Antibodies Formed in Response to Individual Ionization States of Benzenearsonic Acid*

V. Peter Kreiter and David Pressman

From the Department of Biochemistry Research, Roswell Park Memorial Institute, New York State Department of Health, Buffalo 3, New York

Received June 25, 1962

Since the *p*-azobenzenearsonate group exists in both the singly and doubly ionized forms at physiologic *p*H values, a search was made to determine whether antibodies are formed in response to the individual forms. This was done by measuring the change of apparent binding constants with *p*H for the reaction of *p*-(*p*-hydroxyphenylazo)benzenearsonic acid and *p*-(*p*-hydroxyphenylazo)benzenephosphonic acid with rabbit antisera formed in response to the *p*-azobenzenearsonate group (anti-R_p) and in response to the *p*-azobenzenephosphonate group (anti-P_p) over the *p*H range 5 to 10. For anti-P_p this change suggests that antibody has been formed only in response to the doubly charged form of the phosphonate hapten. The change of binding constants for anti-R_p differs from that of anti-P_p and suggests that antibody has been formed in response to the singly charged form of the arsonate hapten as well as in response to the doubly charged form. The data also indicate that perhaps as much as two-thirds of the anti-R_p antibody have been formed in response to the singly charged form.

Rabbit antibodies formed in response to azobenzenearsonate groups have been used in several studies of the interaction of antibody with hapten.[1] However, since azobenzenearsonates exist in two ionic forms at physiologic *p*H values, it is possible that the antibodies formed consist of those formed in response to the singly charged ion and those formed in response to the doubly charged ion. Each antibody would presumably react preferentially with the ion in response to which it had been formed. The extent of combination with hapten of the antibody in an antiazobenzenearsonate antiserum would depend on the relative amounts of the charged forms of the hapten present as well as on the relative amounts of the two types of antibody present and on the respective affinities of the antibodies for the two charged forms of the hapten.

The present study was carried out to determine whether the antibody (anti-R_p antibody) formed in response to the *p*-azobenzenearsonate group will react with the singly charged form as well as with the doubly charged form. If the anti-R_p antibody were formed in response to only one of the two charged forms of the antigen, the binding of hapten (benzenearsonate or derivative) by anti-R_p serum would be expected to be more strongly dependent on *p*H in the range where the ratio of singly to doubly charged ions changes rapidly with *p*H than it would be if the anti-R_p serum contained antibody formed in response to both ionic species. Studies by Epstein and Singer (1958) of light scattering by complexes of the anti-R_p antibody and terephthalanilide-*p,p'*-diarsonate have shown a sharp decrease in the association of antibody and the dihapten substance in the range from *p*H 6 to 11. This parallels the decrease in relative concentration of the doubly charged arsonate ions. However, Epstein and Singer have attributed the observed decrease to buffer ion effects rather than to the increase in the monobasic species. They assumed incorrectly that both ionized forms were equally effective.

We have measured the apparent binding constants at various *p*H values for the reaction of *p*-(*p*-hydroxyphenylazo)benzenearsonic acid (hereinafter called *p*-hydroxyphenyl-R_p) and *p*-(*p*-hydroxyphenylazo)benzenephosphonic acid (hereinafter called *p*-hydroxyphenyl-P_p) with the γ-globulin fraction of anti-R_p sera by the equilibrium dialysis method. The benzenephosphonate derivative was used because the phosphonates are known to cross-react strongly with anti-R_p antibody and would be expected to show a somewhat different dependence of binding on *p*H than the arsonates because of the greater proportion of doubly charged ions present at any *p*H value due to the larger, second dissociation constant. As a control, similar measurements were made with antibody formed in response to *p*-azobenzenephosphonate (anti-P_p antibody). Since *p*-azobenzenephosphonate (pK_a = 6.4) at physiologic *p*H is about 90% in the doubly ionized form, compared with 25% for the *p*-azobenzenearsonate (pK_a = 7.9), anti-P_p antibody might be expected to contain more antibody formed in response to the doubly charged form than would be the case for anti-R_p antibody and, therefore, would show a different dependence of binding on *p*H than would be shown by anti-R_p antibody. The data indicate that anti-P_p antibody was formed almost exclusively in response to the doubly charged form of the hapten and that the anti-R_p antibody was formed in response to both the singly charged and doubly charged forms.

Experimental

Antigens.—The immunizing antigens (R_p-BGG and P_p-BGG) were prepared by coupling diazotized *p*-aminobenzenearsonic acid (543 mg) and diazotized *p*-aminobenzenephosphonic acid (300 mg) with bovine γ-globulin (Fraction II [10 g]). Test antigens were prepared by coupling the diazotized amines (97 mg and 76 mg respectively) to ovalbumin (1 g) and were purified by acetone extraction (Nisonoff and Pressman, 1958).

Antisera.—Rabbits were injected intravenously with 1 ml of 1% R_p-BGG antigen three times a week for 3 weeks. One week after the last injection and weekly thereafter the animals were bled (ca. 30 ml of blood obtained). One ml of antigen was injected after each bleeding. Anti-P_p sera were obtained in a similar manner except that the first bleedings were made after 7–8 weeks of injections. Antisera were pooled according to titer. Only one pool of antiserum for each antibody was used for all the experiments reported here.

γ-Globulin fractions were prepared from pooled sera by three sodium sulfate precipitations at room temperature (Nisonoff and Pressman, 1958). Normal γ-

* Supported in part by Grant E-2342 from the National Institute of Allergy and Infectious Diseases, USPHS.

[1] For example see Landsteiner (1945), Pauling and Pressman (1945), and Epstein and Singer (1958).

globulin was prepared in the same manner from serum of normal rabbits. The γ-globulin preparations examined by free boundary electrophoresis at *p*H 8.6 (20 mg/ml, barbital buffer) showed less than 5% of other proteins.

