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Differences among Antibodies Formed in Response to the *p*-Azobenzenephosphonate and *p*-Azobenzenearsonate Haptens*

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The relative binding constants of various substituted benzenephosphonates and benzenearsonates for the reaction with rabbit antibodies formed in response to the *p*-azobenzenephosphonate (P_p) haptenic group have been measured by hapten inhibition of specific precipitation. These values exhibit a variation with hapten structure that is distinctly different from that shown with antibodies formed in response to the *p*-azobenzenearsonate (R_p) haptenic group. Although anti-R_p antiserum contains two distinct antibodies, one formed in response to the doubly charged ion, the other to the singly charged ion, and anti-P_p antiserum apparently contains primarily antibody formed in response to the doubly charged ion, the difference between the two antisera in reaction patterns with various haptens appears to be attributable, for the most part, to a difference between antibodies formed in response to the doubly charged ions. The data are interpreted to mean that the combining site of the anti-P_p antibody fits more loosely about doubly charged benzenephosphonate than the combining site of anti-R_p antibody fits about doubly charged benzenearsonate.

Recent studies (Kreiter and Pressman, 1963) have indicated that anti-R_p serum contains two populations of antibodies formed in response to the two ionic forms of *p*-azobenzenearsonate. In contrast, anti-P_p serum contains antibody formed only in response to the doubly charged phosphonate ion. Such a difference between the two antisera suggests that their reaction patterns with various haptens should be different.

Although a large amount of information is available on the specificity of antiserum formed in response to the *para*-azobenzenearsonate group (R_p system) (Landsteiner, 1945; Pauling and Pressman, 1945; Erlenmeyer and Berger, 1932), very little information is available regarding the specificity of the antiserum formed in response to the structurally similar *para*-azobenzenephosphonate group (P_p system) except that it reacts strongly with benzenearsonate haptens (Kreiter and Pressman, 1963). The structural similarity between the benzenearsonate and the benzenephosphonate groups was found by Erlenmeyer and Berger (1932) to result in extensive cross reaction of these substances with anti-R_p antibody. On this basis it would appear that the specificity of anti-P_p antibodies should closely resemble the specificity of anti-R_p antibodies.

The relative binding constants for the reactions of several substituted benzenephosphonic acids and benzenearsonic acids with anti-P_p and anti-R_p rabbit γ-globulins have been measured by determining their ability to inhibit precipitation of antibody by the homologous hapten coupled to a foreign protein. A distinct difference in reaction patterns was found between the two systems that could not be attributed to the presence of more than one antibody in anti-R_p serum, but rather to a difference in specificity between antibody formed in response to the doubly charged *p*-azobenzenephosphonate ion and antibody formed in response to the doubly charged *p*-azobenzenearsonate ion.

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EXPERIMENTAL

Antigens.—The antigens (R_p-bovine γ-globulin and P_p-bovine γ-globulin used for immunizing rabbits were prepared by coupling diazotized *p*-aminobenzenearsonic acid and *p*-aminobenzenephosphonic acid to bovine γ-globulin (fraction II) (Kreiter and Pressman, 1963). Test antigens were prepared by coupling the diazotized amines to ovalbumin. The ovalbumin test antigens were purified by acetone extraction (Nisonoff and Pressman, 1958).

Antisera.—Rabbits were injected intravenously with 1 ml of 1% R_p-bovine γ-globulin antigen three times a week for 3 weeks. The animals were bled 1 week after the last injection and weekly thereafter. One ml of antigen was injected immediately after each bleeding. Anti-P_p sera were obtained in a similar manner. The antisera from the first 4 months were pooled according to the titer obtained with the ovalbumin test antigens. A single pool of each antiserum was used in all the experiments. The pool of anti-P_p antiserum and the pool of anti-R_p antiserum used here were the same as those used in our previous study (Kreiter and Pressman, 1963). The anti-P_p pool was obtained from the sera of nine rabbits, three of which contributed equally to about two-thirds of the total

