposes, however, it is to be noted that two well-resolved peaks, the leading one in the

TABLE II
MOBILITIES OF BOUNDARIES IN BSA-S-R₁:ANTI-R-MIXTURES

Solu. ^a	(Ag/ Ab) _w ^b	Mobilities $\times 10^5$, cm./sec./v./cm. ^c								
		Ascending			Descending					
		Ag	α	Ab	Ag	β	γ	δ	Δ	Ab
A	0.17	6.6	4.4	1.7	6.3	4.0	1.1	
B	.24	6.5	4.3	1.6	6.1	4.1	1.1	
C	.28	6.6	4.4	1.8	6.3	4.3	1.2	
D	.32	6.5	4.1	1.9	
E	.40	6.6	4.4	1.8	6.2	4.6	1.1	
F	.50	6.6	4.4	1.7	6.3	4.8	1.2	
G	.62	6.1	4.1	1.8	6.1	5.1	1.3	
H	.93	6.6	4.3	1.5	5.7	1.1	
I	1.63	6.6	4.3	1.5	6.1	3.2	1.3	
J	1.82	6.6	4.3	1.5	6.1	1.2	
K	2.18	6.6	4.4	1.5	6.0	3.1	1.1	
L	2.84	6.7	4.3	1.5	6.1	3.5	1.1	

^a These designations correspond to those of Fig. 5.

^b Weight ratio of total BSA-S-R₁ to total anti-R. ^c See Fig. 5G and L for boundary designations.

ascending pattern and the trailing one in the descending, have the mobilities characteristic of free Ag and free Ab, respectively. Let us for the moment assume that the areas under these peaks are proportional to the equilibrium concentrations of the free Ag and free Ab species, respectively, in the original solution, and determine the consequences of such an assumption.²⁰ These area data are summarized in the first six columns of Table III.

(20) A small correction was first made to these areas. As can be seen from the electrophoresis pattern of BSA-S-R₁ itself (Fig. 3b), small amounts of a faster moving and a slower moving component

Before proceeding to a more quantitative use of these data, we will first show that they are consistent with the Ag and Ab valences in this system. From these estimates of the free Ag and free Ab concentrations ($[Ag]$ and $[Ab]$), and from the known total Ag and total Ab concentrations (Ag_T and Ab_T , respectively) in a solution, the molar ratio, r , of Ag and Ab bound in all the aggregates of that solution may be calculated readily, taking the molecular weights of Ag and Ab as 70,000 and 160,000, respectively. We should expect that as this system changes from large Ab excess to large Ag excess, covering the range from complete reaction of Ag sites to complete reaction of Ab sites, r should increase from 1 to 2. In Fig. 6, $1/r$ is

appear as shoulders on the main peak. If we consider the free Ag peak in the ascending patterns of BSA-S-R₁:anti-R mixtures (Fig. 5), the slower shoulder is in evidence, but the leading shoulder has disappeared. Now, the arsenic content of BSA-S-R₁ was found quite consistently to be 5 to 8% too high for one-to-one reaction at the sulfhydryl group of BSA-SH. If this is not due to a systematic analytical error, it suggests that a small amount of the BSA-S-R₁ had more than one R group substituted per molecule, perhaps on amino functions. At *p*H 8.7, the extra R group should increase the negative electrophoretic mobility of the molecule, and we therefore conclude that the faster moving shoulder in Fig. 3b is due to a doubly R substituted BSA molecule, which because of its higher Ag valence, was preferentially bound by anti-R Ab and is therefore not evident in Fig. 5. Quantitatively, however, it makes no significant difference if we treat this faster shoulder as univalent Ag in our calculations. The small trailing shoulder is probably due to inert BSA which was a contaminant of the BSA-SH preparation, and lacking an SH group could not react with the *N*-(*p*-benzenearsonic acid) iodoacetamide. The area under this shoulder therefore was subtracted from the measured free Ag area and also from the total area in the pattern.

In addition, the usual electrophoretic area anomalies must be considered here. It has been shown,²¹ however, that in mixtures of BSA and γ -globulin at *p*H 8.7, $\Gamma/2$ 0.1, at total protein concentrations such as were used in this study, such anomalies are small, and they have therefore been neglected in our calculations.

(21) S. H. Armstrong, M. J. E. Budka and K. C. Morrison, *THIS JOURNAL*, **69**, 416 (1947).