Haptens.—*p* - Aminobenzenephosphonic acid was kindly supplied by Dr. G. O. Doak.

p-Hydroxyphenyl-R_p was prepared as described previously (Pauling and Pressman, 1945). It was crystallized twice from aqueous ethanol before use. The *p*-hydroxyphenyl-P_p was prepared in a similar manner by coupling diazotized *p*-aminobenzenephosphonic acid (1 g) with a ten-fold excess of phenol at *p*H 9.0–9.5. The reaction mixture was adjusted to a *p*H of 7 and extracted with ether. The *p*H was adjusted to 2–3 and the precipitated material collected and dried. The precipitate (0.5 g) was dissolved in 40 ml of boiling 6 N HCl. Crystals (0.3 g) were obtained from the cooled solution. The product was recrystallized from 40% ethanol and then from glacial acetic acid.

Anal. Calcd. for $C_{12}H_{11}O_4N_2P$: C, 51.80; H, 3.99; P, 11.14. Found, C, 51.37; H, 4.26; P, 10.98.[2]

The *p*-aminobenzenearsonic acid used was Eastman Kodak's White Label material.

Buffers.—The buffers used were adjusted at 25° to a constant ionic strength of 0.16 and the desired *p*H. Borate buffers of *p*H 8, 9, and 10 were prepared by mixing a solution of 0.16 M sodium hydroxide with a solution of 0.2 M boric acid containing 0.16 M sodium chloride. An additional buffer at *p*H 10 was prepared from a solution of 0.02 M boric acid containing 0.16 M NaCl. The buffers used at *p*H 6 and 7 were prepared from sodium dihydrogenphosphate and 1 N sodium hydroxide diluted to give final total phosphate concentrations of 0.05, 0.0125, and 0.005 M plus enough sodium chloride to give an ionic strength of 0.16. The buffer used at *p*H 5 was prepared from 0.2 M acetic acid containing 0.16 M NaCl and 0.16 M sodium hydroxide to give a *p*H of 5.0. At 5°, the temperature at which the binding experiments were carried out, the *p*H values are 0.1 to 0.2 units higher (depending on the buffer) than at 25°, the temperature at which the *p*H determinations were made.

Determination of Binding by Equilibrium Dialysis.—The binding of hapten by antibody was measured by means of equilibrium dialysis. A small dialysis sac made from 0.25 in. cellulose tubing containing 0.5–0.6 ml of γ-globulin, 10 mg/ml, was immersed in 90–100 ml of hapten solution prepared by dissolving the hapten in buffer of the desired *p*H. This system was equilibrated in a water bath at 5° for 1–2 days. At the end of the mixing period, each sac was cut open and the contents analyzed for hapten and for γ-globulin by the spectrophotometric method described below. The free hapten concentration was determined by analysis of the contents of a sac that contained only buffer originally. The bound hapten concentration was given by the difference between the free hapten concentration and the concentration of hapten in the protein solution. That equilibrium was attained by this procedure was shown by adding hapten to antibody γ-globulin at a high free hapten concentration and then equilibrating this mixture and a sample of the same antibody γ-globulin without added hapten at a lower free hapten concentration. The bound hapten concentration was the same in each case. The *p*H and salt concentration of the γ-globulin solutions were adjusted before equilibrium dialysis by extensive dialysis against the desired buffer followed by centrifugation at 14,000 rpm to remove small amounts of precipitate before use. Preparations for use at *p*H 8 were first dialyzed against phosphate buffer at *p*H 6. The small amount of precipitate that was obtained was removed by centrifugation, and the supernatant was adjusted to *p*H 8 by exhaustive dialysis against borate buffer. This procedure prevents the formation of any precipitate during equilibrium dialysis.

To analyze for hapten, 0.2 ml of the contents of a sac were diluted with 0.2 ml of 0.2 N sodium hydroxide. This concentration of base gave a final *p*H of about 13. Since the pK_a of the hydroxyl group is about 8, this is adequate to ionize the phenol completely. The optical density (OD) of this strongly basic solution was determined at 440 mμ in quartz micro cells. After correction of this optical density at 440 mμ for absorption due to the γ-globulin (for blanks dialysis sacs containing γ-globulin were mixed in buffer solution under the same conditions), the molar concentration of total hapten was calculated from the optical density for a 10^{-5} M solution (0.120 ± 0.002 and 0.124 ± 0.002 for *p*-hydroxyphenyl-P_p and *p*-hydroxyphenyl-R_p respectively) diluted with base as described. These values of the optical density calculated for a 10^{-5} M solution are constant within ± 1–2% in the concentration range of 3×10^{-6} to 10^{-4} M. The presence of the γ-globulin was found not to affect the extinction coefficient of the hapten in the basic solutions prepared in this manner.

The precision of measuring the hapten concentration is shown by the fact that the optical density of hapten rarely differed by more than 0.002 units between duplicate samples for a free hapten concentration determination. It is not possible to apply this same test to the determination of bound hapten because each determination is unique in that the protein concentration varies slightly from sac to sac, but a single measurement is not expected to be in error by more than ±0.001–0.002 OD units. The error in determining the bound concentration is larger because the value is obtained by difference of two hapten concentrations and because there is a correction to be applied due to the protein absorption at 440 mμ. The only test of precision for bound hapten concentration is the difference between $1/r$ values ($1/r$ = moles of γ-globulin per mole of bound hapten) for so-called duplicate determinations. This was rarely greater than 2–3% except at low binding levels.

For determination of γ-globulin, 0.1 ml portions of the sac contents were diluted with 0.1 N sodium hydroxide to 5 ml. After about 2 hours the optical densities at 440 mμ and 280 mμ were measured.[3] The precision of this value is about ± 2% based on the precision of the optical density measurement. The optical density of the γ-globulin at 280 mμ was corrected for hapten absorption at this wave length which was calculated from the optical density at 440 mμ and the known ratio (OD_{280}/OD_{440}) for the hapten. The concentration of γ-globulin in mg/ml was calculated from the equation $(OD_{280}/1.5) \times 0.93$ where 1.5 is the OD of a 1 mg/ml solution in borate buffer, *p*H 8, and 0.93 is the factor for converting OD_{280} in base to OD in borate buffer. The

[2] Microanalysis by Spang Microanalytical Laboratory, Ann Arbor, Mich.

