under those experimental conditions where the fraction cleavage does not exceed 33%. The validity of this mechanism, as a partial explanation of the facts, has not yet been determined.

Detailed Chemistry.—The kinetic analysis presented above does not permit a complete determination of the mechanism for the cleavage reaction. However, equations 4a-4e suggest that the cleavage results from the oxidation of the carbinol to a real or potential oxygen cation, which then undergoes cleavage. The kinetic study must be supplemented by stereochemical and other investigations before a detailed mechanism can be presented.

Isotope Effects.—Although the set of reactions in equations 4a-4e explains the kinetics and the cleavage yields satisfactorily, and accounts for the effect of deuterium substitution on the reaction rate, it nevertheless is true that the isotope effect is somewhat larger than usual.[19] Deuterium substitution decreases the rate of the oxidation of isopropyl alcohol by a factor of 8 at 0° in 86.5% acetic acid[4e] and by a factor of 6.9 in water[20] at 25°. Large and unusual isotope effects have, however, occasionally been reported.[21] The usual k_H/k_D ratio is the result of the zero-point energy difference which arises when a C–H vibration, in the reacting molecule, is converted, in the transition state, to translational motion. Possibly in the present example the C–H bending vibrations have considerably lower frequencies in the transition state than in the carbinol; if so, the zero-point energy difference between the hydrogen and the deuterium compounds will be exceptionally large. Tunnelling also has been suggested[22] as a possible cause for large k_H/k_D ratios. But although these explanations may be correct, an alternative explanation is embodied in the schematic equations 4g and 4h below.

$$Cr^5 + H_2A \longrightarrow Cr_4 + HA\cdot \qquad (4g)$$

$$Cr^5 + HA\cdot \longrightarrow Cr^5 + A \qquad (4h)$$

Here HA· represents the free radical $C_6H_5\dot{C}(OH)C(CH_3)_3$ and A represents $C_6H_5COC(CH_3)_3$.

These equations, together with the set of reactions 4a-4e, lead by way of the usual steady-state assumption to a kinetic equation which predicts more chain branching for the hydrogen than for the deuterium compound, and hence an abnormally high k_H/k_D ratio. Although this mechanism is highly speculative, it appears worthwhile to point out that the high isotopic rate ratio here observed may be caused by chemistry rather than physics.

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Physical Chemical Studies of Soluble Antigen–Antibody Complexes. VII. Thermodynamics of the Reaction between Benzenearsonic Acid–Azo-bovine Serum Albumin and Rabbit Antibodies to Benzenearsonic Acid[1,2]

BY MELVIN C. BAKER,[3] DAN H. CAMPBELL, SAMUEL I. EPSTEIN AND S. J. SINGER

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Solutions of antigen (Ag)–antibody (Ab) complexes formed between benzenearsonic acid–azo-bovine serum albumin as Ag and antibodies to benzenearsonic acid, have been subjected to electrophoresis and ultracentrifugation. Two different Ab preparations exhibited some differences in behavior, but for one of these the following thermodynamic data have been obtained. For the reaction Ag + AgAb $\rightleftarrows$ $(Ag)_2Ab$, in veronal–NaCl buffer, *p*H 8.5, $\Gamma/2$ 0.3, at 0°: $K = (7 \pm 3) \times 10^3$ liters/mole, $\Delta F^0 = -4.8 \pm 0.2$ kcal./mole; $\Delta H^0 = 0 \pm 2$ kcal./mole; $\Delta S^0 = +18 \pm 8$ e.u. These data are very similar to those obtained with natural protein antigen–antibody systems and their significance is discussed. A preliminary study of the effect of *p*H on the Ag–Ab equilibria is described.

In previous papers of this series,[4] general methods were developed which permit, for the first time, thermodynamic data to be obtained for the reactions of natural protein antigens (Ag) and their antibodies (Ab). The systems containing rabbit antibodies to bovine serum albumin[4c] (BSA) and ovalbumin[4e] (OA) have been studied. We have now investigated a system containing an artificial antigen, benzenearsonic-acid–*p*-azo-bovine serum albumin (RBSA) and antibodies to benzenearsonic acid (anti-R). This investigation has several purposes. Current concepts of the nature of the antigen–antibody reaction have largely been derived from studies with artificial antigens (usually pre-

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(2) From the thesis submitted by Melvin C. Baker in partial fulfillment of the requirements for the Ph.D. degree at Yale University, June, 1955.