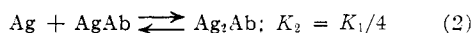
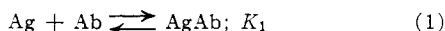
TABLE III
ELECTROPHORESIS DATA FOR BSA-S-R₁:ANTI-R MIXTURES
AT pH 8.7

(Ag/Ab) _w ^a	Total protein concn., mg./ml.	Total Ag, % ^b	Free Ag, % ^b	Total Ab, % ^b	Free Ab, % ^b	K _i × 10 ⁴ , l./mole Ag Data	l./mole Ab Data
0.17	19.4	14.5	3.5	84.3	59.5	1.8	2.1
.24	19.0	19.0	5.7	79.5	52.1	1.5	1.4
.28	18.8	21.7	7.6	76.5	47.1	1.3	1.3
.32	18.6	23.6	9.2	74.5	..	1.2	..
.41	18.3	27.7	11.5	70.1	42.9	1.2	0.7
.51	17.9	32.3	16.4	65.0	35.8	0.9	.7
.62	17.6	37.1	19.2	59.9	32.8	1.1	.6
.93	16.9	46.4	29.8	49.9	23.0	0.9	.6
1.63	16.1	59.0	45.1	36.3	15.2	.8	.9
1.82	16.0	61.3	49.7	33.7	14.5	.6	.4
2.18	15.8	64.9	52.4	29.8	11.7	.8	.5
2.84	15.5	69.8	61.9	24.6	9.9	.4	.4

^a Weight ratio of total BSA-S-R₁ to total anti-R. ^b Expressed as % of total protein in solution, corrected as described.²⁰ To get Ag_T, [Ag], Ab_T and [Ab], multiply columns 3, 4, 5 and 6, respectively, by column 2 and divide by 100.

plotted against both Ab_T/[Ab] and Ag_T/[Ag]. As Ab_T/[Ab] → 1.0, the region of infinite Ab excess is approached, and within the large experimental errors, r may indeed be extrapolated to the value 1.0. Similarly, as Ag_T/[Ag] → 1.0, 1/r → 0.5, r → 2.0. In the latter plot, the data in large Ag excess appear to show some upward curvature, which is probably due to some heterogeneity of Ab binding capacity, as is discussed below.

In view of the fair success obtained with these tests, a more quantitative use of these data may be attempted: the calculation of equilibrium constants for the Ag-Ab reactions. For this purpose the following model is utilized. We assume that all Ag-Ab bonds in the system are intrinsically equivalent and take the Ag as univalent, the Ab as bivalent.¹⁹ The reactions involved are then



K₁ and K₂ are the equilibrium constants characterizing the respective reactions. An intrinsic equilibrium constant, $K_i = K_1/2 = 2K_2$ may also be considered which is independent of the statistical factors affecting K₁ and K₂ due to the bivalence of the Ab. For each solution two independent values of K_i may be obtained from the molar concentrations of total Ag, [Ag_T], and total Ab, [Ab_T], together with either the molar concentration of free Ag, [Ag]_m, or free Ab, [Ab]_m. In the Goldberg notation⁷

$$[Ag]_m = [Ag_T] (1 - p)^f \quad (3)$$

$$[Ab]_m = [Ab_T] (1 - p[Ag_T]/2[Ab_T])^2 \quad (4)$$

$$K_i = p / \{ 2[Ab_T] (1 - p) (1 - f_p [Ag_T]/2[Ab_T]) \} \quad (5)$$

In these equations p is the fraction of antigen sites in the system which have reacted, and f is the valence of Ag, equal to 1 in this case. Using the data from the first six columns of Table III, and equations 3 and 5, we obtain the values of K_i in column 7 of that table; with equations 4 and 5, the K_i values given in column 8.

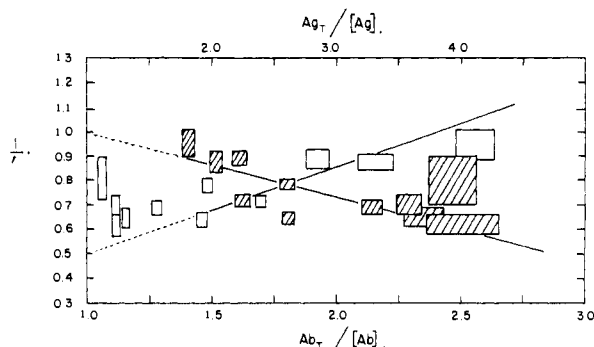


Fig. 6.—Plots of 1/r molar ratio of Ab and Ag bound in all aggregates of a particular solution, against Ab_T/[Ab] (filled rectangles) and Ag_T/[Ag] (open rectangles).

