fragile macromolecular structures has been kept to below 40%. Indeed, experimental uncertainties are such that probably only about half of the differences observed are due to distortions produced in macromolecules.

In addition to achieving a precision in the determination of average dimensions of asymmetric macromolecules nearly comparable with other methods, the electron microscope procedure provides much more detail of the distribution of lengths than heretofore available. Although this too may suffer some from residual distortional effects, it offers direct access to that type of information that is most difficult to obtain by other methods. It is therefore of interest to note that the comparisons made in this study have shown general agreement not only in average values but in breadths of distributions as well: the ichthyocol being quite narrow and the other two being close to the most probable distribution as predicted. With this check on the reliability of the distribution determination as well, the new electron microscope method appears ready to contribute to a number of problems where detailed knowledge of size-distribution of asymmetric macromolecules is desired.

It seems desirable to add a note concerning a quite different method of measuring average molecular weights of macromolecules with the electron microscope that is applicable, independent of shape and capable of much higher accuracy. This method which involves counting the number ratio of standard to unknown particles in a given volume has been adapted to electron microscopy by Williams and Backus[11] to determine the molecular weight of tomato bushy stunt virus (mol. wt. $\sim$ 10,000,000). This is potentially the most accurate method for the determination of molecular weights by electron microscopy, but there are technical difficulties in the application of the method to particles with molecular weights in the range 10^4 to 10^6, although such particles can be seen clearly by the methods used in the present study. These difficulties are mainly: (1) the production of droplets small enough to be entirely included in the field of the microscope at suitable magnifications, (2) availability of standard particles not too different in size from that of the unknowns and (3) adsorption or interaction between standard and unknown species.

(11) R. C. Williams and R. C. Backus, THIS JOURNAL, **71**, 4052 (1949).

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Physical-Chemical Studies of Soluble Antigen–Antibody Complexes. IX. The Influence of *p*H on the Association of a Divalent Hapten and Antibody[1,2]

BY SAMUEL I. EPSTEIN[3] AND S. J. SINGER

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A quantitative study has been made of the light scattering from mixtures of a divalent benzenearsonic acid hapten and anti-benzenearsonic acid (anti-R) antibody (Ab), over a wide range of *p*H. Analysis of the data at alkaline *p*H reveals that a single ionizable group with pK 9.9 $\pm$ 0.2 is present in each Ab site, which must be ionized for association to occur appreciably. Ultracentrifuge experiments with these mixtures are consistent with this interpretation. Acetylation of anti-R Ab, under conditions specific for the blocking of the ϵ-NH_3^+ group of lysine, inactivates it, while an excess of a specific antigen, benzenearsonic acid-azo-bovine γ-globulin (RBG), protects anti-R Ab against this inactivation. Taken together, these experiments demonstrate that a single ϵ-NH_3^+ group of lysine is critically present in each anti-R Ab site and therefore that a salt-linkage between this group and the negatively charged hapten is an essential feature of the specific binding. The light scattering data also suggest that at acid *p*H, anions, especially phosphate, effectively compete with benzenearsonic acid hapten for combination with the Ab sites and that this competition obscures any effect that the titration of the benzenearsonate ion might be expected to have on the association of hapten and Ab. The parallelism in the properties of this hapten-Ab system, and that containing the natural protein antigen bovine serum albumin and its specific Ab is discussed.

It has long been assumed that an important feature of the bonds formed in many antigen (Ag)–antibody (Ab) systems is the coulombic interaction of one or more pairs of oppositely charged groups located in the specific reactive regions of the two molecules. This assumption has derived partly from the fact that those haptens, such as benzenearsonic acid, benzenesulfonic acid, etc., which on being coupled to proteins, are most effective in eliciting antibody production, bear electric charges at physiological *p*H. The inference has been drawn that oppositely charged groups are present in the Ab sites directed against these haptens. Some support for this inference is provided by the experiments of Nisonoff and Pressman,[4] who found that the substitution on a hapten of an uncharged nitro group for a negatively-charged carboxyl group resulted in a marked reduction of the interaction of the hapten with antibodies directed to the *p*-(*p*-azophenylazo)-benzoate ion. While this demonstrates that the negative charge on the hapten is critical, it does not prove, however, that positively charged groups occur in the antibody site.[5] One of the ob-

(1) These studies were supported in part by grants from the National Microbiological Institute, United States Public Health Service, and from the Rockefeller Foundation. Parts of this paper were presented before the meeting of the American Chemical Society in Atlantic City, September, 1956.

(2) The previous paper in this series is F. A. Pepe and S. J. Singer, THIS JOURNAL, **78**, 4583 (1956).

(3) United States Public Health Service Postdoctoral Fellow of the National Microbiological Institute, 1954–1956. Dept. of Chemistry, Tufts University, Medford, Mass.

(4) A. Nisonoff and D. Pressman, THIS JOURNAL, **79**, 1616 (1957). See also D. Pressman, A. L. Grossberg, L. H. Pence and L. Pauling, *ibid.*, **68**, 250 (1946).

(5) It is possible to interpret this result without requiring a complementary charge in the Ab site. If the negatively charged hapten polarizes, or "binds," water molecules, then on formation of the hapten–Ab bond this water might be released, irrespective of the nature of the groups in the Ab site. This would result in a considerable entropy increase favoring the reaction. On the other hand, the uncharged hapten, binding less water to it, would react less favorably with Ab.

jects of the present study is to provide direct evidence concerning the presence of charged groups in the reactive sites of Ab directed to a charged hapten.

Another object of this investigation is connected with recent quantitative studies of the effect of acid pH on the equilibria in *protein* Ag–Ab systems.[6,7] These studies, involving the antigens bovine serum albumin and ovalbumin and their specific rabbit antibodies, have been interpreted to indicate that a single ionized carboxyl group is present in each Ag–Ab bond. The studies reported in this paper, utilizing a simpler system, provide a critical test of this interpretation.

We have performed light scattering and ultracentrifuge measurements of the effect of pH on mixtures of the divalent hapten (Hp), terephthalanilide-p,p'-diarsonic acid (T) and rabbit antibodies directed against benzenearsonic acid (anti-R Ab). Here the hapten is negatively charged near neutral pH. Because of the small molecular weight of T compared to anti-R Ab, the reaction of the two results in the formation of aggregates which are essentially dimers, trimers, etc., of Ab, from the point of view of light scattering and ultracentrifuge measurements. The light scattering behavior of this system at pH 8 previously has been determined.[8] We now find that the effect of alkaline pH in causing dissociation of Hp–Ab bonds is quantitatively accounted for by the hypothesis that there is a single titratable group, with a pK corresponding to the ϵ-ammonium group of a lysine residue, critically present in each Ab site. In the acid pH region, where the dissociation of Hp–Ab bonds might be expected to involve the titration of the benzenearsonate ions, anomalous behavior is observed which appears to be due in part to competitive buffer ion binding to Ab.

Materials and Methods

Hapten–Antibody Mixtures.—The anti-R Ab was taken from the pool designated "54" in an earlier study.[8] After isolation in July, 1954, this Ab was kept at 5° for two months in pH 8.0, μ 0.15 borate buffer; it was then suspended in 40% saturated $(NH_4)_2SO_4$. Most of the measurements at pH above 8.2 described here were made in May, 1955. Within probable experimental error, there was no change in the light scattering from a typical T–anti-R mixture, at pH 8, compared with that found with freshly-prepared material, given in ref. 8. In the ultracentrifuge, 88 (±3)% of the protein sedimented at the rate of γ-globulin; the remainder (see pattern in Fig. 4 below) sedimented somewhat more rapidly and is likely an Ab dimer. Since no ultracentrifuge pattern of the fresh material had been obtained, the origin of the faster-sedimenting component is uncertain. The measurements at pH below 8.2 were made in February and April, 1956. Again, at pH 8, the scattering from a T–anti-R mixture agreed well with earlier values. Moreover, there was no change in the ultracentrifuge pattern. Finally, storage did not change the precipitability of the anti-R with "R_3'-Resorcinol," a trivalent Hp which was used in the purification of the pool.[8]

The stored anti-R sediment was dissolved and dialyzed to prepare anti-R solutions in 0.15 M NaCl. The T used in this work was from the same lot as in the study at pH 8. The concentration of anti-R as well as T solutions was determined spectrophotometrically.[8] T–anti-R mixtures were then made up by weight.