(3) Du Pont Predoctoral Fellow, 1954–1955.

(4) (a) S. J. Singer and D. H. Campbell, THIS JOURNAL, **74**, 1794 (1952); (b) **75**, 5577 (1953); (c) **77**, 3499 (1955); (d) **77**, 3504 (1955); (e) **77**, 4851 (1955); (f) S. J. Singer, L. Eggman and D. H. Campbell, *ibid.*, **77**, 4855 (1955).

pared by coupling simple chemical groups (haptens) to proteins).[5] The relationship between artificial antigen and natural antigen systems is ill-defined, however, and comparative thermodynamic studies may provide a more quantitative basis for comparison. Furthermore, thermodynamic data already have been obtained by entirely different methods for the reactions of anti-R antibodies with small molecules containing the benzene arsonic acid (R) hapten. The effect upon the strength of the same hapten–Ab bond, of attaching the hapten to a large protein molecule, may be studied.

The methods for obtaining thermodynamic data for Ag–Ab reactions have been described in detail.[4b,c,e] Essentially, one determines by electrophoresis the amount of free Ag in equilibrium in a solution containing known total amounts of Ag and Ab, and with the aid of that part of the Goldberg theory[6] which deals with homogeneous equilibria, one evaluates equilibrium constants and corresponding free energy changes. That this approach is valid has been confirmed.[4d] In addition, by ultracentrifugal analyses at different temperatures, the standard heats of reaction, and hence the entropy changes, may be obtained.

Materials and Methods

Preparation of RBSA.—The antigen used in most of these studies was prepared by coupling diazotized *p*-arsanilic acid to BSA. 2.33 grams of once recrystallized *p*-arsanilic acid, 27 ml. 1 *N* HCl and 21.5 ml. of 0.5 *N* $NaNO_2$ were mixed and diluted to 150 ml. After cooling to 0°, this solution was added slowly with continuous stirring to an ice-cold solution of 15 g. of Armour's crystalline BSA in 300 ml. of phosphate buffer, *p*H 7.50, Γ/2 0.1. To maintain a constant *p*H during the addition, 0.2 *N* NaOH was added simultaneously. The final *p*H was 7.9. The mixture was kept at 4° overnight.

Freeing the RBSA from low-molecular weight arsenic-containing impurities was vital to the following studies, since the impurities might have inhibited the Ag–Ab reactions. Any attempt to precipitate RBSA from the reaction mixture, whether by prolonged dialysis against distilled water or against saturated $(NH_4)_2SO_4$, coprecipitated low molecular weight colored impurities (presumably produced by self-coupling of the arsanilic acid).[7] The impurities could be detected by ultracentrifuging a solution of the impure protein; the solution left behind the sedimenting RBSA boundary was colored by their presence. Adequate purification, as judged by ultracentrifugation and electrophoresis, was achieved by 11 days' dialysis against frequent changes of phosphate buffer, *p*H 7.50, Γ/2 0.1. This RBSA solution was then lyophilized and stored at 4° until needed.

The average number of R groups coupled per BSA molecule was 13.4 ± 0.4. This was determined by colorimetric arsenic analyses[8] (precision of ± 3%) combined with micro-Kjeldahl nitrogen analyses (precision of ±0.5%).

Although coupled proteins have been widely used, in only a few instances have their physical properties been extensively studied. In the course of our work, electrophoretic, ultracentrifugal and solubility measurements were performed with RBSA and other BSA derivatives which provide interesting information concerning the nature of these substances; these experiments will be reported elsewhere. For the purposes of this paper, it is sufficient to indicate that near neutral *p*H RBSA migrated as a single boundary both in the ultracentrifuge and electrophoresis; although in the latter an increased spreading of the boundary (compared to BSA) reflected the charge heterogeneity introduced by the coupling of the R groups.