To assess the significance of these numbers, we must first consider to what extent the electrophoresis patterns are affected by re-equilibration reactions. That is, the system containing Ag, Ab and aggregates is originally in equilibrium, but as the components are partially separated by electrophoresis, reactions occur to return the system to equilibrium. If these reactions occur sufficiently rapidly, they will affect the apparent area distributions in the schlieren patterns. In the accompanying paper,¹² an analysis of these effects is presented which shows that the equilibrium constants of Table III are too small, but probably only by a factor of 2 or 3, which is not much greater than the experimental error of the data.

An apparent trend to decreasing values of K_i with increasing Ag content may be noted in Table III. This does not appear to be an artifact produced by re-equilibration.¹² There are at least two possible explanations for this trend. The assumption may not be entirely adequate that a second Ag molecule attaches to Ab with the same intrinsic strength as the first Ag, due to steric or other factors. In other words, $K_2 < K_1/4$. In larger Ag excess, the over-all apparent K_i would therefore decrease. Another possibility is that the trend is a reflection of some heterogeneity in Ab binding capacity. In larger Ag excess, more of a relatively weakly binding Ag, if present, would be bound to Ag, with a consequent diminution in the average value of K_i. The fact that a fractionation of Ab activities was produced in these pooled antisera by precipitation with the hapten R₃'-resorcinol (see section on Properties of anti-R Ab Preparations) suggests that some residual Ab heterogeneity was still present in the purified Ab. Other studies^{19,22} have suggested that anti-hapten antibodies exhibit heterogeneity of binding capacity.

These possibilities can be incorporated readily into a more elaborate theoretical treatment of this system, but in view of the experimental uncertainties in our data and of the effects of re-equilibration on our analyses, it would not be justified to apply such a treatment at the present time. Furthermore, these effects must be of second order in view of the relatively small range of values of K_i. We may therefore calculate from these data an average value of K_i, whose significance is somewhat limited

(22) L. Pauling, D. Pressman and A. L. Grossberg, THIS JOURNAL, 66, 784 (1944).

in view of the above considerations but which is nevertheless meaningful, particularly when compared to similarly calculated K_i values obtained in other systems. From Table III, this average value of K_i is $1.0 \pm 0.5 \times 10^4$ l. mole⁻¹, corresponding to $\Delta F^0 = -5.0 \pm 0.3$ kcal. mole⁻¹ at 0° in barbital buffer, pH 8.7, $\Gamma/2$ 0.1.

Electrophoresis in Tris Buffers.—A series of experiments were performed in a variety of buffers at different pH values with BSA-S-R₁:anti-R solutions recovered from the electrophoresis studies in barbital buffer, pH 8.70, and then pooled. The total Ag and total Ab in such a pool was determined by performing an electrophoresis experiment on an aliquot in barbital buffer, pH 8.70, $\Gamma/2$ 0.1, analyzing for the free Ag area in the ascending electrophoresis pattern, and interpolating in the data relating the free Ag to total Ag given in Table III. Other aliquots were then set to dialyze in different $\Gamma/2$ 0.1 buffers. For the purposes of this paper, only those experiments performed in Tris-HCl buffers, containing no NaCl, will be mentioned here. From the free Ag areas, and equations 3 and 5, the values of K_i listed in Table IV were calculated. In the pH range 7.1–8.6 in Tris-HCl buffers, K_i is essentially constant at 0.5×10^4 compared to the value 0.9×10^4 in a solution of corresponding composition in barbital buffer (Table III, column 7). Since this calculation is derived from an examination of the same solution in both Tris-HCl and barbital buffers, this small difference may be significant and possibly reflects a non-specific buffer ion effect in this system. Effects due to buffer ions in a related hapten R:anti-R system have been reported.²

TABLE IV
ELECTROPHORESIS DATA AT DIFFERENT pH VALUES IN
 $\Gamma/2$ 0.1 BUFFERS

pH	Buffer	(Ag/Ab) _w ^a	$K_i \times 10^4$
8.60	Tris-HCl	0.89	0.7
8.26	Tris-HCl	.83	.5
7.60	Tris-HCl	.83	.5
7.18	Tris-HCl	.83	.5
6.30	Acetate	.87	.3

^a Weight ratio of total BSA-S-R₁ to total anti-R.