Light Scattering Measurements.—Several stock solutions which were about 0.1% in protein and had a T–anti-R mole ratio (r) of about 1 were prepared. The r-value was calculated on the assumption, used throughout these Hp-Ab studies, that all protein in the purified solutions used is active anti-R, at suitable pH. Such a stock solution was clarified and the protein concentration in it determined as described earlier.[8] Portions with a volume of 2.5 ml. were diluted by weight in a 10 mm.-square cell with 1 ml. of a series of buffers which had been clarified by filtration. The cell was stoppered with a clean, tight-fitting Teflon cap and by slowly inverting a few times, the contents were mixed without introducing appreciable optical impurity. The scattering measured just after mixing, which required about a minute, lay within less than 3% of that found after 1 to 4 hr., except as noted below, and presumably represents an equilibrium state. The final pH always was determined since it generally differed from that of the buffer used.

Most pH above 8 were attained with either 0.2 M H_3BO_3, 0.15 M in NaCl, titrated with 0.15 M NaOH; or μ 0.15 Na_2HPO_4–Na_3PO_4 mixtures; or 0.001 to 0.01 M NaOH solutions brought to μ 0.15 with NaCl. Other diluents, used chiefly for attaining acid pH, were μ 0.15 acetate, arsenate, maleate, phosphate, phthalate and tris-(hydroxymethyl)-aminomethane (THAM)–HCl buffers, or solutions 0.00015 to 0.003 M in HCl, brought to μ 0.15 with NaCl. In a few cases, more concentrated diluents were used to attain a final μ of about 1. Sodium salts were used throughout.

The light scattering measurements were made in a Brice–Speiser absolute photometer. Light of wave length 436 mμ was used and dn/dc of Ab was taken as 0.200 at all pH, although it was determined[8] only at pH 8.

Ultracentrifuge Measurements.—For examination in the Spinco Model E ultracentrifuge, a stock mixture in 0.15 M NaCl was made in which the initial protein concentration was 1.03% and r was 1.06. The mixture became cloudy and flocculation soon occurred, but after the mixture stood overnight and the precipitate was removed, the supernatant solution now remained clear for at least two weeks. Such flocculation was never observed after making up the dilute mixtures used in light scattering. The optical density of the supernatant of this solution, at 280 mμ, was 95(±2)% of the value one would have expected if no precipitation had occurred. If we assume that the precipitate contains equal moles of T and anti-R, this number gives, very nearly, the percentage of anti-R which remained in solution. An almost identical mixture was made up (initial protein concn., 1.05%; initial r, 0.99) to demonstrate reversibility of the pH effects. In this case, only 80(±2)% of the anti-R, estimated in this way, remained in solution. The pH of these T–anti-R solutions was not measured. Although the T solution had been adjusted to pH 8 in the course of dissolving the free acid and the anti-R had been dialyzed against saline adjusted to pH 7 with NaOH, absorption of CO_2 by these unbuffered solutions probably resulted in a pH below 7. Since the mixtures were unbuffered, it is possible that their pH differed and that this affected the extent of precipitation.

Portions of the stable supernatant were diluted by weight using two parts T–anti-R solution to one of buffer, with some of the buffers described earlier, and the pH was then noted. The mixtures were run at 50740 r.p.m. at temperatures near 25°.

Area measurements were made essentially as described elsewhere.[9]

Other **Methods.**—Electrophoretic analyses were performed in a Perkin–Elmer Model 38 Tiselius apparatus.

pH was determined with a Beckman Model G meter, with correction for the sodium error.

A Beckman Model DU or a Cary spectrophotometer was used for ultraviolet absorption measurements.

Experimental Results and Discussion

Light Scattering Results at pH above 8.2.—Two preliminary observations are pertinent. First, the slope of a light scattering plot of Kc/R_{90} *vs.* concentration up to 0.082% for anti-R in pH 10.73, μ 0.15 phosphate buffer is zero, within probable ex-

(6) S. J. Singer and D. H. Campbell, THIS JOURNAL, **77**, 3504 (1955).

(7) S. J. Singer, L. Eggman and D. H. Campbell, *ibid.*, **77**, 4855 (1955).

(8) S. I. Epstein, P. Doty and W. C. Boyd, *ibid.*, **78**, 3306 (1956).

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

perimental error (±3% in Kc/R_{90}), that is, the virial coefficient B is zero, just as it is at pH 8.0. At any intermediate pH, B will also be zero, since only electrostatic repulsions might reasonably be expected to affect B, and these must be less important than at pH 10.73. We can thus plausibly take M_w, the weight-average aggregate weight, in T–anti-R mixtures throughout this range as R_{90}/Kc. Secondly, the effect of pH on the extent of association is essentially reversible, at least up to pH 10.99, which bounds the region of interest. This will be discussed in more detail further on.

Most of the light scattering observations at pH above 8.2 were made on mixtures derived from a common stock solution and are listed in Table I. In the third column, we record the values of Kc/R_{90}, determined by light scattering, and in the fourth, $R_{90}/160000Kc$ that is, since B is zero, M_w/M_A, where M_A is the antibody molecular weight. The molecular weight of anti-R determined from light scattering at pH 10.73 was 162000 ± 10000, which agrees with the value obtained at pH 8.0 for the fresh material.[10]

In order to evaluate these data, we may consider at this juncture the following simple model for the T–anti-R system, a model which is essentially identical to that used by Singer and Campbell with protein Ag–Ab systems.[6,7] It is assumed that: (a) each of the two reactive sites of anti-R contains a single group which must be positively charged in order for the bond to form; (b) these groups are characterized by a single intrinsic acid association constant,[11] K_H; (c) the negatively charged haptenic groups undergo no significant changes in the pH range where the groups in the anti-R sites are titrated; and (d) non-specific electrostatic repulsive forces between Ab molecules do not appreciably influence the Hp–Ab association.[11] It is also assumed, as in the studies at pH 8, that all anti-R sites are identical and equally accessible to T.

Typical equilibria in basic solution may then be written

$$Hp + Ab^{+2} \rightleftharpoons HpAb^{+1};\ 4K_i \quad (1)$$

$$Hp + Ab^{+1} \rightleftharpoons HpAb;\ 2K_i \quad (2)$$

$$Hp + HpAb^{+1} \rightleftharpoons HpAbHp;\ K_i \quad (3)$$

$$Ab^{+1} + H^+ \rightleftharpoons Ab^{+2};\ K_H/2 \quad (4)$$

$$Ab^0 + H^+ \rightleftharpoons Ab^{+1};\ 2K_H \quad (5)$$

$$HpAb^0 + H^+ \rightleftharpoons HpAb^{+1};\ K_H \quad (6)$$

The symbol at the right is the equilibrium constant characterizing each reaction; the superscript indicates the number, j, of Ab sites per species which are both free and charged. Hp, in this context, represents a T molecule, and Ab, an anti-R molecule. All other equilibria are characterized, in the chosen model, by the same intrinsic constants, K_i and K_H, with a suitable entropy factor. Light scattering observations, we shall see, yield p, the equilibrium fraction of reacted haptenic groups. Let T_0 and A_0 be the total concentration of haptenic groups and anti-R sites, respectively. If we were unaware that some anti-R sites were discharged and inactive, we would then compute an apparent association constant

$$K_{app} \equiv \frac{(\text{hapten–antibody bonds})}{(\text{free haptenic groups})(\text{free Ab sites, charged or not})} \quad (7)$$

$$= \frac{pT_0}{(T_0 - pT_0)(A_0 - pT_0)} \quad (8)$$

K_{app} is related to K_i through K_H, for

$$K_i \equiv \frac{(\text{hapten–antibody bonds})}{(\text{free, charged haptenic groups})(\text{free, charged Ab sites})} \quad (9)$$

$$= \frac{pT_0}{(T_0 - pT_0)\dfrac{(A_0 - pT_0)}{\left(1 + \dfrac{1}{K_H[H^+]}\right)}} = K_{app}\left(1 + \frac{1}{K_H[H^+]}\right) \quad (10)$$

since

$$K_H \equiv \frac{(\text{free, charged Ab sites})}{(\text{uncharged Ab sites})(H^+)} \quad (11)$$

If we recast (10) to obtain a form more appropriate for treating data obtained over a range of pH, we have[11]

$$\log\left[\frac{1}{K_{app}} - \frac{1}{K_i}\right] = \log\frac{1}{K_H K_i} + p\text{H} \quad (12)$$

Thus for the model chosen, a plot of $\log [1/K_{app} - 1/K_i]$ *versus* pH must have a slope of +1.0, and from the intercept K_H may be computed. If two such critical groups were present in each Ab site, the relation equivalent to equation 12 would be quadratic in pH, with a coefficient of 2.0 for the term linear in pH.