[3] The optical density at 280 mμ of γ-globulin in 0.1 N NaOH changes with time. There is a rapid rise in the optical density during the first hour after mixing to a value that remains constant within 1%, for at least one hour. Two hours after mixing there is a slow decrease in optical density of about 1% or less per hour. The measurements reported here were made during the third and fourth hours after mixing, so the errors between samples due to changes in optical density with time were not greater than 1–2% of the total values.

TABLE I
BINDING DATA FOR ANTI-R_p γ-GLOBULIN[a]

pH	Concentration (M × 10^5) Free	Bound	Concentration γ-Globulin (M × 10^5)	$\frac{1}{c}$	$\frac{1}{r}$	
			Binding of *p*-Hydroxyphenyl-R_p			
9	1.020	0.879	6.28	9.80	7.15	Borate buffer, 0.13 M total borate, 0.055 M borate ion
	0.508	0.750	6.36	19.7	8.48	
	0.290	0.638	6.20	34.5	9.71	
8	0.932	1.220	6.47	10.7	5.30	Borate buffer, 0.17 M total borate, 0.023 M borate ion; cycled through *p*H 6
	0.468	1.089	6.59	21.4	6.05	
	0.274	0.936	6.68	36.5	7.15	
8	0.931	1.287	6.81	10.7	5.29	Borate buffer, 0.17 M total borate; *not* cycled through *p*H 6
	0.572	1.126	6.69	17.5	5.94	
	0.379	1.008	6.58	26.4	6.52	
	0.282	0.919	6.59	35.5	7.22	
7	0.960	0.536	5.95	10.4	11.1	Phosphate buffer, 0.0125 M total phosphate
	0.472	0.423	6.05	21.2	14.3	
	0.319	0.331	5.99	31.4	18.2	
6	0.964	0.532	7.61	10.4	14.3	Phosphate buffer, 0.0125 M total phosphate
	0.580	0.387	7.64	17.3	19.7	
	0.391	0.306	7.63	25.6	25.0	
	0.298	0.250	7.81	33.6	31.3	
			Binding of *p*-Hydroxyphenyl-P_p			
9	1.000	0.783	6.13	10.0	7.83	Borate buffer, 0.13 M total borate
	0.500	0.688	6.45	20.0	9.39	
	0.308	0.538	6.26	32.5	11.6	
8	0.950	1.148	6.39	10.5	5.57	Borate buffer, 0.17 M total borate
	0.484	1.004	6.44	21.7	6.42	
	0.288	0.862	6.34	34.7	7.36	
7	0.992	0.883	6.02	10.1	6.82	Phosphate buffer, 0.0125 M total phosphate
	0.500	0.696	6.07	20.0	8.73	
	0.317	0.554	6.05	31.6	10.9	
7	0.908	0.701	6.79	11.0	9.68	Phosphate buffer, 0.05 M total phosphate
	0.566	0.534	6.75	17.7	12.5	
	0.371	0.443	6.69	27.0	15.3	
	0.279	0.367	6.82	35.8	18.6	
6	0.970	0.934	6.40	10.3	6.85	Phosphate buffer, 0.0125 M total phosphate
	0.592	0.758	6.40	16.9	8.44	
	0.400	0.637	6.34	25.0	9.94	
	0.308	0.559	6.29	32.5	11.3	

[a] The values given for free hapten concentrations are the averages of duplicate determinations. The values for the bound concentrations are averages of two determinations which are not strictly duplicates, since the protein concentration varies from one sample to another (see Experimental). The protein concentrations for a given free hapten concentration, however, usually did not differ by more than 2–3%. The values of $1/c$ and $1/r$ are also averages of two values. The binding constants, however, were obtained from plots made from individual values (unaveraged).

molar concentration was calculated using 160,000 for the molecular weight of γ-globulin.

Ionization Constants.—Values for the ionization constants (pK_a) of the phenolic hydroxyls in *p*-hydroxyphenyl-P_p and *p*-hydroxyphenyl-R_p were determined by a spectroscopic method (Flexser *et al.*, 1935) based on change of spectrum with *p*H.

RESULTS

The amounts of *p*-hydroxyphenyl-R_p and *p*-hydroxyphenyl-P_p bound at various free hapten concentrations by the γ-globulin fractions of anti-R_p and anti-P_p antisera were determined at several *p*H values. The results are given in Tables I and II.

For the interaction of antibody combining sites with hapten, Ab + HP ⇄ AbHp, equation (1) (Nisonoff and Pressman, 1958) is obtained from the mass action equation $K = [AbHp]/[Ab][Hp]$, where b is the concentration of bound hapten, c is the concentration of

$$\frac{1}{b} = \frac{1}{cKA} + \frac{1}{A} \qquad (1)$$

free hapten, A is the total concentration of antibody combining sites, and K is the binding constant.

This becomes equation (2), which is used when bindings are carried out with the whole γ-globulin fraction instead of specifically purified antibody. This equation also permits correction for variations in protein concentration. Here r is the ratio of concentration of bound hapten to the concentration of γ-globulin, α is the fraction of the γ-globulin that is antibody, and n is the number of binding sites per antibody molecule. The concentration of binding sites is obtained from extrapolation of plots of $1/r$ against $1/c$. It is the product of the reciprocal of the intercept on the ordinate and the γ-globulin concentration. The binding constant, K, is calculated from the slope with the value of αn obtained from the intercept. Deviations from linearity of plots of $1/r$ *vs.* $1/c$ are interpreted as due to heterogeneity of antibody populations.

$$\frac{1}{r} = \frac{1}{cK\alpha n} + \frac{1}{\alpha n} \qquad (2)$$

At *p*H 8 plots of *p*-hydroxyphenyl-R_p and of *p*-hydroxyphenyl-P_p with either anti-R_p or anti-P_p γ-globulin were essentially linear in the free hapten concentration range of 3×10^{-6} to 10^{-5} M, thus showing very little heterogeneity. In some experiments at *p*H 8 binding was carried to a concentration of free hapten of 10^{-4} M and the $1/r$ *vs.* $1/c$ plots for anti-R_p and anti-P_p showed some curvature at concentrations greater