Light Scattering Results.—The determination of an intrinsic equilibrium constant in this system by the independent method of light scattering provides an important check on the reliability of the values obtained by electrophoresis, since the former measurement does not disturb the state of equilibrium of the system. On the other hand the increase in the light scattering from BSA-S-R₁:anti-R solutions due to the presence of Ag-Ab aggregates is small and difficult to evaluate accurately. Since aggregation in this system is appreciable only in concentrated protein solutions, effects on light scattering due to non-ideality of the solutions must be minimized and accounted for. To minimize these effects, Tris-NaCl buffer, pH 7.0, $\Gamma/2$ 0.5, was used for the experiments, the pH chosen to be close to the isoelectric point of Ab, and the high ionic strength, to reduce intermolecular electrostatic potentials.²³ To account for the relatively small non-ideal ef-

fects remaining in this buffer, control experiments were necessary. It might ordinarily have been considered adequate to take the light scattered from the hypothetical *unaggregated* Ag-Ab solution as equal to that of a solution of BSA-SH and anti-R Ab of the same composition. However, because the two solutions, the Ag-Ab and the BSA-SH:Ab, might not have precisely the same composition and would have to be separately centrifuged and examined, it was felt that an additional control experiment was required to achieve the greatest precision. Accordingly, we also examined by light scattering two aliquots of the same centrifuged Ag-Ab solution, one with and one without 0.1 *M* Na *p*-arsanilate. In the latter solution, the molar excess of this hapten over the BSA-S-R₁ Ag was 1000-fold at the highest Ag concentration examined, and the BSA-S-R₁:Ab bonds were essentially completely replaced by arsanilate:Ab bonds. From the point of view of light scattering, therefore, the solution of Ag and Ab in 0.1 *M* arsanilate served as the unaggregated control for the same solution without arsanilate, except for any non-specific thermodynamic or refractive index effects caused by the presence of arsanilate. These latter effects were independently determined by the difference in light scattering of the unaggregated BSA-SH:Ab mixture with and without arsanilate.

As a preliminary to these experiments, to test our light scattering technique and to determine what effects were produced by the presence of arsanilate, we examined solutions of BSA-SH itself in Tris-NaCl buffer, pH 7.0, $\Gamma/2$ 0.5, with and without 0.1 *M* Na *p*-arsanilate (Fig. 7). The same K value is used in both sets of data to evaluate Kc/R_{90} , although dn/dc was not determined in the presence of arsanilate. The intercept of the line through the data without arsanilate corresponds to a molecular weight of 76000 ± 2000 ; with arsanilate, the entire line is displaced slightly upward. This molecular weight is somewhat higher than the accepted value of about 69000, but this may be due to a small amount of albumin aggregate in the solution.²⁴

The results obtained in the same Tris-NaCl buffer with a mixture of BSA-S-R₁ and anti-R Ab at a weight ratio of 0.80 without arsanilate (solution L1) and with 0.1 *M* arsanilate (L2); and with a mixture of BSA-SH and anti-R Ab at a weight ratio of 0.81 without arsanilate (L3) and with 0.1 *M* arsanilate (L4); and dilutions of these solutions are shown in Fig. 8. A single experiment with the BSA-S-R₁:Ab solution in 0.05 *M* arsanilate, the Kc/R_{90} value for which is indistinguishable from that in 0.1 *M* arsanilate, demonstrates that the Ag-Ab bonds are essentially all converted to arsanilate-Ab bonds in 0.1 *M* arsanilate. This is further indicated by the essentially identical value of the intercept obtained with solutions L2 and L4. From the intercept obtained with solution L3, the weight average molecular weight, $M_w^0 = 117,000$, is obtained for the BSA-SH:Ab mixture and hence for the hypothetical unaggregated BSA-S-R₁:Ab mixture in the absence of arsanilate. If we take the molecular weight of BSA-S-R₁ as 76,000, the value we obtained for BSA-SH, then M_w^0 for the mixture

(23) P. Doty and J. T. Edsall, in "Advances in Protein Chem.," Vol. VI, Academic Press, Inc., New York, N. Y., 1951, p. 69.

(24) S. N. Timasheff, H. M. Dintzis, J. G. Kirkwood and B. D. Coleman, *Proc. Nat. Acad. Sci. U. S. A.*, **41**, 710 (1955).