If we express M_w in terms of the concentration of aggregates, the relation of M_w to p follows. Neglecting the weight of free and bound T

$$M_w \cong \frac{\sum_n \left\{ \sum_{j=0}^{+2} (Hp_{n-1}Ab_n^j) + \sum_{j=0}^{+1} (Hp_nAb_n^j) + (Hp_{n+1}Ab_n) \right\} (nM_A)^2}{\sum_n \left\{ \sum_{j=0}^{+2} (Hp_{n-1}Ab_n^j) + \sum_{j=0}^{+1} (Hp_nAb_n^j) + (Hp_{n+1}Ab_n) \right\} (nM_A)} \quad (13)$$

(10) If the rapidly-sedimenting component noted in the ultracentrifuge is dimer, then we estimate from the areas under the two peaks that M_A is 179000 ± 5000 (weight average). We use the usually accepted value of 160000 because it agrees with the light scattering value: if the latter is in error, it is likely on account of factors which enter into R_{90}/Kc for a T–anti-R mixture in the same way, so that the ratio $R_{90}/160000Kc$ is correct. Moreover, traces of the R_3'-resorcinol used to purify the anti-R were present in the pool and may cause aggregation only at the much higher concentrations used in the ultracentrifuge.

(11) More correctly, account should be taken of two factors: (1) the electrostatic effect of the other charges of the Ab molecule on the apparent K_H of the critical group; and (2) the non-specific electrostatic repulsion of the Ab molecules on the apparent K_i of the Hp–Ab association. If we use the functions of φ and ψ to express these effects respectively, equation 12 should be written

$$\log\left[\frac{1}{K_{app}} - \frac{1}{K_i\psi}\right] = \log\frac{1}{K_H\varphi K_i\psi} + p\text{H} \quad (12a)$$

φ and ψ depend on ionic strength; on the location of the Ab sites on the highly asymmetric γ-globulin molecule; and on the net charge on the Ab molecule and hence on pH. At present it is not possible to give correct explicit form to these functions in this system, but it is clear that their effects are opposed and tend to cancel. An increase in alkaline pH and in net negative charge Z on the Ab molecule makes it more favorable for the critical group to accept a proton and must therefore increase φ. On the other hand, an increase in Z increases the mutual repulsion of Ab molecule and hence decreases ψ. Furthermore, if the critical groups are located near the poles of the elongated Ab molecules, both φ and ψ will certainly be close to unity at μ 0.15 and at all values of Z which are encountered. We assume, therefore, that $\varphi\psi \cong 1$ and varies by no more than about 15% between pH 9 and 10.5 in this system.

Combining (13) with (1) – (6) and equations for analogous equilibria, and noting that

$$(Ab^{+2}) = \frac{A_0}{2}\frac{(1 - pr)^2}{\left(1 + \frac{1}{K_H[H^+]}\right)^2}$$

and that

$$(Hp) = \frac{T_0}{2}(1 - p)^2$$

we obtain

$$\frac{M_w}{M_A} = \frac{1 + 4K_i^2(Ab^{+2})(Hp)}{1 - 4K_i^2(Ab^{+2})(Hp)} = \frac{1 + p^2r}{1 - p^2r} \qquad (14)$$

In these equations, r is defined as T_0/A_0. The final relation is identical with the one obtained for mixtures in which all Ab sites are charged.[8]

Application of equations 14 and 8 to the data of Table I results in the values of K_{app} listed in column 5. In Fig. 1, these results are plotted in accordance with (12), using $K_i = 4.0 \times 10^5$ l./mole, the value found at pH 8.23, where essentially all of the anti-R sites are presumably active. The points are shown fitted with a line of slope 1.15, although a line with slope 1 fits nearly as well. This result indicates that the data are indeed compatible within probable experimental error with the simplified model discussed above. Furthermore, from equation 12 we obtain the result that log $K_H = 9.9 \pm 0.2$, that is, pK_a of the group in the Ab site which is being discharged is 9.9, where $K_a = 1/K_H$ is the acid ionization constant for the group. The only acid groups in proteins with $pK_a = 9.9$ are cysteine –SH, tyrosine –OH, and the ϵ-NH_3^+ of lysine.[12] The guanidinium group of arginine and the α – NH_3^+ group are normally characterized by pK_a values of about 14 and 8, respectively. Of these possible acid groups, only that of lysine has the correct pK_a and bears a positive charge in the pH range where Hp–Ab association is maximal. It is possible, however, that instead of a positively charged lysine residue a tyrosine –OH in the Ab site is hydrogen-bonded to the benzenearsonate ion. This hydrogen bond would be unstable either upon the ionization of the tyrosine –OH or upon protonation of the benzenearsonate ion, and hence the dissociation behavior of such a linkage would be the same as that of an ion-pair interaction. The data up to this point cannot distinguish between these two possibilities, but the results of chemical modification studies to be discussed later on indicate that it is a lysine residue which is present in the Ab site.

TABLE I

DEPENDENCE OF LIGHT SCATTERING FROM T–ANTI-R MIXTURES ON pH ABOVE pH 8.23

Conditions: r = 1.15, μ 0.15 buffers, borate below pH 10, phosphate above pH 10.

pH	Protein concn., %	$10^6Kc/R_{90}$	M_w/M_A	Range[a] of $10^{-5}K_{app}$
8.23	0.067	3.00 ± 0.09	2.08 ± 0.06	4.0
8.99	.067	3.19 ± .10	1.96 ± .06	3.2 –3.7
9.48	.067	3.37 ± .10	1.85 ± .06	2.7 –3.2
9.68	.070	3.49 ± .10	1.79 ± .05	2.4 –2.8
9.90	.067	3.72 ± .11	1.68 ± .05	2.1 –2.5
10.22	.067	4.70 ± .14	1.33 ± .04	0.96–1.2
10.30	.069	4.80 ± .14	1.30 ± .04	.84–1.1
10.42	.069	4.92 ± .15	1.27 ± .04	.75–0.99
10.54	.067	5.25 ± .16	1.19 ± .04	.54– .78
10.68	.067	5.55 ± .17	1.13 ± .03	.40– .58

[a] These are the probable extreme values of $10^{-5}K_{app}$ if we assign a probable experimental error of ±3% to $10^6Kc/R_{90}$ and hence, also, to M_w/M_A. The possible presence of a small amount of dimer in the anti-R pool was ignored in computing these values: all the protein was assumed to be monomeric as well as divalent anti-R.

(12) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publ. Corp., New York, N. Y., 1943, p. 445.

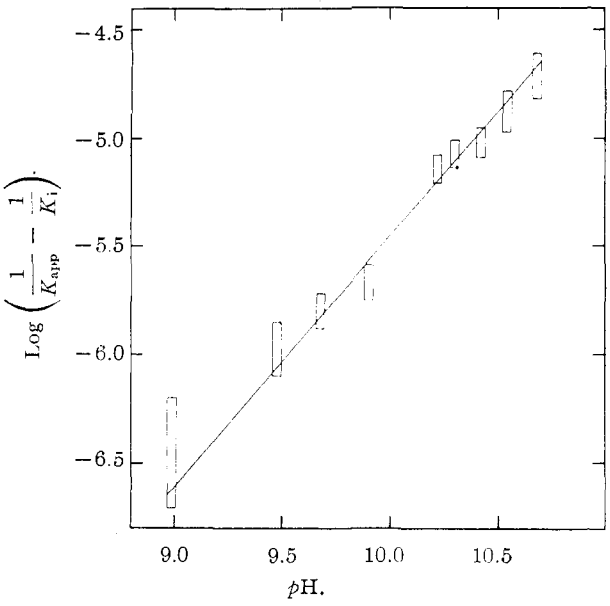


Fig. 1.—The variation of the apparent equilibrium constant data of Table I.