TABLE I
COMBINATION OF HAPTENS WITH ANTI-P_p ANTIBODY

Hapten	K_0' (rel)	σ	$\Delta F°$ (rel) (kcal)	Amount of Precipitate[a,b] at Concentration of Hapten × 10^5 M		
				10.4	41.7	167
		Set 1				
Benzenephosphonic acid						
Unsubstituted	1.00	3		66	41	20
o-Chloro-	0.76	3	+0.15	71	44	22
m-Chloro-	1.05	2.5	−0.03	68	38	16
p-Chloro-	1.37	2.5	−0.17	61	33	12
3,5-Dichloro-	0.79	3	+0.13	68	44	21
		Set 2				
Benzenephosphonic acid						
Unsubstituted	1.00	3		69	43	21
m-Nitro-	0.66	3	+0.23	73	50	25
p-Nitro-	1.23	3	−0.11	82[c]	64[c]	39[c]
p-Nitro-*o*-methoxyl-	0.71	4	+0.19	72[d]	60[d]	39[d]
m-Amino-	0.68	2.5	+0.21	75	51	26
p-Amino-	1.63	3	−0.27	58	35	15
		Set 3				
Benzenephosphonic acid						
Unsubstituted	1.00	2		58	27	5
p-Nitro-	1.32	2.5	−0.15	49	28	5
p-(*p*-Hydroxyphenylazo)-	7.1	1	−1.1	100[e]	79[e]	30[e]
Benzenearsonic acid						
Unsubstituted	0.31	2	+0.65	86	47	22
p-Nitro-	0.69	2.5	+0.20	63	36	10
		Set 4				
Benzenearsonic acid						
Unsubstituted	1.00	3		76	55	34
o-Nitro-	0.65	3	+0.24	80	61	40
m-Nitro-	0.93	4	+0.04	72	57	36
p-Nitro-	1.62	3	−0.27	70[f]	48	26
		Set 5				
Benzenearsonic acid						
Unsubstituted	1.00	3		70	60	35[g]
p-(*p*-Hydroxyphenylazo)-	12.3	2.5	−1.38	79[h]	55[h]	27[h]
p-(*p*-Hydroxyphenylazo)-benzenephosphonic acid	26.4	2.5	−1.81	58[h,i]	37[h]	15[h]

[a] Percentage of the amount of precipitate obtained in the absence of hapten. The amounts of precipitate obtained in the absence of hapten for sets 1–5 are 303 μg, 275 μg, 106 μg, 274 μg, and 274 μg, respectively. [b] Average deviation from the mean values for the amounts of precipitate obtained with duplicates for sets 1–5 are ±4%, ±2%, ±8%, ±1%, and ±4%, respectively. [c] Concentrations are 1/4 of those in the heading. [d] Concentrations are 1/2 of those in the heading. [e] Concentrations are 1/50 of those in the heading. [f] At a concentration of 8.1×10^{-5} M a value of 80 was observed [g] At a concentration of 1×10^{-2} M a value of 24 was obtained. [h] Concentrations are 1/10 of those in the heading. [i] At a concentration of 2.5×10^{-5} M a value of 71 was observed.

pool. The anti-R_p pool was obtained from the sera of eight rabbits, two of which contributed equally to two-thirds of the pool. γ-Globulin fractions were prepared from the pooled antisera by three sodium sulfate precipitations at room temperature (Nisonoff and Pressman, 1958). γ-Globulin prepared in this manner showed less than 5% of other proteins by free-boundary electrophoresis at *p*H 8.6 in barbital buffer.