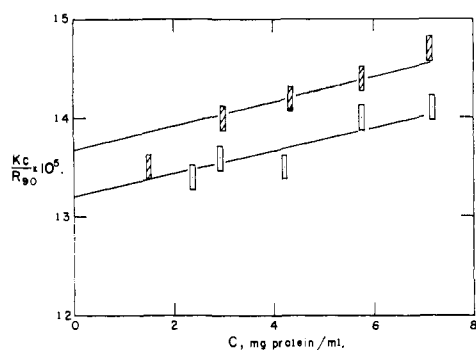


Fig. 7.—Light scattering data for BSA-SH in Tris-NaCl buffer, pH 7.0, $\Gamma/2$ 0.5, with 0.10 M Na arsenilate (shaded rectangles) and without (open rectangles).

is consistent with a molecular weight of 150,000 for Ab.²⁵ Not enough Ab was available for separate light scattering study.

From a comparison of the parallel data for solutions L3 and L4, a line may be drawn (dotted line L', Fig. 8) parallel to the data for solution L2, which represents the hypothetical light scattering results that would be obtained for the particular BSA-S-R₁:Ab solution if it were unaggregated and contained no arsenilate. Therefore, at any concentration c , the difference between Kc/R_{90} for curves L1 and L' is equal to $1/M_w - 1/M_w^0$, where M_w is the weight average molecular weight in the Ag-Ab solution.

To evaluate an intrinsic equilibrium constant from these data, we make use of the same model system employed in connection with the electrophoresis data of Table III. In the Goldberg notation,¹ the following relation should apply in this system

$$(M_w - M_w^0)(C_A + C_G) = C_G M_G p^2 r + 2M_A C_G p \quad (6)$$

In this equation C_A and M_A are the concentrations in grams/liter and the molecular weight of Ab (150,000), respectively, and C_G and M_G (76,000) the corresponding quantities for the Ag; r is $C_G M_A / 2C_A M_G$, and p has been defined earlier. A knowledge of M and M_w^0 thus enables us to calculate p , and K_i by equation 5. The best single value of K_i which fits the data is $0.2 \pm 0.1 \times 10^4$ (Fig. 9) and applies in the Tris-NaCl buffer, pH 7.0, $\Gamma/2$ 0.5, at 25°.

Comparison of Equilibrium Constants Determined by Electrophoresis and Light Scattering.—The average value of K_i determined by electrophoresis, $1.0 \pm 0.5 \times 10^4$, is reasonably close to the value $0.2 \pm 0.1 \times 10^4$ obtained by light scattering. Too close a comparison of these results, however, is not strictly justified, since in order to achieve the most satisfactory conditions for each technique, buffers of different pH and ionic strength were employed. In addition, we have observed that the value of K_i from electrophoresis varies with the Ag-Ab ratio in the solution. On the other hand, we showed in a previous section that electrophoresis experiments

(25) The molecular weights of Ag and Ab obtained in these light scattering experiments are somewhat different from these we have used in the calculations of the electrophoretic results. In order to treat the light scattering data in a consistent manner, however, the light scattering values are used in the calculation of light scattering equilibrium constants.

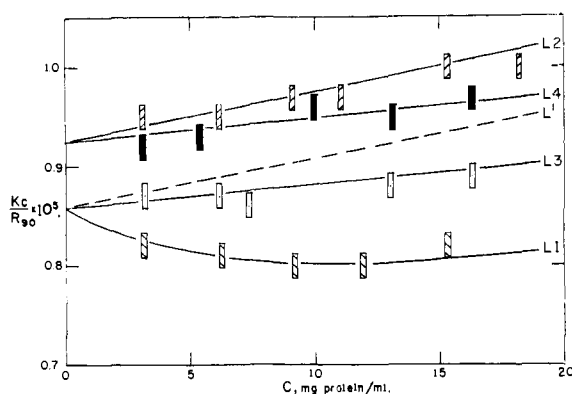


Fig. 8.—Light scattering data for BSA-S-R₁:anti-R and BSA-SH:anti-R mixtures with designations referred to in text.