A complicating feature of the T–anti-R system is that arsonic acids are dibasic, and the second dissociation occurs at alkaline pH. In order to determine to what extent the alkaline dissociation of Hp–Ab bonds is affected by this ionization, the second dissociation constant of T was determined spectrophotometrically. The method was based on the fact that the ultraviolet absorption of T at the 280 mμ maximum drops about 12% when the pH changes from 6.5 to 9.5, without noticeable change in λ_{max}. Three-ml. portions of μ 0.15 NaCl solution containing 70 μg.T/ml. were diluted by weight with 1-ml. portions of μ 0.15 borate or phosphate buffers. The optical density of each solution was measured against a blank consisting of a solution brought to pH 11.2, where all the arsonic acid groups are doubly ionized. If we write, schematically, for the dissociation of the second H^+ from a single haptenic group

$$As^- \rightleftharpoons As^= + H^+, K_2 \qquad (15)$$

we have

$$pH = pK_2 + \log\frac{(As^=)}{(As^-)} \qquad (16)$$

The (As^-) at any pH is proportional to the observed difference in optical density, m; the $(As^=)$ at the same pH is proportional to $m_{max} - m$, where m_{max} is the maximum difference in optical density observed, that is, for the case where the solution contains T almost all of whose groups are singly-

charged (pH 6.5). Thus

$$\log \frac{m}{m_{max} - m} = pK_2 - p\mathrm{H} \qquad (17)$$

We assume in this derivation that the contribution to the optical density of a T molecule with one singly ionized group and one doubly ionized group is exactly midway between that of a T molecule with both groups singly ionized and one with both groups doubly ionized. The symmetry of the plot of m *vs.* pH, Fig. 2, is consistent with this assumption.

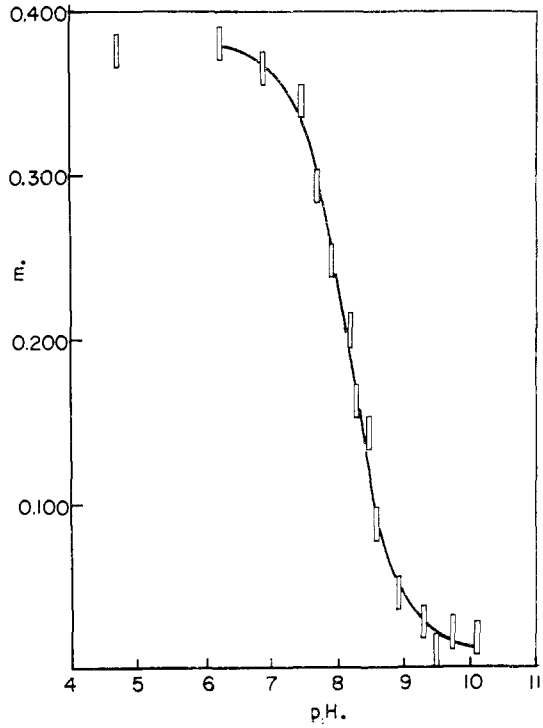


Fig. 2.—The variation of optical density of T with pH; m is the difference between the optical density of a solution and that of a solution at pH 11.2; concentration of T, 52 μg./ml.; μ 0.15 borate or phosphate buffers; uncertainty in m, $\pm$0.010.

In Fig. 3, the data of Fig. 2 are plotted according to (17). The line which fits the data has a slope of -1, in accordance with (17); $pK_2 = 8.2 \pm 0.1$. This finding is consistent with the values found by Pressman and Brown[13] in an extensive study of aromatic arsonic acids. The fact that pK_2 is much less than pK_H of the Hp–Ab dissociation demonstrates that the second ionization of the arsonic acid is not involved in the dissociation. The data of Fig. 1 begin at pH 9.0, by which point about 90% of the arsonic acid groups are already doubly ionized. Indeed, it is surprising that the second ionization has an apparently negligible effect on the Hp–Ab reaction. One might expect it to increase the coulombic interaction of Hp and Ab sites and hence to increase the association and the turbidity of T–anti-R mixtures; on the contrary, a 10% drop in turbidity occurs between pH 8.2 and 9.2, which can be entirely accounted for in our model by the partial titration of the group in the Ab site with pK 9.9. For the same reason, the use of K_1 calculated from a turbidity at pH 8.2, where half the haptenic

(13) D. Pressman and D. H. Brown, THIS JOURNAL, **65**, 540 (1943).

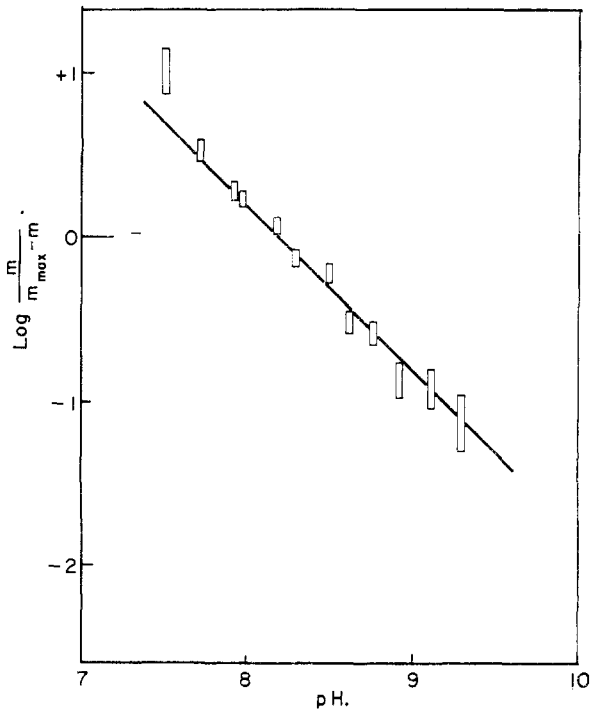


Fig. 3.—Variation of optical density of T with pH: comparison of data of Fig. 2 with model for the effect of the second ionization of haptenic groups on optical density.

groups are still singly charged, is justified, even though essentially all the haptenic groups are doubly charged in the pH range where the largest fraction of the turbidity change occurs.

Ultracentrifuge Results.—For the purpose of testing the pH dependence of aggregation by another method, we have also subjected T–anti-R mixtures to ultracentrifugation. It is not possible to resolve by electrophoresis anti-R and its aggregates with T, which are essentially anti-R polymers. The sedimentation patterns are presented in Figs. 4 and 5, along with that of anti-R itself. Three not well-resolved peaks are distinguishable, at most, in the T–anti-R mixtures. These peaks have s_{20} of approximately 6.5–7, 9–10, and 12–13 svedbergs; we shall refer to these peaks as m, d and t, respectively. Presumably m represents the species Ab, AbHp and AbHp$_2$, since it sediments at the rate of γ-globulin. The sedimentation rates of peaks d and t are consistent with those which would be anticipated for anti-R "dimer" and anti-R "trimer," the sum of species containing two anti-R molecules and three anti-R molecules, respectively.[14]

(14) These s_{20} values are the observed sedimentation rates, corrected only for the temperature dependence of the solvent viscosity, this being the principal correction applicable in these dilute buffers, excepting concentration dependence. At infinite dilution, s^w_{20} will exceed these values. Taking s^w_{20} for anti-R as 7, f/f_0 for the molecule is found to be 1.5 from the Svedberg equation. If the departure of f/f_0 from unity is ascribed solely to asymmetry, and we assume the prolate ellipsoid model, the Ab molecule has an axial ratio of 9. If side-by-side aggregation occurs and we assume that the aggregate is an ellipsoid as long as the monomer, and whose diameter is chosen so that the volume of the aggregate equals the degree of polymerization multiplied by the volume of monomer, we find that s^w_{20} is 12.6 and 17.3, for the dimer and trimer, respectively. The same method of calculation, using a complementary assumption, yields s^w_{20} of 8.8 and 9.6, respectively, for end-to-end dimer and trimer. The observed sedimentation rates of peaks d and t lie between these limiting estimates.