TABLE II
BINDING DATA FOR ANTI-P_p γ-GLOBULIN[a]

pH	Concentration (M × 10^5) Free	Bound	Concentration γ-Globulin (M × 10^5)	$\frac{1}{c}$	$\frac{1}{r}$	
			Binding of *p*-Hydroxyphenyl-P_p			
10	1.041	0.722	6.58	9.60	9.13	
	0.625	0.554	6.43	16.0	11.6	Borate buffer, 0.095 M total borate; 0.084 M borate ion
	0.433	0.413	6.55	23.1	15.9	
	0.321	0.333	6.45	31.2	19.4	
10	1.117	1.175	7.44	8.95	6.33	
	0.566	0.843	7.30	17.7	8.66	Borate buffer, 0.018 M total borate; 0.017 M borate ion
	0.346	0.666	7.38	28.9	12.0	
9	1.020	1.456	6.81	9.80	4.68	
	0.618	1.219	6.77	16.2	5.78	Borate buffer, 0.13 M total borate
	0.408	1.013	6.77	24.5	6.69	
	0.317	0.849	6.82	31.6	8.04	
8	0.983	1.896	6.55	10.2	3.46	
	0.475	1.760	6.63	21.1	3.77	Borate buffer, 0.17 M total borate, 0.023 M borate
	0.296	1.547	6.66	33.8	4.31	
7	0.916	0.780	6.29	10.9	8.08	
	0.471	0.521	6.36	21.2	12.2	Phosphate buffer, 0.05 M total phosphate
	0.283	0.383	6.42	35.3	16.8	
7	0.966	1.242	6.75	10.4	5.44	
	0.479	0.930	6.77	20.9	7.29	Phosphate buffer, 0.0125 M total phosphate
	0.288	0.766	6.78	34.7	8.86	
7	1.038	1.392	6.18	9.63	4.44	
	0.517	1.138	6.03	19.4	5.30	Phosphate buffer, 0.005 M total phosphate
	0.325	0.934	6.23	30.8	6.64	
6	0.928	1.227	6.32	10.8	5.16	
	0.433	0.981	6.31	23.1	6.43	Phosphate buffer, 0.0125 M total phosphate
	0.258	0.767	6.13	38.8	7.99	
5	1.075	1.200	6.61	9.30	5.50	
	0.541	0.880	6.66	18.5	7.57	Acetate buffer, 0.105 M total acetate
	0.334	0.687	6.75	30.0	9.83	
			Binding of *p*-Hydroxyphenyl-R_p			
8	0.952	1.836	6.82	10.5	3.71	
	0.467	1.565	6.73	21.4	4.31	Borate buffer, 0.17 M total borate
	0.278	1.295	6.72	36.0	5.19	
7	0.972	0.456	6.36	10.3	14.0	
	0.488	0.270	6.40	20.5	23.7	Phosphate buffer, 0.0125 M total phosphate
	0.306	0.166	6.50	32.7	39.0	
6	0.943	0.250	6.49	10.6	26.0	
	0.480	0.121	6.39	20.8	52.9	Phosphate buffer, 0.0125 M total phosphate
	0.294	0.069	6.37	34.0	92.0	

[a] See footnote[a] of Table I.

than 10^{-5} M. Some, if not most, of this curvature for anti-R_p γ-globulin was found to be due to nonspecific binding (measured with normal rabbit γ-globulin). No correction for nonspecific binding in the 3×10^{-6} to 10^{-5} M range was needed. Moreover, at all *p*H values examined between 6 and 10 the nonspecific binding was found to be essentially zero in this concentration range. The binding curves in the 10^{-5} to 3×10^{-6} M range were used for measuring binding constants. Because the curve is linear, it would appear that a homogeneous population is being considered in this range. At higher hapten concentrations errors in measurements become larger. Figures 1 and 2 show $1/r$ *vs.* $1/c$ plots for the binding of *p*-hydroxyphenyl-R_p to anti-R_p and to anti-P_p respectively. Values for binding constants obtained in the manner described above and $1/r$ intercepts are given in Tables III to VII.

The binding constants obtained in this manner are apparent binding constants only. They were obtained without correction for degree of ionization of the haptens or for buffer effects. In most cases the uncertainty in the apparent binding constants was found to be less than ±10% either by duplicate measurements or by determining the error in estimating the slope of the line drawn through the points. In a few cases the possible range of values is given.

At *p*H 7 the apparent binding constants of *p*-hydroxyphenyl-P_p with anti-R_p or with anti-P_p were found to be dependent on the total phosphate concentration of the buffers used (Table V). In the case of the binding to anti-P_p γ-globulin, the data show that the phosphate competes for the binding site. The combination of hapten and antibody as affected by

TABLE III
APPARENT BINDING CONSTANTS FOR ANTI-R_p AT *p*H VALUES OF 6, 7, AND 8

pH	$K \times 10^{-4}$	$1/r$ Intercept	Antibody Sites Present per Mole of Total Globulin[a]
	p-Hydroxyphenyl-R_p		
6[b]	9.5	6.8	0.15
7[b]	23	7.6	0.13
8	65	4.6	0.22
	p-Hydroxyphenyl-P_p		
6[b]	23	4.8	0.21
7[b]	27	4.9	0.20
8	65	4.8	0.21

[a] Maximum uncertainty is ±0.02 (and is usually not greater than ±0.01). [b] At 0.0125 M total phosphate.

TABLE IV

APPARENT BINDING CONSTANTS FOR ANTI-P_p AT *p*H VALUES OF 5, 6, 7, AND 8

*p*H	$K \times 10^{-4}$	1/*r* Intercept	Antibody Sites Present per Mole of Total Globulin[a]
	p-Hydroxyphenyl-P_p		
5 Acetate	17	3.6	0.28
6 Phosphate[b]	43	4.2	0.24
7 Phosphate[b]	30	4.2 ± 0.2	0.24
8 Borate	91	3.1	0.32
	p-Hydroxyphenyl-R_p		
6 Phosphate[b]	1–2	—[c]	—
7 Phosphate[b]	4–5	3.8 ± 0.4	0.26 ± 0.03
8 Borate	58	3.2	0.31

[a] Maximum uncertainty is ±0.02 except where otherwise noted. [b] At 0.0125 M total phosphate. [c] Indeterminate owing to a relatively large error in extrapolation from low binding levels.