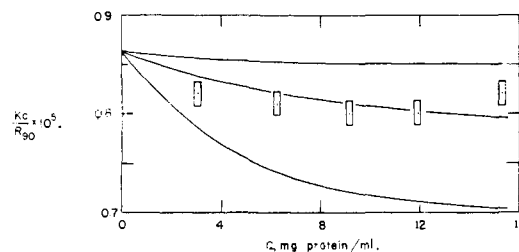


Fig. 9.—The determination of the value of K_1 which best fits the light scattering data for solution L1. The three theoretical curves correspond to $K_1 = 0.1, 0.2$ and 0.4×10^4 from top to bottom, respectively, and the rectangles represent the experimental data.

in barbital and Tris buffers at the same ionic strength led to similar values of K_i , and the results of an earlier light scattering investigation of a related system² also indicate that the buffer effects to be expected in this case are relatively small. A particularly significant comparison may be made between the value $K_i = 0.5 \pm 0.2 \times 10^4$ obtained by electrophoresis of a solution containing 43.8% Ag in Tris-HCl buffer, pH 7.2, $\Gamma/2$ 0.1 at 0°, and the value $0.2 \pm 0.1 \times 10^4$ from light scattering of a solution containing 44.5% Ag in Tris-NaCl buffer, pH 7.0, $\Gamma/2$ 0.5 at 25°. As was discussed earlier, the electrophoresis values are, if anything, too small if re-equilibration effects play a significant role. On the other hand, the light scattering values are true equilibrium values. It follows from the similarity of the two results that the electrophoretic value must be affected only relatively slightly by re-equilibration, within present experimental error.

Comparison with Other R:anti-R System.—In an earlier paper of this series,¹¹ thermodynamic data were obtained for a system containing as Ag benzene-arsonic acid-azo-bovine serum albumin (R-BSA) and anti-R Ab. The RBSA contained an average of 13 R groups per BSA molecule. An interesting comparison therefore may be made between the thermodynamic properties associated with the reaction of a multivalent Ag and a univalent Ag with the same Ab. To be more certain of this comparison, in view of the fact that different Ab pools were involved, we first studied mixtures of RBSA with anti-R IV. The RBSA was the same preparation described previously,¹¹ which had been

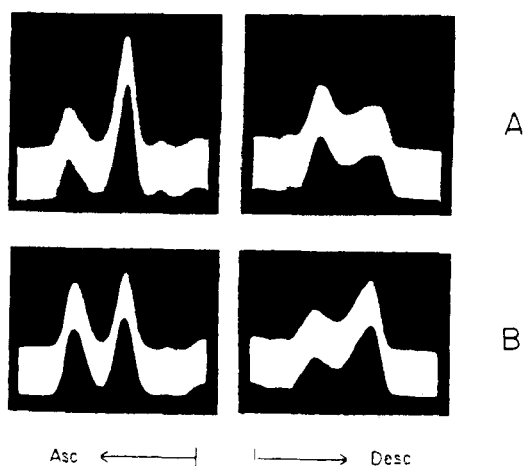


Fig. 10.—Electrophoresis patterns of two mixtures of RBSA and anti-R IV in barbital-NaCl buffer, pH 8.5, $\Gamma/2$ 0.3, after 9000 sec. at 4.6 v./cm. Weight ratios of Ag to Ab were (A) 1.03 and (B) 1.86. The arrows indicate the starting positions and directions of migration.

lyophilized and stored at 4° for 2 years. Two mixtures of RBSA and anti-R IV were prepared by weight from dialyzed and refractometrically analyzed solutions of the two proteins and were examined electrophoretically (Fig. 10) in the barbital-NaCl buffer, pH 8.5, $\Gamma/2$ 0.3, used earlier.¹¹ From the free Ag area in the ascending limb, corrected for electrophoretic anomalies, and from the total Ag in the solution, values of K_i (Table V) were calculated with the aid of the Goldberg theory, from equations 3 and 5, taking $f = 13$. The results are very similar to those obtained with the other Ab pools.^{11,26}

Ab Pool	(Ag/Ab) _w	Total Ag, %	Free Ag, %	$K_i \times 10^3$
I ¹²	1.07	51.8	30.2	1.2 ^a
	1.94	66.0	49.2	0.9 ^a
II ¹²	0.98	49.6	22.8	3.8 ^a
	1.91	65.6	45.0	2.2 ^a
IV	1.03	50.7	26.4	2.1
	1.86	65.0	44.6	2.0

^a These values are obtained from K_2 values in ref. 12 by dividing by 13/2. Only two of the eight values obtained with pool I are given here for purposes of comparison.