If we interpret the patterns in this way, those at neutral pH are most interestingly consistent with the earlier light-scattering studies of this system[8]: aggregation is maximal when r is about 1, and in excess of either reactant, aggregation is diminished. At alkaline pH above 10, aggregation diminishes abruptly, again reflecting light scattering behavior.

The sedimentation pattern can provide a pictorial proof that the effect of pH is reversible under the conditions used. A T–anti-R solution was brought to pH 10.99 by the slow addition of pH 11.8, μ 0.15 phosphate buffer. After 0.5 hr., it was neutralized to pH 7.55 by adding pH 4.85, μ 0.15 acetate buffer. The sedimentation pattern (Fig. 6) is nearly superposable on that of a mixture identical except that the acid and alkaline buffers were first mixed and then added to the T–anti-R solution. Thus appreciable denaturation of anti-R was absent in our experiments.

Upon attempting to obtain a more quantitative comparison of the ultracentrifugal and light scattering results, we encounter the problem of the poor resolution of the peaks in the sedimentation patterns. This is due, in part, to the small difference in sedimentation rates of the peaks but may also be affected by the occurrence of re-equilibration reactions as an adjustment to loss of larger aggregates in various parts of the cell. For example since Ab, AbHp and HpAbHp sediment at the same rate, reactions of the type Ab + HpAbHp $\rightarrow$ HpAbHpAb may occur as dimer sediments out of the region of the monomer peak. In contrast, the free Ag concentration can be obtained unequivocally in systems like bovine serum albumin (BSA)–anti-BSA, since the sedimentation of aggregates leaves a region in the cell which contains only BSA, so that there is no possibility of further reaction. While such reequilibration reactions, if they were of an appropriate rate, would be expected to diminish progressively the area ascribed to monomer in successive patterns of a single experiment, this was not observed within the errors of analysis. Because of the poor resolution, corrections for the Ogston–Johnston anomalies, and for the fast-sedimenting component present in the anti-R itself, were not considered warranted.

With these reservations on the accuracy of the data, we may proceed to interpret the results of Table II, which lists the areas under each peak in the T–anti-R patterns of Figs. 5 and 6. If peak m indeed represents anti-R monomer, then the area under it permits evaluation of K_{app}. We note first that according to our model, (total Ab n + l-mer) is given by (p^2r) (total Ab n-mer), where parentheses indicate concentration in moles/liter where appropriate. For example

$$\sum_{=0}^{+2}(\mathrm{HpAb_2}^j) + \sum_{j=0}^{+1}(\mathrm{Hp_2Ab_2}^j) + (\mathrm{Hp_3Ab_2}) = \left\{\sum_{j=0}^{+2}(\mathrm{Ab}^j) + \sum_{j=0}^{+1}(\mathrm{HpAb}^j) + (\mathrm{Hp_2Ab})\right\}(p^2r) \quad (18)$$

The area under an n-mer peak will be proportional to the weight concentration of total n-mer, that is, to n(total n-mer). Thus, where k is a constant relating area under peak m to molar concentration of total monomer (M), and A_{tot} is the total area under all peaks

$$A_{tot} = k(\mathrm{M}) + 2kp^2r(\mathrm{M}) + 3k(p^2r)^2(\mathrm{M}) + \ldots\ldots \quad (19)$$

Then

$$\frac{k(\mathrm{M})}{A_{tot}} = \frac{A_m}{A_{tot}} = (1 - p^2r)^2 \quad (20)$$

where A_m is the area under the peak m. We have used this relation to obtain p and hence K_{app} from the data listed in Table II; the results are recorded in column 6. The values are consistent within the large experimental error with those estimated from light scattering, column 7, although they are uniformly lower. This may be explained by the previous finding[8] that the equilibrium constant appar-

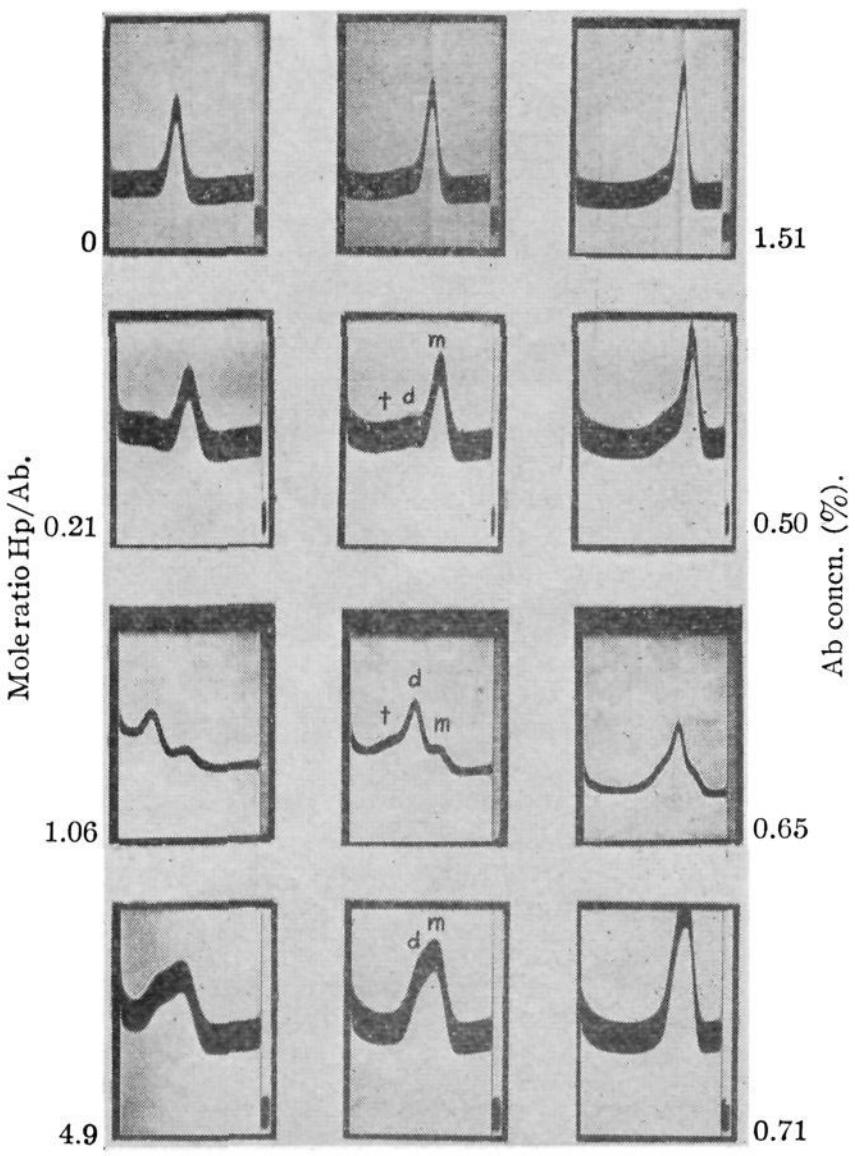
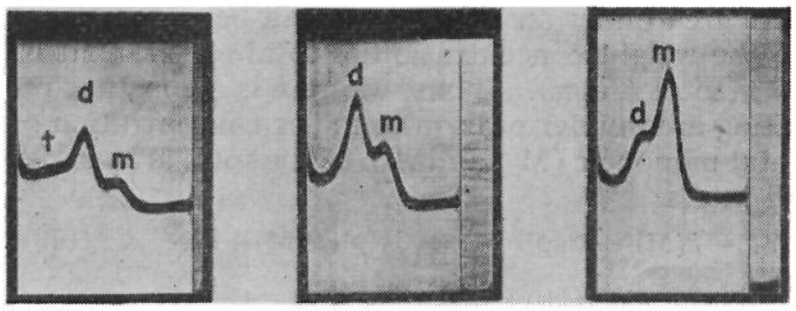


Fig. 4.—Ultracentrifuge diagrams of T–anti–R mixtures with different r-values, in μ 0.15 borate buffers, pH near 8, compared with that of anti-R (top row). The patterns at three different times in a single experiment are presented, from right to left, chronologically. Sedimentation proceeds to the left. Peaks labelled m, d, and t refer to monomer, dimer, trimer, as discussed in text.

ently decreased with increasing total concentration, and the solutions examined in the ultracentrifuge were about ten times more concentrated than those studied by light scattering. There is a sharp drop in K_{app}, which coincides with the abrupt change observed by light scattering, between pH 10.11



*p*H 8.27 10.11 10.64

Fig. 5.—Ultracentrifuge diagrams demonstrating the effect of alkaline *p*H on T-anti-R association, μ 0.15 buffers. Mole ratio Hp/Ab = 1.06, Ab concn. = 0.65%. Single patterns from each experiment were observed at roughly comparable times of sedimentation. Sedimentation proceeds to the left. Peaks labelled m, d, and t refer to monomer dimer and trimer, as discussed in text.

and 10.64, and which reflects the shift in the proportions of anti-R in the two dominant peaks (Fig. 5).