Haptens.—Benzenearsonic acid, benzenephosphonic acid, *o*-nitrobenzenearsonic acid, and *m*-nitrobenzenearsonic acid, obtained from Distillation Products Industries, were crystallized from ethanol-water mixture before use. *p*-Aminobenzenearsonic acid, benzoic acid, and sodium benzenesulfonate were used as received from Distillation Products Industries. *p*-Nitrobenzenearsonic acid was prepared by reaction of diazotized *p*-nitroaniline with sodium arsonite and purified by repeated precipitations from basic solution with hydrochloric acid and finally by crystallization from water. The preparation of *p*-(*p*-hydroxyphenylazo)benzenearsonic acid and *p*-(*p*-hydroxyphenylazo)-benzenephosphonic acid has been described previously (Kreiter and Pressman, 1963). *m*-Nitro-, *p*-nitro-, *p*-nitro-*o*-methoxy-, *m*-amino, *p*-amino, *o*-chloro, and *p*-chlorobenzenephosphonic acids were kindly supplied by Dr. G. O. Doak. *m*-Chloro-, and 3,5-dichlorobenzenephosphonic acids were obtained from Dr. R. K. Ingham. Stock solutions, 0.01 M, of these compounds were prepared by dissolving a weighed sample in sufficient 0.16 M NaOH to give a *p*H of 8.0–8.1. These solutions were then diluted with sodium borate buffer, *p*H 8. Stock solutions, 0.0001 M, of the two azo compounds were prepared by dissolving the compounds directly in borate buffer, *p*H 8. Solutions of the desired concentrations were prepared from the stock solutions by diluting with borate buffer.

Buffer.—The borate buffer used was prepared by mixing 165 ml of a solution of 0.16 M sodium hydroxide with 1 liter of a solution of 0.2 M boric acid containing 0.16 M sodium chloride. The *p*H of this solution was 7.97 ± 0.02 at 25°.

Reactions of Antibody with Antigen and Haptens.—Solutions of the test antigen were prepared by diluting the stock solution with borate buffer. The concentration (optimum) of test antigen required for maximum precipitation of antibody from a 20 mg/ml solution of γ-globulin was determined.

TABLE II
COMBINATION OF HAPTENS WITH ANTI-R_p ANTIBODY

Hapten	K_0' (rel)	σ	$\Delta F°$ (rel) (kcal)	Amount of Precipitate[a,b] at Concentration of Hapten × 10^5 M		
				10.4	41.7	167
		Set 1				
Benzenephosphonic acid						
Unsubstituted	1.00	2.5		60	36	9
m-Nitro-	1.20	2	−0.10	56	26	5
p-Nitro-	3.46	2	−0.69	65[c]	34[c]	8[c]
		Set 2				
Benzenephosphonic acid						
Unsubstituted	1.00	2.5		68	45	15
o-Chloro-	0.55	2.5	+0.33	75	58	26
m-Chloro-	1.52	2.5	−0.23	64	35	11
p-Chloro-	2.40	2	−0.48	51	21	4
3,5-Dichloro-	1.10	2.5	−0.05	67	41	17
		Set 3				
Benzenearsonic acid						
Unsubstituted	1.00	2		81[d]	58[d]	24[d]
o-Nitro-	0.54	2	+0.34	80	56	22
m-Nitro-	1.74	2	−0.31	85[e]	60[e]	27[e]
p-Nitro-	4.69	2	−0.85	89[f]	66[f]	35[f]
		Set 4				
Benzenearsonic acid						
Unsubstituted	1.00	2		77	52	20
p-Nitro-	4.68	2.5	−0.85	71[g]	47[g]	19[g]
p-(*p*-Hydroxyphenylazo)-	12.1	2	−1.38	76[h]	46[h]	18[h]
Benzenephosphonic acid						
Unsubstituted	1.00	2.5	0.0	74	51	25
p-(*p*-Hydroxyphenylazo)-	12.3	2.5	−1.37	73[i]	47[i]	20[i]

[a] Percentage of the amount of precipitate obtained in the absence of hapten. The amounts of precipitate obtained in the absence of haptens for sets 1–5 are 188 μg, 272 μg, 254 μg, and 322 μg, respectively. [b] Average deviation from the mean values for the amounts of precipitate obtained with duplicates for sets 1–4 are ±3%, ±4%, ±3%, and ±2%, respectively. [c] Concentrations are $^1/_4$ of those in the heading. [d] Concentrations are $^1/_2$ of those in the heading. [e] Concentrations are $^1/_4$ of those in the heading. [f] Concentrations are $^1/_{15}$ of those in the heading. [g] Concentrations are $^1/_4$ of those in the heading. [h] Concentrations are $^1/_{10}$ of those in the heading. At a concentration of 10^{-3} M a value of 7 was observed. [i] Concentrations are $^1/_{10}$ of those in the heading. At a concentration of 10^{-3} M a value of 11 was observed.