The average electrophoretic values of K_i for the RBSA:anti-R system, $0.2 \pm 0.1 \times 10^4$, is quite similar to that for the BSA-S-R₁:anti-R system, $1.0 \pm 0.5 \times 10^4$. Any significant difference might be attributable in part to quantitatively different re-equilibration effects occurring during the electrophoresis of these two systems or to the larger content of NaCl in the buffer used with the former system. Since RBSA is a multivalent Ag, capable of forming large aggregates with anti-R Ab, whereas BSA-S-R₁ is only univalent, the similarity of these

(26) The close correspondence of these equilibrium constants obtained with different Ab pools is an important demonstration of their significance as well as their reproducibility. The three Ab pools were entirely independent, made with different injecting antigens in different laboratories. Furthermore, whereas anti-R II and IV were purified by similar procedures, anti-R I was not first isolated but was converted directly into soluble Ag-Ab complexes.

results demonstrates that it is a fairly good approximation to consider the intrinsic Ag-Ab bond strength as independent of the size of the aggregate in which the bond occurs, at least up to the aggregate size found among the soluble RBSA:anti-R complexes. (Whether this approximation is still satisfactory for aggregate sizes found in Ag-Ab precipitates is another question, not answered by our results). The assumption of equal intrinsic bond strengths is central to the Goldberg theory of Ag-Ab reactions,⁷ and our previous use of this theory in the determination of the thermodynamics of protein Ag-Ab reactions⁸⁻¹¹ is therefore further validated by the present results.

Thermodynamic data obtained with several different R:anti-R systems are collected in Table VI. It is striking that $-\Delta F^0$ is the same in the systems containing small molecule R haptens, whether univalent or bivalent, but is considerably larger than $-\Delta F^0$ in those systems with R groups attached to the relatively large BSA molecule, whether univalent or multivalent. The data are as yet too few and inaccurate to decide whether the difference is largely enthalpic or entropic in origin. A number of factors might be suggested to account for this difference. One is that there is a free energy contribution of about 3 kcal./mole due to the electrostatic repulsion between the protein Ag and anti-R Ab molecules.²⁷ However, if this were the case, about a thousand-fold increase in K_i should occur in the BSA-S-R₁:anti-R system between pH 8.7 and pH 6.0. At the latter pH , the isoelectric point of the Ab, the electrostatic free energy contribution should be essentially zero. On the contrary, electrophoresis experiments in Tris-HCl buffers at different pH values (Table IV) as well as other experiments not reported in this paper show that no significant increase in K_i occurs in this pH range. Another possible explanation is that steric hindrance of the protein Ag and anti-R Ab molecules results in a weaker bond than in the hapten-Ab case. However, in the bivalent hapten:anti-R system studied by Epstein, Doty and Boyd,¹⁴ two Ab molecules attached to the same hapten molecule must encounter a degree of steric hindrance not much less than exists in the BSA-S-R₁:anti-R aggregates, yet this is not reflected in $-\Delta F^0$.

TABLE VI
THERMODYNAMICS OF BENZENEARSONIC ACID-ANTIBODY SYSTEMS

Antigen	ΔF_{298}^0 , kcal./mole	ΔH^0 , kcal./mole	ΔS_{298}^0 , cal./mole
R hapten (univalent) ¹⁹	-7.7
Terephthalanilide diarsonic acid hapten (bivalent) ¹⁴	-7.7	1 ± 2	23 ± 7
RBSA (13 valences) ¹¹	-4.5	0 ± 2	17 ± 7
BSA-S-R ₁ (univalent)	-5.0

We suggest that the factor which may be responsible for a large part of this difference is the larger loss of translational entropy accompanying the combination of the high molecular weight protein Ag than of the low molecular weight hapten with Ab. A crude estimate of the magnitude of this effect may be made using the Sackur-Tetrode equa-

(27) F. Karush, THIS JOURNAL, **78**, 5519 (1956).

tion, which however applies strictly only in the gas phase. Neglecting all internal degrees of freedom of the molecules, the difference between ΔS^0 for the protein Ag-Ab reaction and for the hapten-Ab reaction is written as²⁸

$$\Delta(\Delta S^0) = (3R/2) \ln (M_{AgAb}M_{Hp}/M_{HpAb}M_{Ag}) \quad (7)$$

where M_{Ag} , M_{AgAb} , M_{Hp} , M_{HpAb} are the molecular weights of the protein Ag, the Ag-Ab aggregate, the hapten, and the hapten-Ab aggregate, respectively. In the case of BSA-S-R₁, $M_{Ag} = 70,000$, and for the bivalent hapten $M_{Hp} = 564$. This gives $\Delta(\Delta S^0)$ of -14 e.u.; *i.e.*, ΔS^0 for the protein Ag-Ab reaction would be 14 e.u. less positive, and ΔF^0 4 kcal./mole