TABLE II

SEDIMENTATION PATTERNS OF T–ANTI-R MIXTURES

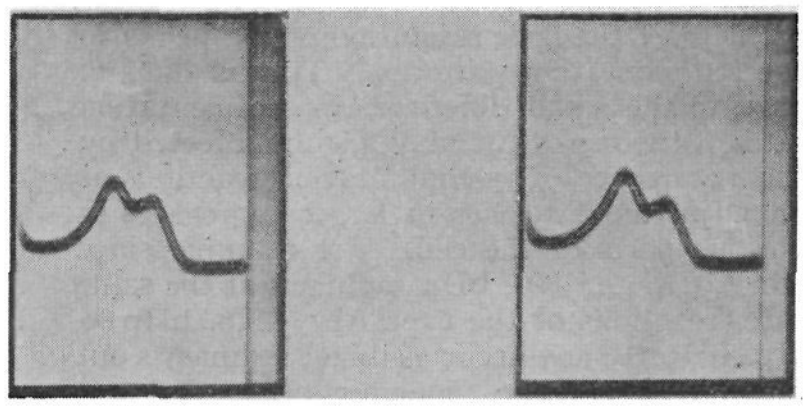
μ 0.15 buffers; r = 1.06 for the first three solutions; r = 0.99 for the others. Buffer composition-UC data: first two solutions, μ 0.05 phosphate, μ 0.10 NaCl; third solution, μ 0.05 borate (contains NaCl), μ 0.10 NaCl; last two solutions, μ 0.015 acetate, μ 0.04 phosphate, μ 0.095 NaCl. The uncertainty in the LS (light scattering) values for K_{app} is about ±10%; in the UC (ultracentrifuge) values, *because of the poor resolution,* ±50%.

*p*H	% Protein	% Total area under peak m	d	t	10^{-5} K_{app} UC	LS[a]
10.64	0.65	67 ± 10	33 ± 10		0.2	0.5
10.11	.65	31 ± 10	69 ± 10		0.9	1.4
8.27	.65	16 ± 5	57 ± 10	27 ± 10	2.2	4.0
7.55	.50	35 ± 10	52 ± 10	13 ± 5	0.9	2.1
7.50	.50	30 ± 10	55 ± 10	15 ± 5	1.2	2.1

[a] Values estimated from data of Fig. 6, which pertain to solutions of slightly different buffer composition.

There are, however, some inconsistencies in the ultracentrifuge diagrams. If we obtain p^2r from equation 20, we can compute the expected distribution of the remaining area among the other peaks. In the case of the pattern at *p*H 8.27, the fraction of the total area which should appear as dimer and trimer, presumably under the d and t peaks, is 19 and 17%, respectively; the other 48% of the anti-R should be involved in larger aggregates. On the other hand, the observed areas under the d and t peaks are much larger than expected. The explanation for this discrepancy is not clear at present. It may be that the assignment of the d and t peaks to dimer and trimer species only, is an oversimplification. Furthermore, re-equilibration reactions may seriously distort the area distribution in these patterns.

Light Scattering from T–anti-R Mixtures below *p*H 8.2.—The scattering from T–anti-R mixtures at *p*H below 8.2 was observed, and a few more mixtures at *p*H above 8.2 were examined as a check on the previous result. The data obtained are shown in Fig. 7, together with all the measurements from which Fig. 1 was derived.[15] We plotted $R_{90}/160000Kc$, that is, if the virial coefficient is zero, M_w/M_A, against *p*H. To guarantee that the virial coefficient is indeed zero for all the solvents in which the mixtures were examined, would require measurements of the molecular weight of antibody in all buffers used over the *p*H range 3–8. Actually, one would anticipate that the virial coefficient, at least at these low concentrations and moderate ionic strengths, would have only a negligibly small effect on the turbidity. The few data that were obtained on 0.06% solutions of anti-R itself supported this expectation. Thus, $10^6Kc/R_{90}$ was 6.02, 5.75, 6.55 and 6.34 in *p*H 8.2 borate, *p*H 6.7 phosphate, *p*H 3.9 acetate and *p*H 3.0 phthalate buffers, respectively, all at μ 0.15. The data suggest a small decrease in the turbidity at low *p*H, but the average deviation of the four results from the mean value 6.17 (expected value for $B = 0$ and $M_A = 160000$, 6.25) is about 5%, which is only a little larger than the probable experimental error.



(a) (b)

Fig. 6.—The reversibility of the effect of alkaline *p*H on T–anti–R association. Pattern (a) is of a stock solution exposed to *p*H 10.99, and returned to *p*H 7.55; (b) stock solution diluted to have same concentration and buffer composition as (a). The Hp/Ab mole ratio is 0.99, final Ab concn. 0.50%. The buffer contains phosphate ions, see text.

As was indicated earlier, it was originally expected that the acid dissociation of Hp–Ab bonds would be determined by the titration of the negatively charged benzenearsonate ion. If K_{Hh} is the reciprocal of the first ionization constant of the haptenic group and we adopt a model entirely analogous to the one previously discussed for the alkaline Hp–Ab dissociation, it follows that

$$\log\left(\frac{1}{K_{app}} - \frac{1}{K_i}\right) = \log\frac{K_{Hh}}{K_i} - pH \qquad (21)$$

This equation is identical in form to that derived by Singer and Campbell.[6,7] In this context

$$K_{app} \equiv \frac{\text{(hapten–antibody bonds)}}{\text{(free haptenic groups, charged or not)(free Ab sites)}}$$

From the value of $K_i = 4 \times 10^5$, and the assumed value of $K_{Hh} = 10^4$, which is at the upper limit of values found for typical benzenearsonic acids,[13] we may calculate, from equations 21, 8 and 14, the values of $R_{90}/160000Kc$ as a function of acid *p*H which are predicted by this model. The calculations were made for two different sets of r-values and anti-R concentrations: r = 1.15, anti-R concn. = 0.067%; and r = 1.00, anti-R concn. = 0.060%; to correspond closely to the mixtures actually stud-

(15) The turbidity of the mixtures which were brought to μ 1.2 with sodium phosphate was 10% lower, 4 hr. after mixing, than the turbidity just after mixing. In these cases, no observation was made 1 hr. after mixing, so it is not known whether the drop occurred gradually or during the first hour.