Preparation of Antibody.—Two Ab preparations were used in this study. Anti-R-I was produced by injecting rabbits with highly coupled R-azo-bovine γ-globulin. High titer hyper-immune sera were pooled and titrated with RBSA. The titration curve (Fig. 1) is typical, except for the persistence of a small amount of precipitation in heavy Ag excess. However, since the anti-R Ab was subsequently precipitated in its equivalence zone (see next section), no significant interference from this source is to be expected. Anti-R-II was a sample of purified Ab. It was produced by immunization of rabbits with R-azo-horse serum. The purified Ab was over 60% precipitable with the trivalent hapten $(R')_3$-resorcinol $\left(R' = -N{=}N-\langle\bigcirc\rangle-N{=}N-\langle\bigcirc\rangle-AsO_3H_2\right)$. Further details of its preparation and characterization are given elsewhere.[9,10]

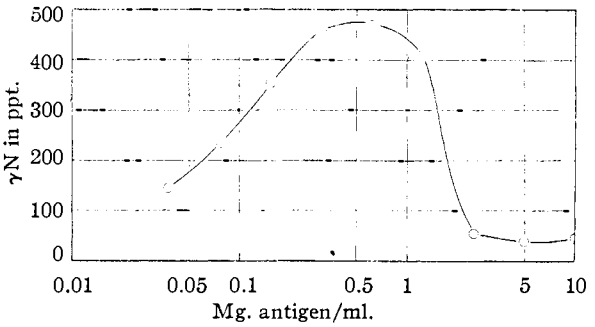

Fig. 1.—Precipitin curve for anti-R antibodies and RBSA antigen. For each point, 1.0 ml. of a serial dilution of RBSA was added to 1.0 ml. of a solution of a γ-globulin fraction containing the antibody.

Solutions of Soluble Complexes.—The γ-globulin fraction of the antisera containing anti-R-I was precipitated with 1/3 saturated $(NH_4)_2SO_4$, was redissolved in saline at *p*H 7.5, and anti-R was precipitated in the equivalence zone with RBSA. The precipitate, after the usual cold saline washings, contained 300 mg. of anti-R and 93 mg. of RBSA; it was suspended in 35 ml. of 2% RBSA solution, agitated at 4° for 24 hr., and the small insoluble residue was centrifuged off and discarded. The solution was shipped air express from Pasadena to New Haven. In order to remove some of the large excess of Ag required to redissolve the Ag–Ab precipitate,[4a] the solution was brought to 45% saturation with respect to $(NH_4)_2SO_4$, the precipitate was centrifuged and redissolved in phosphate buffer, *p*H 7.5, Γ/2 0.1. This process was repeated twice. The final solution was dialyzed thoroughly against the phosphate buffer, and labeled R-A-I. Since RBSA is colored, a convenient and accurate analysis for total Ag and total Ab in R-A-I was achieved by combining color analyses for Ag with measurements of refractive increment for total protein. The color analyses were performed over a range 330–400 mμ at three different *p*H values, 3.3, 7.5, 11.5, and were calibrated with similar measurements with RBSA itself. It was separately determined that normal γ-globulin does not affect the absorption properties of RBSA, and that the specific refractive increment of RBSA is, within experimental error, that of BSA. These measurements gave the value 0.85 ± 0.01 for the ratio of total Ag to total Ab in R-A-I. Seven solutions in greater RBSA excess were made up by weighing together aliquots of R-A-I and a standardized RBSA solution.[4c]

Anti-R-II was stored at 4° as a precipitate under 37% saturated $(NH_4)_2SO_4$, and aliquots were redissolved when needed in phosphate buffer, *p*H 7.5, Γ/2 0.1, were dialyzed several days to remove the $(NH_4)_2SO_4$, and were centrifuged to remove small amounts of insoluble matter. The concentration of the solution was determined by refractive increment measurements, and mixtures were prepared by weight with standardized RBSA solutions.

(5) K. Landsteiner, "The Specificity of Serological Reactions," 2nd Edition, Harvard University Press, Cambridge, Mass., 1947.

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

(7) E. W. Gellewitz, W. L. Riedeman and I. M. Klotz, *Arch. Biochem. Biophys.*, **53**, 411 (1954).

(8) H. J. Magnuson and E. B. Watson, *Ind. Eng. Chem., Anal. Ed.*, **16**, 339 (1940).

(9) S. I. Epstein, Ph.D. Thesis, Harvard University, 1954.