TABLE V

EFFECT OF PHOSPHATE ON APPARENT BINDING CONSTANTS OF *p*-HYDROXYPHENYL-P_p WITH ANTI-P_p AND WITH ANTI-R_p ANTIBODIES AT *p*H 7

Phosphate Conc. (M)	$K \times 10^{-4}$	1/*r* Intercept	Antibody Sites Present per Mole of Total Globulin[a]
	Anti-P_p		
0.05	12	4.4	0.23
0.0125	30	4.2	0.24
0.005	35	3.5	0.29
	Anti-R_p		
0.05	16	5.7	0.18
0.0125	27	4.9	0.20

[a] Maximum uncertainty is ±0.02 and is usually not greater than ±0.01.

TABLE VI

APPARENT BINDING CONSTANTS OF ANTI-P_p WITH *p*-HYDROXYPHENYL-P_p AT *p*H 8, 9, AND 10

*p*H	$K \times 10^{-4}$	1/*r* Intercept	Antibody Sites Present per Mole of Total Globulin[a]
8	91	3.1	0.32
9	19	3.0	0.33
10[b]	9	4.4	0.23
10[c]	13	3.8	0.26

[a] Maximum uncertainty is ±0.01. [b] 0.084 M borate ion concentration. [c] 0.018 M borate ion concentration.

TABLE VII

APPARENT BINDING CONSTANTS FOR ANTI-R_p AT *p*H 8 AND 9

*p*H	$K \times 10^{-4}$	1/*r* Intercept	Antibody Sites Present per Mole of Total Globulin[a]
	p-Hydroxyphenyl-R_p		
8	65	4.6	0.22
9	54	6.0	0.17
	p-Hydroxyphenyl-P_p		
8	65	4.8	0.21
9	36	6.0	0.17

[a] Maximum uncertainty is ±0.02.

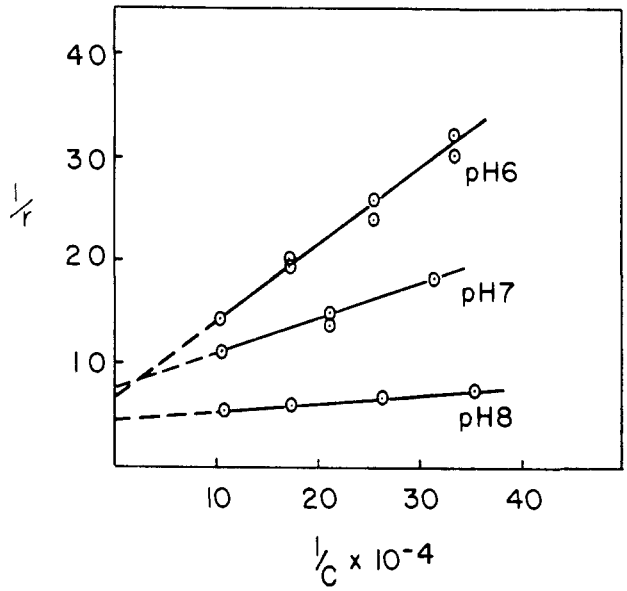

FIG. 1.—Binding of *p*-hydroxyphenyl-R_p by anti-R_p γ-globulin at different *p*H values. Points at each concentration of hapten are in duplicate and appear as single points where deviation is too small to be seen.

the binding of $HPO_4^=$ ion with the antibody combining site is given by equation (3).[4] This is a modification

$$\frac{1}{r} = \frac{1}{\alpha n} + \frac{1}{cK\alpha n} + \frac{Kp}{\alpha nK}(HPO_4^=) \qquad (3)$$

of equation (2), where K_p is the binding constant of $HPO_4^=$ and K is the binding constant of the hapten. A plot of $1/r$ values for a given free phosphonate hapten concentration against the concentration of $HPO_4^=$ ion at *p*H 7 gives a straight line. Since the hapten does not bind nonspecifically, $HPO_4^=$ ion appears to be competing for the binding site.

Although values at only two phosphate concentrations were used in the anti-R_p system, it appears that phosphate also competes for the site of anti-R_p antibody, as has been suggested by Epstein and Singer (1958) from studies on anti-R_p.

Ionization constants (pK_a values of the hydroxyl groups) of *p*-hydroxyphenyl-R_p and *p*-hydroxyphenyl-P_p were obtained from the effect of *p*H on the spectra of these compounds. The values are 7.9–8.0 and 8.2–8.3 respectively. Measurements of the second ionization constants of the arsonic acid and phosphonic acid were not made because the compounds were too insoluble to be titrated and the changes observed in the spectra as the *p*H changed appeared due to ionization of the phenolic hydroxyls. The pK_{a_2} values for the second dissociation of these two compounds were estimated by means of Hammett's free energy equation, $\log K - \log K^0 = \rho\sigma$ (Hammett, 1940).[5] For the arsonic acid the pK_{a_2} was estimated to be 7.9 and for the phosphonic acid the pK_{a_2} was 6.4.

[4] Equation (3) is derived from the mass action equations for the reactions Ab + Hp ⇄ AbHp and Ab + $HPO_4^=$ ⇄ Ab($HPO_4^=$).

[5] Data for this calculation were taken from the papers by Hammett and Paul (1934), Pressman and Brown (1943), and Jaffe *et al.* (1953). The influence of the phenolic hydroxyl is neglected, but on the basis of the small effect of hydroxyl on the pK_{a2} of benzenephosphonic acid (Jaffe *et al.*, 1953) it would appear to be within the uncertainty of the estimation from σ and ρ values.