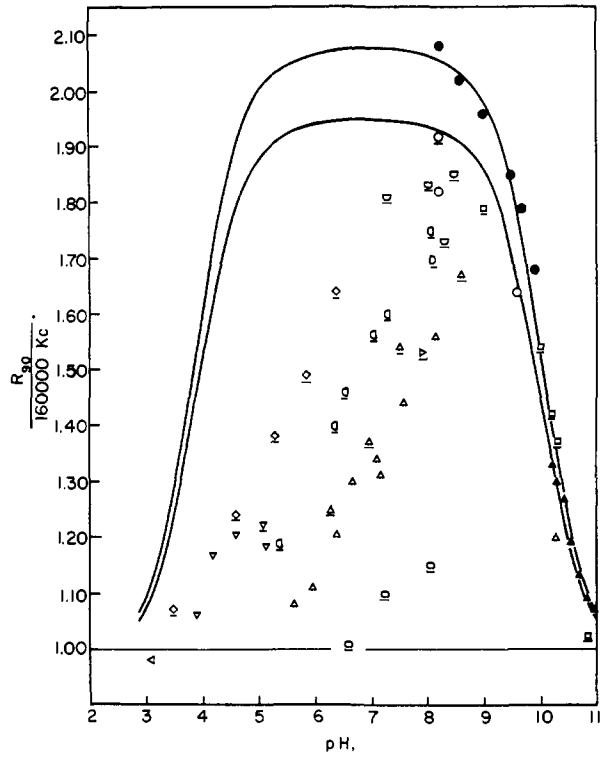


Fig. 7.—Turbidity of T–anti-R solutions as a function of pH. Full curves calculated as described in text; lower bell-shaped curve, for r = 1.00 and anti-R concn. 0.060%; upper bell-shaped curve, for r = 1.15 and anti-R concn. = 0.067%. Code to experimental data: solid figures, r = 1.15, anti-R concn. = ~0.067%, data of May, 1955; open plain figures, r = 1.02, anti-R concn. = 0.065%, data of February, 1956; open underlined figures, r = 1.02, anti-R concn. = 0.061%, data of April, 1956. Δ, μ 0.04 phosphate, μ 0.11 NaCl; ∇, u 0.04 acetate, μ 0.11 NaCl; ▷, μ 0.01 borate, u 1.36 NaCl; ◁, μ 0.04 phthalate, μ 0.11 NaCl; ○, μ 0.04 borate, μ 0.11 NaCl; ⏢, μ 1.1 phosphate, μ 0.11 NaCl; ⏣, μ 0.04 THAM, μ 0.11 NaCl; ⏥, μ 0.04 arsenate, μ 0.11 NaCl; ⏧, μ 0.04 maleate, μ 0.11 NaCl; □, μ 0.15 NaCl, NaOH; ◇, μ 0.15 NaCl, HCl.

ied. These theoretical curves are shown in Fig. 7, together with similar curves for the alkaline pH region constructed by use of equations 12, 8 and 14 and the previously determined value of $K_H = 8 \times 10^9$. As follows from the results discussed above, the alkaline pH data agree very well with the theoretical curve. On the other hand, the acid pH data clearly do not. Not only does the Hp–Ab dissociation occur at higher pH than expected for this hapten, but the dissociation occurs more gradually with pH than predicted. Furthermore, and most significantly, whereas the alkaline pH data are essentially independent of the nature and concentration of the buffer medium, those at acid pH are very strongly dependent on these variables.

The most plausible explanation for these results is that the buffer ions compete, to a varying extent, with hapten for the Ab site.[16] It is particularly striking that the turbidity at a given acid pH is lowest in phosphate buffer and decreases with increasing concentration of phosphate. These effects observed by light scattering are apparent in certain of the ultracentrifuge patterns as well. Thus the pattern at pH 7.5 (Fig. 6) in a buffer containing phosphate shows a large increase in the m component, at the expense of the d and t components, compared with the pattern at pH 8.2 in borate buffer. It has long been known that arsenate itself inhibits the precipitation of anti-R Ab by specific substances,[17] clearly because of a competition for Ab sites. Our one light scattering measurement with arsenate present in the buffer indicates that at the same pH and molar concentration phosphate and arsenate are essentially equally effective in reducing the turbidity of T–anti-R mixtures. We conclude that phosphate can also react with anti-R Ab sites, which is not surprising in view of the close structural relationship between phosphate and arsenate ions. This effect of phosphate in causing the dissociation of Hp–Ab bonds takes precedence over any similar effect that might be produced by the titration of the hapten groups. Between pH 8 and 5.5, the turbidity of mixtures in phosphate buffer drops more than 30%, to a value nearly equal to that of a solution containing anti-R alone; yet T remains essentially fully charged under these conditions.

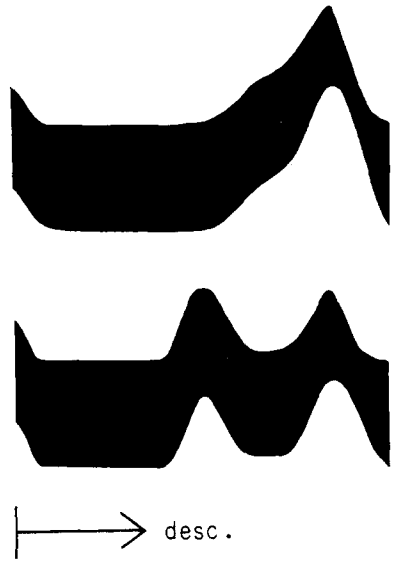


Fig. 8.—The demonstration of the protection of anti-R by an excess of RBG, against inactivation by acetylation. Descending electrophoresis patterns of: top, acetylated RBG–anti-R mixtures; bottom, a mixture of separately acetylated RBG and rabbit γ-globulin. Both experiments in barbital buffer, pH 8.6, μ 0.1, under identical conditions. The fast peak in each diagram is due to acetylated RBG, and the arrow indicates the starting position and direction of migration.

The light scattering data suggest, moreover, that in the acid pH region where Ab molecules bear a net positive charge, maleate, acetate and chloride ions, in order of decreasing effectiveness, can also com-

(16) Some difficulty may be encountered in this system at pH below 4.5 from the limited solubility of T in acid solution. Rough measurements which we made indicate, however, that at pH above 4.5, the solubility of T exceeded 2.2 μg./ml., its concentration in the T–anti-R mixtures examined by light scattering.

(17) K. Landsteiner, *Biochem. Z.*, **104**, 280 (1920). See also F. Haurowitz and F. Breinl. *Z. physiol. Chem.*, **214**, 111 (1933).

pete for the anti-R site. The influence of *p*H, in a given buffer system, might therefore be very complicated, involving at least the following factors: (a) the net positive charge on anti-R increases as the *p*H is lowered below 7, which should increase the binding of buffer anions to the specific, as well as numerous other, sites on the anti-R molecule; (b) T hapten is an anion, and at acid *p*H may bind non-specifically to anti-R at other than the reactive sites of the Ab molecule, thus increasing the extent of Hp–Ab association by effectively increasing the Ab "valence" beyond 2; and (c) finally, the titration of the charged groups on the hapten. The factors (a) and (b) should oppose one another; as the *p*H is progressively decreased, the former should decrease, the latter increase, the apparent extent of Hp–Ab association, and the resultant variation might be complex indeed. This could account qualitatively for the observed variation of the turbidity of T–anti-R mixtures with acid *p*H. An attempt to treat this situation theoretically does not seem warranted at the present time.

At first sight it might seem unjustified to regard the light scattering data obtained at alkaline *p*H as relatively free of complications because they conform to simple theoretical expectations, and those at acid *p*H as anomalous because they do not, but our interpretation is supported by a number of internal consistencies. It is, in fact, logical that at alkaline *p*H, where Ab molecules bear a net negative charge, competition of buffer anions for anti-R sites should be much less significant than at acid *p*H, where Ab is positively charged. (Non-specific ion-binding by bovine γ-globulin at *p*H about 7 is very small.[18]) Therefore the alkaline *p*H studies might be expected to be freer of such complications. Furthermore, experimentally, the turbidity results at alkaline *p*H are essentially the same in borate, phosphate or NaCl–NaOH solutions. In addition, the order of effectiveness of the buffer ions in reducing the extent of Hp–Ab dissociation at a given acid *p*H is correlated roughly with the extent of their structural similarity to the benzenearsonate group, and hence their capacity to react with anti-R sites. It follows from these arguments that considerable interest would attach to a similar study of a Hp–Ab system in which the Hp is positively charged. In this case, the alkaline *p*H range might yield anomalous results due to the possible competition of cations with the Hp for the Ab site, while the acid *p*H range might simply reflect the titration of a negatively charged group in the Ab site.

A question which this discussion provokes deserves comment: is the value of K_i obtained in earlier studies[8] in *p*H 8 borate buffer affected by either buffer inhibition or non-specific binding of T? The most abundant anion in *p*H 8 borate buffer is chloride, since the pK of boric acid is about 9: the coincidence of the results in borate and THAM buffers presumably reflects this common property. From the results at acid *p*H, it is plain that chloride ion is the weakest inhibitor among the ions used here. Thus, while an estimate of the effect on K_i cannot be given, it should be comparatively small. The non-specific binding of T to anti-R should be negligible at *p*H 8 in comparison with the specific binding, since it was found earlier[8] that data over a range of r-values were all compatible with an anti-R valence of two. However, while the effect is small, generally lying within probable experimental error, it is perhaps significant that the aggregation in mixtures in which r was about 5 persistently exceeded that expected from the data for mixtures with r about 1. Weak non-specific binding of T would indeed be favored by high T concentration, and the resultant increased possibility for aggregation would only become apparent at high r values where aggregation by the completely specific mechanism is diminished because of T excess inhibition.