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

Electrophoretic Analyses.—Electrophoresis experiments were performed in a Perkin–Elmer model 38 Tiselius apparatus. Seven R-A-I solutions, and two solutions, R-A-II-1 and R-A-II-2 made from anti-R-II, were examined at a total protein concentration of about 16 mg./ml. in veronal–NaCl buffer (0.1 *M* in veronal–0.2 *M* in NaCl), *p*H 8.51, Γ/2 0.3, for 9000 sec. at a field strength of 4.6 volts/cm.

Ultracentrifugal Analyses.—A Spinco model E instrument was used at 59,780 r.p.m. for the ultracentrifuge experiments, which were performed in phosphate buffer, *p*H 7.5, Γ/2 0.1 at a total protein concentration of about 16 mg./ml. The area measurements were made as previously described.[4a,c]

For measurements of the heat of reaction, ultracentrifuge experiments were carried out at 38, 25 and 2°, on a number of solutions containing RBSA and anti-R-II, and careful comparative analyses of the areas under the free Ag peak were made, as in our earlier studies.[4c,e]

Auxiliary Techniques.—A Beckman Model B spectrophotometer was used for color analyses, and refractive increment measurements were made with a modified Brice Phoenix differential refractometer.

Experimental Results and Discussion

Effects of Re-equilibration during Electrophoresis and Ultracentrifugation.—The various species in these Ag–Ab solutions are initially in equilibrium, but as the boundaries resolve, the distribution of species readjusts to new equilibrium conditions. These effects have been analyzed[4a] and methods have been developed to test their significance in the anti-BSA[4a,c] and anti-OA[4e] systems. In the RBSA–anti-R system, however, it appears that re-equilibration is considerably more rapid than in the others. One indication of this is the discontinuity which exists at the boundary due to Ag–Ab complexes in the descending electrophoresis patterns of solutions containing anti-R-I (Fig. 2). We may interpret this effect as follows.[4a] In the descending limb, the free Ag migrates away from the complexes, leaving behind a solution whose over-all composition is in the zone of precipitation. The complexes therefore undergo disproportionation reactions, producing still larger complexes and free Ag. The pronounced light scattering of the larger complexes reduces the light intensity transmitted through that region of the cell, and the discontinuity in the pattern results. In addition, the free Ag produced during re-equilibration causes an elevation of the base-line behind the free Ag peak[4a]; the area of the free Ag peak therefore appears larger in the descending than in the ascending patterns. In the anti-BSA system under the same conditions, the descending patterns show no discontinuity, and the ascending and descending patterns are nearly mirror images, indicating that re-equilibration occurs more slowly. Anti-R-II, however, behaves differently than anti-R-I. Solutions containing anti-R-II re-equilibrate at rates intermediate to those of the anti-R-I and anti-BSA systems; they show no discontinuity in the descending patterns (Fig. 3), but the two patterns in an experiment are far from mirror images. This difference between the two anti-R preparations may be paralleled by a difference in their thermodynamic constants to be discussed.

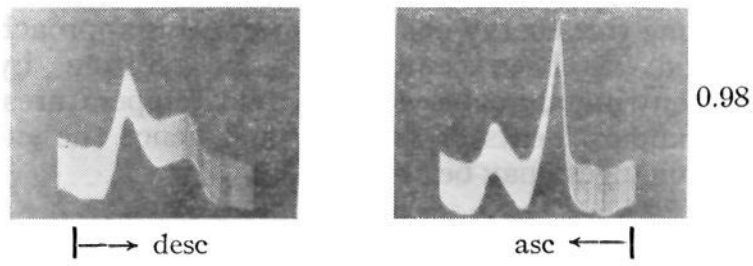

Fig. 3.—Electrophoresis diagrams of solution R-A-II-1 in veronal–NaCl buffer, *p*H 8.51, Γ/2 0.3.