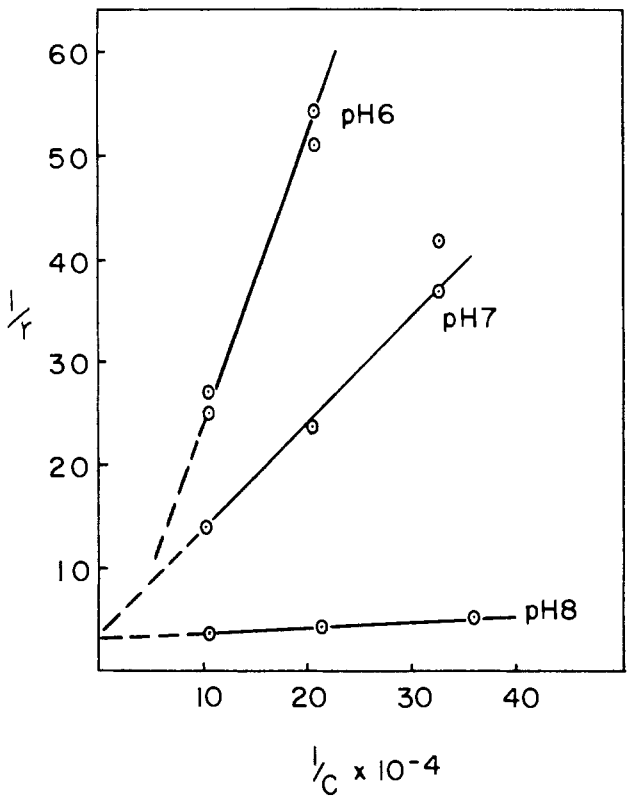

FIG. 2.—Binding of *p*-hydroxyphenyl-R_p by anti-P_p γ-globulin at different *p*H values. Points at each concentration of hapten are in duplicate and appear as single points where deviation is too small to be seen.

DISCUSSION

Change of Apparent Binding Constants with pH.—If only the doubly charged forms of the hapten, *p*-hydroxyphenyl-R_p or *p*-hydroxyphenyl-P_p, were bound significantly by antibody, the change in the ratio of apparent binding constants, K_P/K_R, would parallel the change in the ratio of the percentages of doubly charged ions, $\%P_p^=/\%R_p^=$, as the *p*H changes. For a given total free hapten concentration the amount of bound hapten is determined by the free concentration of the doubly charged ion. The change in binding with *p*H then parallels the change in the free concentration of the doubly charged ion as the degree of ionization changes with *p*H. Any consideration of changes in binding due to changes in the buffer composition from one *p*H value to another or to changes in the protein are avoided by a treatment of this sort, as long as the bindings of the two haptens are compared in buffers of the same composition.[6]

For anti-P_p γ-globulin the ratios of apparent binding constants at *p*H 8, 7, and 6 parallel the ratios of the percentages of doubly charged ions (Table VIII), indicating that only the doubly charged ions are being bound and that only antibody formed in response to the doubly charged ion is present in the anti-P_p γ-globulin. These data also mean that there cannot be more than a relatively weak cross-reaction between the singly charged arsonate hapten and antibody formed in response to the doubly charged form of the phosphonate, whereas a strong cross-reaction occurs between this antibody and the doubly charged form of arsonate hapten.

TABLE VIII
RATIOS OF APPARENT BINDING CONSTANTS AT DIFFERENT *p*H VALUES

*p*H	$\%P_p^{=a}/\%R_p^{=}$	K_P/K_R[b] Anti-P_p	K_P/K_R[b] Anti-R_p
8	1.75	1.56	1.0
7	7.3	6.7	1.2
6	24	29	2.4

[a] Calculated from data in Table IX. [b] Calculated from data in Tables III and IV.

The ratios of binding constants did not parallel the percentages of doubly charged ions for anti-R_p from *p*H 8 to 6 (Table VIII). The change of apparent binding constant with *p*H for binding of *p*-hydroxyphenyl-P_p to anti-R_p (Table III) is about the same as for the binding to anti-P_p (Table IV), but the binding of *p*-hydroxyphenyl-R_p hapten to anti-R_p γ-globulin at *p*H 6 and 7 is considerably greater than would be expected if only the doubly charged ion were binding. This can be interpreted as meaning that the singly charged ion of *p*-hydroxyphenyl-R_p is being bound by a second antibody present which has been formed in response to the singly charged *p*-azobenzenearsonate ion. Although it is possible that a single antibody formed in response to *p*-azobenzenearsonate binds significantly both ionic forms of *p*-hydroxyphenyl-R_p, in view of the structural similarity between arsonates and phosphonates and the fact that anti-P_p γ-globulin does not appear to bind the singly charged form of *p*-hydroxyphenyl R_p very much, the presence of two antibodies in anti-R_p γ-globulin, each reacting preferentially with only one of the charged forms of the hapten, appears to be a more suitable interpretation at this point.

Comparison of the binding constants for the combination of *p*-hydroxyphenyl-R_p and of *p*-hydroxyphenyl-P_p with anti-R_p at *p*H 9 with those at *p*H 8 (Table VII) suggests that a significant proportion (at least one third, *vide infra*) of the antibody of anti-R_p is specific for the doubly charged arsonate ion. The binding of *p*-hydroxyphenyl-R_p to an antibody formed in response to the doubly charged ion of azobenzenearsonate would be expected to increase more rapidly with an increase of *p*H from 8 to 9 or 10 than would the binding of *p*-hydroxyphenyl-P_p, because the proportion of doubly charged ion increases to a greater extent for *p*-hydroxyphenyl-R_p than for *p*-hydroxyphenyl-P_p over this *p*H range (Table IX). However, there seems to be an opposing effect of increased *p*H which decreases binding similar to the decrease in binding of *p*-iodobenzoate by anti-*p*-azobenzoate antibodies observed upon changing the *p*H from 8 to 9 to 10 (Pressman *et al.*, 1961b).[7] In this latter case the decrease was attributed to the ionization of a tyrosine in the antibody site. A similar effect is observed here, probably for the same reason. Anti-R_p does have a tyrosine in its site, according to work in this laboratory (Grossberg *et al.*, 1962; Pressman and Sternberger, 1951) (see also Koshland *et al.*, 1959) and binding decreases in this range as a result (Table VII). A decrease is likewise found for binding of *p*-hydroxyphenyl-P_p by anti-P_p (Table VI) from *p*H 8 to 9 to 10. The decrease with anti-R_p, however, is less for *p*-hydroxyphenyl-R_p than for *p*-hydroxyphenyl-P_p and is in accord with the relatively larger

[6] The degree of ionization of the hydroxyl group of the azo haptens will vary greatly in the *p*H range examined, and some effect on binding, probably small, might be expected. As long as only the ratio of the binding constants of the two haptens is being compared and because the change of ionization of the hydroxyl with *p*H is about the same for each hapten (pK_a is about 8 for each compound), variations in binding due to variations in ionization of the phenolic hydroxyls would probably cancel each other.