It should be noted finally that our results suggest that light scattering might be used effectively to determine absolute values of hapten inhibition constants in systems in homogeneous equilibrium.

Chemical Modification Studies.—In order to investigate further the nature of the groups present in anti-R Ab sites, some preliminary studies were made of the effect of acetylation of the Ab under conditions which are specific for the blocking of free amino groups[19,20] and which do not cause the reaction of tyrosine–OH groups. For these experiments the antigen employed was a heavily-coupled benzenearsonic acid-azo-bovine γ-globulin (RBG) used in other studies.[21] The anti-R was not pure Ab but was part of a preparation containing mostly inert γ-globulin.[22] Treatment of RBG with acetic anhydride at 0° in half-saturated sodium acetate[19] permitted it to retain 100% of its capacity to precipitate anti-R Ab. This is as expected, since the acetylation reaction should have no effect on the benzenearsonic acid hapten. On the other hand, the anti-R γ-globulin fraction treated in the same manner, with the resultant blocking of about 75% of its free amino groups,[23] lost its capacity to precipitate any measurable amount of RBG, as determined 24 hr. after mixing the two protein preparations. The detailed methods used in these experiments are essentially those described previously.[20]

The inactivation of anti-R by acetylation may be due either to a non-specific denaturation of the molecule consequent to the blocking of 75% of its normally free amino groups or to the blocking of essential amino groups in the two reactive sites, the blocking of all the other amino groups being inconsequential. That a non-specific denaturation is not involved is shown by the fact that an excess of RBG protects anti-R from inactivation by acetylation, presumably due to the protection of the Ab sites by the Ag.[24] This protection was demonstrated as follows.[20] A precipitate of RBG and

(18) I. M. Klotz, in the "Proteins," ed. by H. Neurath and K. Bailey, Vol. IB, Academic Press, New York, N. Y., 1953, p. 787.

(19) H. Fraenkel-Conrat, R. S. Bean and H. Lineweaver, *J. Biol. Chem.*, **177**, 385 (1949).

(20) S. J. Singer, *Proc. Natl. Acad. Sci. U. S.*, **41**, 1041 (1955).

(21) F. Pepe and S. J. Singer, to be published.

(22) M. C. Baker, D. H. Campbell, S. I. Epstein and S. J. Singer, THIS JOURNAL, **78**, 312 (1956). This preparation is referred to as anti-R-1 in this paper.

(23) As determined by Van Slyke analyses: D. D. Van Slyke, *J. Biol. Chem.*, **83**, 425 (1929).

(24) D. Pressman and L. A. Sternberger, *J. Immunol.*, **66**, 609 (1951).

anti-R was dissolved in excess RBG to give a mixture of soluble Ag–Ab aggregates. This mixture was acetylated and subsequently examined electrophoretically (Fig. 8, top). As a comparison, a mixture of *separately* acetylated RBG and rabbit γ-globulin was also examined (Fig. 8, bottom). It is evident that a peak due to Ag–Ab aggregates, of mobility intermediate between that of acetylated RBG and acetylated γ-globulin, is present in Fig. 8, top, indicating that the anti-R retained considerable activity after acetylation in the presence of RBG. Therefore, the inactivation of anti-R which takes place upon acetylation in the absence of RBG would appear to be due to the blocking of essential amino groups in the specific Ab sites. This information complements the light scattering results with T–anti-R mixtures at alkaline *p*H discussed earlier and we conclude that in each Ab site there is critically present a single ϵ-NH_3^+ group of a lysine residue, complementary to the benzenearsonate hapten.

Comparison with Protein Ag–Ab Systems.—In the systems containing bovine serum albumin (BSA) and ovalbumin (OA) and their respective rabbit antibodies, quantitative studies have been made[6,7] in the ultracentrifuge of the effect of acid *p*H of the extent of Ag–Ab association. It was found that the data in both systems obeyed a relation equivalent to equation 21, with $K_h \cong 10^5$, and it was therefore concluded that a single ionized carboxyl group was critically present in each Ag–Ab bond formed in these systems. The studies at alkaline *p*H in the T–anti-R system provide another independent example of such behavior and lend considerable support to the conclusions reached in these earlier studies.

The behavior of the BSA:anti-BSA system toward acetylation is also quite parallel to that of the RBG:anti-R system.[20] BSA retains most (70%) of its activity upon acetylation, but its specific Ab is inactivated under these conditions. Furthermore, an excess of BSA protects its Ab from inactivation by acetylation. We conclude, therefore, that the two apparently unrelated systems have a great deal in common: a single ion-pair is involved in each of the bonds of BSA:anti-BSA and of R:anti-R, with one of the groups being an ϵ-NH_3^+ in each Ab site. This parallelism is very likely reflected in the very similar thermodynamic parameters characterizing these systems.[9,22]

Acknowledgments.—We thank Drs. W. C. Boyd and P. M. Doty for permission to use the pool 54 anti-R and Drs. D. Pressman and D. H. Campbell for the gift of the necessary haptens.

NEW HAVEN, CONN.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF MICHIGAN]

The Polarography of Histidine Complexes of Cobalt(II) and Cobalt(III)

BY BRUNO JASELSKIS

RECEIVED JULY 25, 1957

Unlike most cobalt(II) complexes bihistidinatocobalt(II) shows an anodic wave in buffer solutions above *p*H 5.5; the potential of which becomes more negative with an increasing *p*H until a limiting value of −0.25 v. *vs.* S.C.E. is reached. The presence of the anodic wave is attributed to the uncharged bihistidinatocobalt(II) species being oxidized in a reversible manner to bihistidinatocobalt(III) ion. The anodic wave for bihistidinatocobalt(II) and the cathodic wave, the first wave for bihistidinatocobalt(III) ion, are for the same couple $Co(III)(hi)_2^+ + e \rightleftarrows Co(II)(hi)_2$. The cobalt(II) complexes of histidine methyl ester and histamine show a similar anodic wave above *p*H 8, presumably due to the oxidation of uncharged hydroxo complexes.

The polarographic reduction of cobalt(II) in the presence of histidine has been reported previously.[1,2] It has been observed that histidine depressed the cobalt(II) reduction maximum producing a similar maximum at a more negative potential.

In the present paper the polarographic behavior of bihistidinatocobalt(II) and of bihistidinatocobalt(III) ion is reported in greater detail. In particular the polarograms have been examined in the region between −0.1 v. and −0.40 v. *vs.* S.C.E. in various buffer solutions.

The presence of a well-defined anodic wave for bihistidinatocobalt(II) in buffered solutions above *p*H 5.5 and the lack of an anodic wave for the cobalt(II) complexes of histidine methyl ester, and histamine in buffer solutions below *p*H 8 is attributed to the oxidation of the uncharged complexes. This is substantiated by the studies of cobalt(II) complexes of histidine, histidine methyl ester and histamine at various *p*H's.

Furthermore, the relationship between the first cathodic wave for the reduction of bihistidinatocobalt(III) ion and the anodic wave for the oxidation of bihistidinatocobalt(II) is established.

Experimental Work

Materials.—The crystalline bihistidinatocobalt(II) was prepared by the reaction of 0.01 mole of cobalt(II) hydroxide with 0.02 mole of pure histidine in 200 ml. of deaerated water. Subsequent vacuum evaporation yielded the crystalline complex having an empirical formula $C_{12}H_{16}N_6O_4Co \cdot H_2O$. The results of analysis of this crystalline complex are summarized in Table I.

The bihistidinatocobalt(III) aqueous solution was pre-

TABLE I

SUMMARY OF ANALYSIS OF CRYSTALLINE BIHISTIDINATOCOBALT(II)

Analysis of	Percentage found	Percentage calcd.
Co (total)	15.35	15.33
Co(II)	15.24	15.33
N (Dumas)	22.02	21.82
C	36.81	37.40
H	4.68	4.67

(1) J. Sladek and M. Lipschütz, *Coll. Czech. Chem. Comm.*, **6**, 487 (1934).

(2) E. R. Roberts, *Trans. Faraday Soc.*, **37**, 353 (1941).