Further evidence of relatively rapid re-equilibration in RBSA–anti-R solutions appears in the ultracentrifuge diagrams (Fig. 4). Only two peaks are evident in solutions containing RBSA and either anti-R-I or anti-R-II. The slower peak is clearly

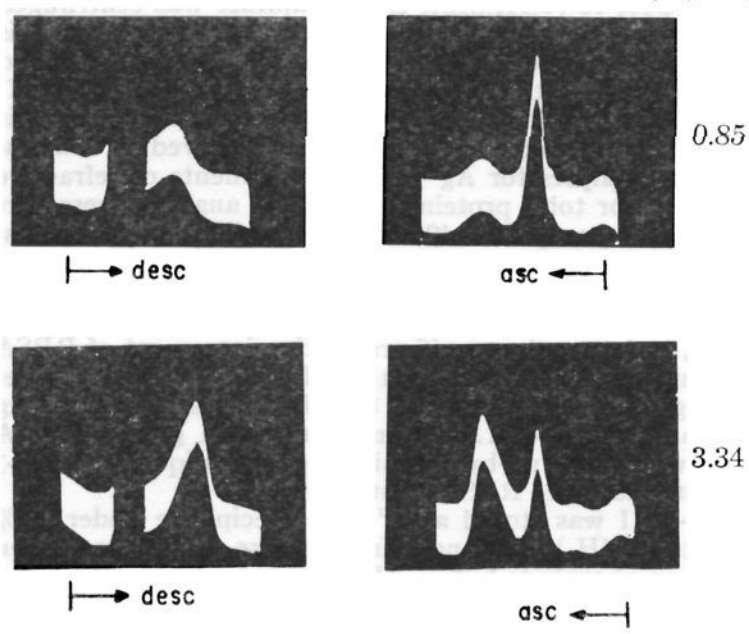

Fig. 2.—Electrophoresis diagrams of solution R-A-I (upper patterns) and R-A-I-6 (lower patterns) in veronal–NaCl buffer, *p*H 8.51, Γ/2 0.3. Starting positions in the ascending and descending patterns are indicated by the arrows.

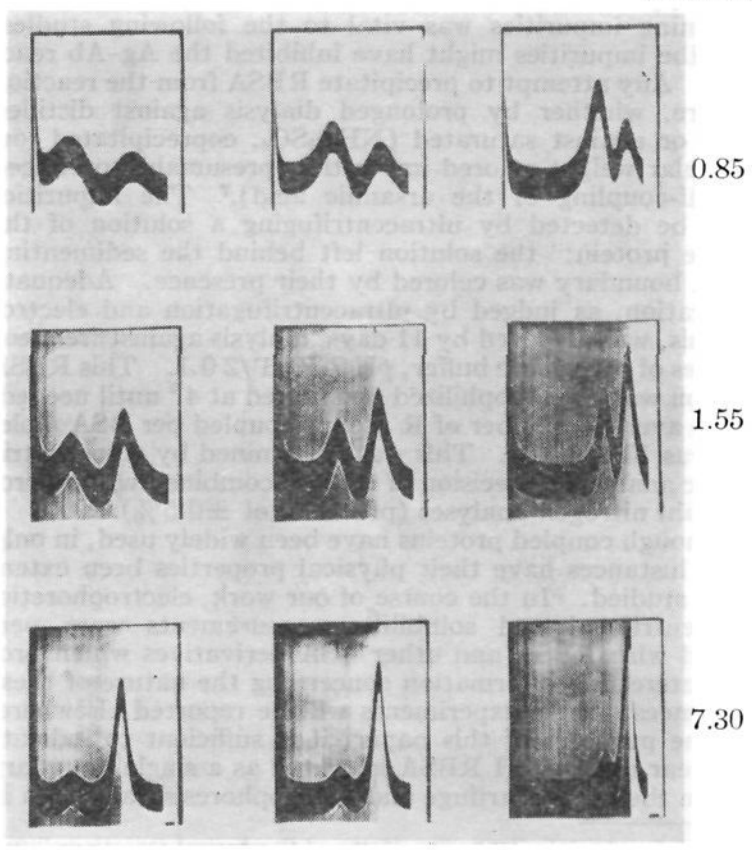

Fig. 4.—Ultracentrifuge diagrams of RBSA–anti-R-I solutions. Three patterns in each experiment are shown, sedimentation proceeding to the left. The top patterns are for solution R-A-I, the middle for R-A-I-3, and the lower for R-A-I-7.

due to free RBSA. Its sedimentation rate is that of RBSA, and its relative area corresponds with that of the free Ag in the ascending electrophoresis patterns[11] (Table I). The faster peak sediments faster than free Ab, and must be due to Ag–Ab complexes. In the anti-BSA and anti-OA systems,[4a,e] on the other hand, the complexes, due to their different masses, are resolved into a number of discrete peaks. It has been shown,[13] however, that if a system of components is in very rapidly adjusted equilibrium, only a single constituent peak may be observed, with a constituent sedimentation rate which is a weighted average of those of the components. The faster peak in RBSA–anti-R solutions is therefore considered to be a constituent peak due to Ag–Ab complexes which are rapidly re-equilibrating. Since the larger complexes predominate in low Ag excess, and smaller complexes in large Ag excess,[4a] we expect the constituent sedimentation constant to decrease with increasing Ag excess. This is indeed the case (Table I, column 3).