TABLE IX

APPROXIMATE PERCENTAGE OF DOUBLE CHARGED FORM PRESENT IN HAPTEN AT CRITICAL *p*H VALUES

*p*H	*p*-Hydroxyphenyl-P_p[a]	*p*-Hydroxyphenyl-R_p[b]
9	100	93
8	98	56
7.4	91	24
7	80	11
6	29	1.2
5	3.8	0.13

[a] $pK_{a_2} = 6.4$. [b] $pK_{a_2} = 7.9$.

percentage increase in doubly charged ions over the *p*H range 8 to 9 for the *p*-hydroxyphenyl-R_p.

Effect of Buffer Composition on Apparent Binding Constants.—Changes in buffer composition at a given *p*H are found to affect the value of the binding constant. Increase in the concentration of phosphate in buffers at *p*H 7 from 0.005 to 0.05 M was found to result in relatively large decrease in the apparent binding constant for binding of *p*-hydroxyphenyl-P_p to anti-P_p (a factor of 3 for increase of phosphate from 0.005 to 0.05 M) and to anti-R_p (a factor of 1.6 for increase of phosphate from 0.0125 to 0.05 M) (Table V). This change is apparently due to competition for the binding site (see Results) by a buffer ion structurally similar to the haptens. Borate ion also appears to be weakly bound to anti-P_p γ-globulin. At *p*H 10 in borate buffer the apparent binding constant for anti-P_p γ-globulin and *p*-hydroxyphenyl-P_p is dependent on the total borate concentration (Table VI). The borate ion is structurally less related to the azobenzenephosphonate group, and the effect may be considered as a specific anion effect in much the same manner as suggested for the effect of various anions on the binding of *p*-iodobenzoate to anti-X_p (Pressman *et al.*, 1961a). As a result of these buffer effects, close correlation between degree of ionization of a hapten and apparent binding constants at different *p*H values cannot be expected; however, the buffer effects can be minimized by comparing ratios of binding constants to ratios of degrees of ionization as carried out above.

Other differences between observed and expected changes in the apparent binding constants as the *p*H changes can be explained also in terms of buffer competition for the binding sites. Thus the binding constant for *p*-hydroxyphenyl-P_p and anti-P_p γ-globulin at *p*H 6 is somewhat larger than that at *p*H 7 (Table IV) instead of being smaller, as expected from the conversion of the doubly charged phosphonate into the singly charged ion. This difference can be interpreted as being due to a greater inhibition of binding of *p*-hydroxyphenyl-P_p by the higher concentration of the doubly charged phosphate, $HPO_4^=$, present at *p*H 7 than at *p*H 6, since this ion is structurally more related to the doubly charged hapten in response to which the antibody appears to be formed than is the singly charged phosphate ion $H_2PO_4^-$.

The Effect of pH on Apparent Concentration of Combining Sites.—Theoretically, the value of $1/r$ intercept which gives the number of binding sites should be the same for all conditions where the binding site has not been altered regardless of the extent of ionization of the hapten or presence of any competing ions. We would expect to find no difference between intercepts at any *p*H or for any buffer composition if extrapolation is made from high enough hapten concentration. Only a change in binding constant due to altered protein (other than at the binding site) would be seen. As carried out here, extrapolation is made from hapten concentrations (10^{-6} to 10^{-5} M free hapten) which are only sufficiently high to give information about the sites showing a free energy of combination of over −7.25 Kcal. However, because the $1/r$ intercepts used to calculate K and the concentration of binding sites are obtained by extrapolation from partial binding curves, variations in the observed number of sites might well be expected. Since it is unlikely that the complete binding curves are linear under all the conditions studied, a change in slope of the binding curve due to variations in buffer composition or *p*H could result in different $1/r$ intercepts when extrapolation is made from short portions of the binding curves.

In fact, there does appear to be a shift in concentration of binding sites with *p*H (Tables III, IV, VI, and VII) and with buffer composition (Table V). However, whether these shifts are due to errors in the extrapolations or to changes in the effective number of sites as a result of examining only part of the antibody present by use of only part of the binding curves is not clear. Most of the shifts appear to be small and possibly within experimental error. Only in the case of binding of *p*-hydroxyphenyl-R_p by anti-R_p γ-globulin is a change in concentration of sites large enough to appear significant. At *p*H 6 or 7 the concentration of sites is about two-thirds what it is at *p*H 8 (Table III) (Fig. 1).[8] At *p*H 6 and 7 there is relatively little doubly charged hapten (1% and 10% respectively) and the extent of binding of the doubly charged ion at any particular total hapten concentration in consequence is greatly reduced. Since anti-R_p γ-globulin is thought to contain antibody formed in response to the singly charged azobenzenearsonate, the singly charged form of *p*-hydroxyphenyl-R_p is the form mainly bound by the antibody at these *p*H values.

If it is assumed that the observed shift in concentration of sites is not due to errors, then the extrapolated $1/r$ intercept at *p*H 6 (Table III) can be considered to give a value for the concentration of sites in anti-R_p that is 68% of the number measured at *p*H 8. This value may be somewhat larger than the number of sites formed in response to the singly charged ion, because some binding of doubly charged ions may occur, but the data do indicate an upper limit of two-thirds for the anti-R_p antibody formed in response to the singly charged ion. The smaller variation in intercepts in the P_p system was apparently due to the fact that anti-P_p antibody was formed almost exclusively in response to the doubly charged phosphate ion and

[7] Epstein and Singer (1958) found by light scattering that the association of a bihaptenic simple substance and antibody decreases in the range *p*H 9 to 10 and attributed the effect to dissociation of an ionizable group in the combining site of the antibody. However, the system they studied would be expected to show large dissociation effects due to increased repulsion of the antibody molecules for each other because of increasing negative charge. This type of repulsion causes a decreased precipitability of antibody (Nisonoff and Pressman, 1960; Marrack and Orlans, 1954). Although part of the decrease in light scattering observed by Epstein and Singer may well be due to dissociation of a group in the site, the phenomenon of the increased solubility due to charge on the antibody does not permit the conclusion that decreased light scattering is due to dissociation of a group in the site.