(28) *Cf.*, S. Glasstone, "Textbook of Physical Chemistry," 2nd Ed., D. Van Nostrand Co., New York, N. Y., 1946, p. 874.

more positive, than for the hapten-Ab reaction. The effect is therefore of the right order of magnitude. It should be pointed out that the existence of this effect is due to the particular choice of standard states, in calculations of ΔS^0 and ΔF^0 , as solutions containing 1 mole/l. of each of the components of the reaction.

Acknowledgments.—We are greatly indebted to Dr. Samuel I. Epstein for having performed preliminary light scattering measurements with the BSA-S-R₁:anti R system and for his advice and guidance with light scattering techniques. We are also grateful to Drs. Alfred M. Holtzer and E. P. Geiduschek for their many helpful suggestions.

NEW HAVEN, CONNECTICUT

[CONTRIBUTION NO. 1514 FROM THE STERLING CHEMISTRY LABORATORY, YALE UNIVERSITY]

Physical Chemical Studies of Soluble Antigen-Antibody Complexes. XI. An Analysis of the Resolution by Electrophoresis and Ultracentrifugation of a Univalent Antigen-Bivalent Antibody System¹

BY S. J. SINGER, FRANK A. PEPE AND DAVID ILTEN

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An analysis is presented, with the aid of the Gilbert-Jenkins theory, of the re-equilibration effects attending the resolution by electrophoresis and ultracentrifugation of a univalent antigen (Ag)-bivalent antibody (Ab) system. The calculations show that apparent equilibrium constants calculated from the free Ag area in the ascending electrophoresis patterns and the free Ab area in the descending are smaller than the true values, but by factors not much greater than the experimental error. An independent criterion is evolved for determining the magnitude of the reequilibration effects by a comparison of the free Ag areas in the electrophoresis and ultracentrifuge patterns, which should be widely different if the effects are important.

Introduction

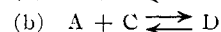
In the accompanying paper² an extended physical chemical study is presented of the interaction of a univalent protein antigen (Ag), BSA-S-R₁, with bivalent anti-R antibodies (Ab). This Ag-Ab system is relatively simple in that only two aggregates, AgAb and Ag₂Ab, may form. Estimates of equilibrium constants for the reactions forming these aggregates are obtained from light scattering experiments, which do not disturb the state of equilibrium of the system, and also from an analysis of the schlieren patterns of electrophoresis experiments over a wide range of ratios of BSA-S-R₁ and anti-R Ab. In the electrophoresis experiments, however, partial separation of the components results in a continual disruption of the state of equilibrium, and reactions may be expected to occur throughout the experiment to return the system to that state. These re-equilibration effects may therefore considerably influence the distribution of species in the schlieren patterns. The major purpose of this paper is to assess the significance of the apparent equilibrium constants which we have obtained from the electrophoresis experiments on the BSA-S-R₁:anti-R system, by investigating these re-equilibration effects with the aid of the theory recently developed by Gilbert and Jenkins.³ Furthermore, the results of this investigation are useful in the interpretation of similar electrophoresis experi-

ments with more complicated protein Ag-Ab systems.⁴⁻⁷

The Gilbert-Jenkins theory considers a system of three species in equilibrium, represented by the equation



Now the BSA-S-R₁:anti-R system is more complicated than this: it may be denoted by the simultaneous equilibria



where A, B, C and D represent the Ag, Ab, AgAb and Ag₂Ab species, respectively. Our justification for using the theory in this case is that it is the most nearly adequate treatment available and should provide at least reliable estimates of the effects produced by reequilibration reactions, particularly since under most conditions in the BSA-S-R₁:anti-R system, the molar concentration of Ag₂Ab turns out to be less than that of AgAb. Furthermore, the mobilities and sedimentation constants of the two species are fairly similar,^{2,5,8} and since the species are therefore not well resolved from one another, they may effectively be treated as one, to a first approximation. We shall therefore ignore the effects of reaction 2b in the calculations, and consider them qualitatively subsequently.

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

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

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