TABLE I

Solution	Sed. constants S^w_{20}. svedbergs RBSA[a]	Complex[b]	Total Ag, %	Free Ag, % Ultracent.	Electroph. App.[c]	Cor.[d]	K[e] × 10^{-3}
R-A-I	2.84	7.65	45.9	27	26.2	24.8	9.1
R-A-I-1	3.35	7.63	51.8	39	32.1	30.2	7.9
R-A-I-2	3.62	7.47	55.0	38	37.1	35.1	6.6
R-A-I-3	3.50	7.31	61.0	44	44.2	41.8	7.2
R-A-I-4	3.68	7.31	66.0	53	51.6	49.2	5.8
R-A-I-5	3.43	6.99	73.1	62	61.0	58.9	6.1
R-A-I-6	3.44	7.06	77.1	68	67.3	65.3	5.0
R-A-I-7	3.47	6.85	88.0	85	82.0	80.5	7.4
R-A-II-1	..	..	49.6	..	24.6	22.8	25
R-A-II-2	..	..	65.6	..	47.3	45.0	15

[a] S^w_{20} for RBSA is about 0.5 unit less than BSA under these conditions. [b] Constituent complex peak, see text. [c] Relative free Ag area in ascending patterns. [d] Corrected for electrophoretic area anomalies, see text. [e] For reaction Ag + AgAb ⇄ $(Ag)_2Ab$, in veronal–NaCl buffer, *p*H 8.5, 0°.

The Free Ag Concentration.—The apparent percentage free Ag in equilibrium in a solution is taken as the relative area under the leading peak in the ascending electrophoresis pattern[4a,c] (Table I, column 6). This area is somewhat too large, however, because of electrophoretic anomalies,[4c,14] approximate corrections for which may be made with the aid of the Dole theory[4c,15] (Table I, column 7). Any residual uncertainty in the free Ag area is within experimental error.

The Valence of Antibody.—From equilibrium dialysis studies of hapten–anti-R systems,[16] it has been shown that anti-R Ab is bivalent. The results of the present investigation are consistent with this conclusion. Knowing the amounts of free Ag, total Ag and total Ab, we may calculate $(Ag/Ab)_{B,N}$, the average number of moles of Ag bound per mole of Ab in all the complexes in a given solution. As the Ag excess increases, this number should approach the valence of Ab. From a plot of $1/(Ag/Ab)_{B,N}$ against $1/[Ag]$, (the reciprocal of the concentration of free Ag) extrapolation to $1/[Ag] = 0$ should give the reciprocal of the Ab valence as the intercept. This plot is similar to that used by Klotz[17] in studies of the binding of small ions to proteins. Data for the RBSA–anti-R, and for comparison, the anti-BSA systems are plotted in Fig. 5. Because of the larger slope (which is related to the smaller free energy of binding, to be discussed), and because of the experimental difficulty of obtaining accurate data in large Ag excess, the curve for the RBSA–anti-R system cannot be as unambiguously extrapolated as that for anti-BSA. Nevertheless, the bivalence of Ab is entirely consistent with the data.