[8] The increase in the $1/r$ intercept with *p*H does not mean that antibody sites are being lost by denaturation or otherwise at *p*H 6 or 7 compared with *p*H 8. The binding curve at *p*H 8 for *p*-hydroxyphenyl-R_p or *p*-hydroxyphenyl-P_p and anti-R_p γ-globulin cycled through *p*H 6 gives the same intercept (and the same binding constant) that the binding curve does for anti-R_p γ-globulin that had not been exposed to *p*H 6 or 7.

that a significant concentration of the doubly charged phosphonate ion remains at *p*H 6 and 7.

REFERENCES

Epstein, S. I., and Singer, S. J. (1958), *J. Am. Chem. Soc. 80*, 1274.

Flexser, L. A., Hammett, L. P., and Dingwall, A. (1935), *J. Am. Chem. Soc. 57*, 2103.

Grossberg, A. L., Radzimski, G., and Pressman, D. (1962), *Biochemistry 1*, 391.

Hammett, L. P. (1940), Physical Organic Chemistry, New York, McGraw-Hill Book Co., chap. VII.

Hammett, L. P., and Paul, M. A. (1934), *J. Am. Chem. Soc. 56*, 827.

Jaffe, H. H., Freedman, L. D., and Doak, G. O. (1953), *J. Am. Chem. Soc. 75*, 2210.

Koshland, M. E., Englberger, F., and Koshland, D. E., Jr. (1959), *Proc. Nat. Acad. Sci. U. S. 45*, 1470.

Landsteiner, K. (1945), The Specificity of Serological Reactions, Cambridge, Mass., Harvard University Press.

Marrack, J. R., and Orlans, E. S. (1954), *Brit. J. Exp. Path. 35*, 389.

Nisonoff, A., and Pressman, D. (1958), *J. Immunol. 80*, 417.

Nisonoff, A., and Pressman, D. (1960), *J. Immunol. 83*, 138.

Pauling, L., and Pressman, D. (1945), *J. Am. Chem. Soc. 67*, 1003.

Pressman, D., and Brown, D. H. (1943), *J. Am. Chem. Soc. 65*, 540.

Pressman, D., Nisonoff, A., and Radzimski, G. (1961a), *J. Immunol. 86*, 35.

Pressman, D., Nisonoff, A., Radzimski, G., and Shaw, A. (1961b), *J. Immunol. 86*, 489.

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

Effects of the Amidination Reaction on Antibody Activity and on the Physical Properties of Some Proteins*

LEON WOFSY†‡ AND S. J. SINGER‡

From the Department of Chemistry,§ Yale University, New Haven, Connecticut

Received July 26, 1962

Essentially all lysine residues of proteins can be amidinated under mild conditions without introducing gross changes in protein structure and physical properties. Partially and exhaustively amidinated bovine serum albumin and similarly modified rabbit antibody to benzenearsonic acid have been studied with respect to electrophoretic properties, sedimentation in the ultracentrifuge, optical rotation, and ultraviolet absorption. Six rabbit antibodies—three of them to antigenic or haptenic determinants bearing negatively charged groups—have been exhaustively amidinated to determine whether the particular antibody sites might involve complementary, positively charged ϵ-ammonium lysine residues. In each instance, antibody binding capacity was largely retained despite such modification. It has been concluded that lysine residues cannot be vital components of the reactive sites of the antibodies examined.

It has been suggested that in certain antigen-antibody systems a positively charged lysine ammonium group on the antibody molecule might be complementary to a negatively charged antigenic function (Heidelberger and Kabat, 1929; Chow and Goebel, 1935; Kleinschmidt and Boyer, 1952; Singer, 1955; Epstein and Singer, 1958). Previous investigations into this possibility have proceeded by chemically modifying the free amino groups of certain rabbit antibodies, and determining the effects of such modification on the antibody activity. Such studies have made use of either or both of two modification reactions: acetylation (Chow and Goebel, 1935; Marrack and Orlans, 1954; Singer 1955; Nisonoff and Pressman, 1958) and guanidination (Singer, 1955; Habeeb *et al.*, 1959). Each of these reactions, however, presents serious problems and limitations. In neither case can all lysine residues be reacted under conditions that do not cause nonspecific inactivation or denaturation of antibody. Extensive acetylation of amino groups results in a substantial increase in the net negative charge on a protein at neutral *p*H, since neutral functions replace positively charged groups. Intramolecular electrostatic repulsions may become sufficiently pronounced to produce gross conformational changes in the antibody molecules (Habeeb *et al.*, 1959). Such nonspecific conformational alterations in antibody molecules inevitably confuse the issue of whether any observed inactivation of antibody may conceivably be attributed to a specific chemical change within the binding sites. (Thus, it has been shown by Nisonoff and Pressman [1958] that antibody to a conjugated benzoate protein undergoes a severe loss in precipitating capacity after only 20% acetylation of amino groups—but the reactive sites have not lost their ability to bind a benzoate hapten up to the point of some 70% acetylation. If more extensive acetylation is produced, all antibody activity is eventually lost.)

Guanidination does not significantly change the charge on protein molecules at neutral *p*H, and does not appear to produce gross conformational changes in the molecules. However, in certain cases it is necessary to subject a protein to highly undesirable reaction conditions, a *p*H of 10.0–10.5 for 72 hours, in order to guanidinate a maximum of only about 70% of the amino groups (Hughes *et al.*, 1949).

By utilizing the amidination reaction, it has been possible in the present studies to modify chemically under mild conditions essentially all of the ϵ-NH_2-lysine residues of rabbit antibodies, while producing

* The studies in this paper were taken in part from the thesis submitted by L. W. in partial fulfillment of the requirements for the Ph.D. degree in Chemistry, Yale University, June, 1961. The work was supported in part by grants from the United States Public Health Service [RG-3207(C5) and E-1204(C4)].

† Monsanto Chemistry Fellow, 1961.

‡ Present address: Department of Biology, University of California, San Diego, La Jolla, Calif.

§ Contribution No. 1704.