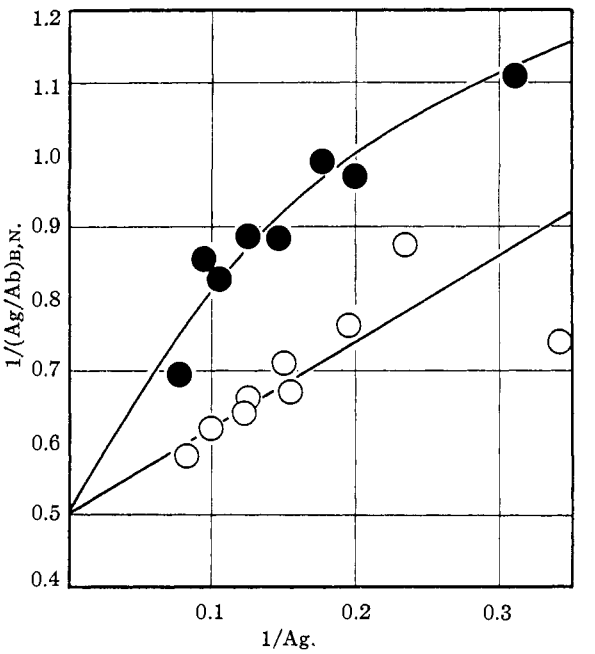

Fig. 5.—The binding of antigen by antibody for two systems: O, BSA-rabbit anti-BSA; ●, RBSA–rabbit anti-R.

Equilibrium Constants and Thermodynamic Data.—For the determination of equilibrium constants, K, the free Ag and total Ag concentrations are used in conjunction with part of the Goldberg theory,[6] as completely described elsewhere.[4b,c] K for the reaction Ag + AgAb ⇄ $(Ag)_2Ab$ may be calculated (Table I, column 8) taking the valence of Ab as 2, and of Ag as 13. (For Ag valence > 4, K is essentially independent of Ag valence.) For solutions containing anti-R-I, K does not vary with the degree of Ag excess, within experimental error, and its average value[18] is $(7 \pm 3) \times 10^3$

(11) The relative free Ag areas in the sedimentation patterns are consistently somewhat larger than those in the ascending electrophoresis patterns. This is due to the Johnston–Ogston anomaly.[12,4a,c]

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

(13) T. Svedberg and K. O. Pedersen, "The Ultracentrifuge," Oxford University Press, 1940, p. 28.

(14) In veronal–NaCl buffer, *p*H 8.5, Γ/2 0.3, the area corrections in anti-BSA solutions are within experimental error.[4c] They are somewhat larger in RBSA–anti-R solutions, however, because the mobility of RBSA is 30% larger than that of BSA at *p*H 8.5.

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

(16) H. Eisen and F. Karush, *ibid.*, **71**, 363 (1949).

(17) I. M. Klotz, in "The Proteins," Vol. 1, part B, edited by H. Neurath and K. Bailey, Academic Press, Inc., New York, N. Y., 1954, pp. 771–773.

(18) Our results do not preclude the possibility that a small amount of univalent Ab may be present in these solutions. Even if as much as 10% of the Ab is univalent, however, the change in K, calculated from the general equations of Goldberg,[6] is less than the experimental error.

liters/mole. Two experiments with solutions containing anti-R-II resulted in $K = (20 \pm 8) \times 10^3$ liters/mole. The standard free energy changes, ΔF^0, are -4.8 ± 0.2 and -5.2 ± 0.2 kcal./mole, respectively. The difference between the constants for the two Ab preparations is probably within experimental error, but it is interesting that a definite difference is observed in their rates of reaction with RBSA (see section on Effects of Re-equilibration). Possibly, differences in the immunizing antigens used to make the two Ab preparations are involved.

From the ultracentrifuge experiments performed at different temperatures, no significant change in the free Ag area was observed. We calculate,[4c] therefore, that $\Delta H^0 = 0 \pm 2$ kcal./mole. This leads to $\Delta S^0 = +18 \pm 8$ and $+19 \pm 8$ e.u. for anti-R-I and anti-R-II solutions, respectively.

These data are similar to those obtained by a light scattering investigation[10] of the reaction of a bivalent arsonic acid hapten, terephthalanilide-*p*,*p*'-diarsonic acid (Hp), with anti-R antibodies. For the corresponding reaction Hp + HpAb $\rightleftarrows$ $(Hp)_2Ab$, $\Delta F^0 = -7.5$ kcal./mole, $\Delta H^0 = -2.5$ kcal./mole, and $\Delta S^0 = +16$ e.u. The difference in ΔF^0 for the two systems is probably significant,[19] but before the data may be compared, two factors must be considered. First, due to the different valences of RBSA and Hp, a statistical factor must be introduced which makes the difference in ΔF^0 still larger by 1.0 kcal./mole. Second, quite different protein concentration ranges were examined in the two studies, and the quantitative importance of this factor is not known. At present, any interpretation of this difference in ΔF^0 would be premature. ΔS^0 has essentially the same positive value for the two systems, in spite of a large difference between the molecular weights of RBSA and Hp (70,000 and 564, respectively). This is consistent with the suggestion that a major factor contributing to ΔS^0 is the release of polarized water molecules from the reactive sites upon formation of the Ag–Ab bond,[4c] and that the size of the Ag molecule is only of secondary importance.

It is remarkable that the thermodynamic data for the anti-BSA,[4c] anti-OA[4e] and RBSA–anti-R systems are quite similar (ΔF^0 for the last is somewhat less negative than for the first two, which may be related to the different rates of reaction observed with these systems (see section on Effects of Re-equilibration)). This similarity suggests that the detailed mechanisms of many, if not all, Ag–Ab reactions are fundamentally similar.

The Effect of *p*H.—In the anti-BSA[4d] and anti-OA[4f] systems, a quantitative ultracentrifugal investigation has been made of the effect of acid *p*H on Ag–Ab equilibria. Strong evidence has thus been obtained that the protonation of a single carboxylate ion in each Ag–Ab bond is sufficient to disrupt the bond. A similar study of the RBSA–anti-R system would be of great interest, since it is likely that the single arsonate ion in each Ag–Ab bond is similarly involved. Unfortunately, the low solubility of RBSA in the *p*H region 3.6–5.0 makes such a study impossible. At lower *p*H than 3.6,[20] the ultracentrifuge diagrams indicate that the solution contains mainly free Ag and free Ab, and that dissociation of the Ag–Ab bonds is extensive. At *p*H 5.0, the diagrams are essentially the same as at *p*H 7.5. These results are qualitatively consistent with those obtained for the anti-BSA and anti-OA systems.

It is suspected that there is a positively charged group on the antibody site which is complementary to the arsonate ion. With the object of detecting such a group, ultracentrifuge studies were performed with the RBSA–anti-R system in the *p*H range 7 to 12. In order to avoid denaturation of antibody, an RBSA–anti-R-II solution in 0.2 *M* KCl at *p*H 7 was rapidly adjusted to the desired *p*H with 0.2 *M* KOH, and was then immediately injected into an ultracentrifuge cell with a plastic centerpiece. A quantitative analysis of these experiments, however, was made difficult by the fact that only two peaks, the free RBSA and the constituent complex peak, were observed at all *p*H values. No separate free Ab peak appeared. Two measures of the extent of dissociation were therefore investigated; (a) the relative sedimentation constant of the complex peak, and (b) the ratio of apparent areas of the complex and free Ag peaks. These proved to be quantitatively not satisfactory, however, the first because the sedimentation constants of RBSA and γ-globulin decrease markedly at *p*H values above 10.5 in 0.2 ionic strength solutions, and the second because, as a result of this change in sedimentation constants, the Johnston–Ogston area anomalies[12] cause considerable changes in the relative areas of the two peaks with change of *p*H. These effects were demonstrated by independent experiments with mixtures of RBSA and normal γ-globulin.[21]

A successful study of the effect of *p*H on the anti-R system requires, therefore, first, that an antigen be prepared which is sufficiently soluble over a wide *p*H range and, second, that the system be one in which the rates of re-equilibration be sufficiently slow that the complexes and free Ab may be resolved ultracentrifugally, and equilibrium constants determined from the areas in the diagrams.[4d,f] Investigations of suitable antigens are under way.

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(19) The preparation anti-R-II was used in both the RBSA–anti-R and the Hp–anti-R studies; the difference in ΔF^0 is therefore not due to different Ab preparations.

(20) Between *p*H 3.3 and 3.6, ultracentrifuge diagrams of RBSA itself demonstrate considerable aggregate formation which is not present at lower or higher *p*H.

(21) The following are representative data. In an RBSA-normal γ-globulin mixture containing 55% RBSA at a total protein concentration of 17.2 mg./ml., the sedimentation constants, S^{W}_{20}, at *p*H 7 were 4.1 *S* and 6.0 *S* for RBSA and γ-globulin, respectively, while at *p*H 11.5, these had changed to 2.8 *S* and 4.8 *S*, respectively. At *p*H 7, the ratio of apparent areas of the RBSA and γ-globulin peaks was 54%, while at *p*H 11.5, it was 74%.