Experiments on the hapten inhibition of precipitation were carried out by adding 0.20 ml of hapten solution, 0.20 ml of test antigen (optimum concentration), and 0.20 ml of γ-globulin to a small tube in that order. Duplicate samples with each hapten were run at three or four concentrations. In order to obtain the amount of precipitate in the absence of the hapten, duplicate samples were run using 0.20 ml of borate buffer, *p*H 8, in place of the hapten solution. Blanks containing borate buffer, 0.40 ml, and 0.20 ml of γ-globulin were run at the same time to correct for small amounts of protein that precipitate from γ-globulin solutions standing at 3–5°. The mixtures were allowed to stand at 37° for 1 hour and then for 5 days at 3–5°. At the end of this time the mixtures were centrifuged, and the precipitates were washed twice with borate buffer and twice with 0.16 M sodium chloride. The washed precipitates were dissolved in 0.02 ml of 0.02 N sodium hydroxide. The solutions were transferred to Klett tubes and diluted with 0.8 ml of water. The protein solutions were analyzed by a modified Folin method (Lowry *et al.*, 1951). The intensity of the color after 1–1.5 hours was measured with a Klett colorimeter using a No. 66 filter. A calibration curve was prepared from γ-globulin solutions standardized by measuring the optical density of the γ-globulin in borate buffer at 280 mμ and taking the optical density of 1 mg/ml to be 1.5.

RESULTS

Anti-P_p Antibody.—The effects of various haptens on the precipitation of anti-P_p and anti-R_p antibody by P_p-ovalbumin and R_p-ovalbumin, respectively, are given in Tables I and II. The average relative binding constants K'_0 (rel) (relative to that of the reference haptens, taken as 1.00) and the heterogeneity constant, σ, for the combination of hapten and antibody were calculated as described previously, using the theory of heterogeneity of combining sites of antibody which assumes a Gaussian distribution of combining energies (Pauling *et al.*, 1944). The values of K'_0 (rel) have, in general, a precision of about ±10% as shown by duplicate measurements and by the error found in fitting calculated curves (from which the K'_0 (rel) values are computed) to the experimental points (i.e., plots of the percentage of the amount of precipitate obtained in the absence of hapten vs. the log of the moles of hapten added). The relative $\Delta F°$ values calculated for 5° are listed.

The charge specificity of anti-P_p antibody is similar to that found for various other antihapten antibodies (see Landsteiner, 1945) in that anti-P_p antibody did react with the structurally similar benzenearsonate haptens but not with the other charged haptens examined. Neither benzoic acid nor benzenesulfonic acid inhibited the precipitation of anti-P_p antibody by P_p-ovalbumin (Table III) at 0.0017 M, the highest concentration used.

As shown in Table I, *ortho* and *meta* substituents on the benzene ring of benzenephosphonate have little effect on the binding energy of the reaction of the hapten with anti-P_p antibody. An *ortho*-chloro group reduced the binding energy by only 0.15 kcal. Even the larger *ortho*-methoxy group of *p*-nitro-*o*-methoxybenzenephosphonic acid reduced the binding by only

TABLE III

EFFECT OF DIFFERENT ANIONS (1.7 mM) ON THE PRECIPITATION OF ANTI-P_p ANTIBODY BY P_p-OVALBUMIN AT pH 8

Anion	Precipitate Found in Absence of Anions (%)
Benzenephosphonate	23
Benzenearsonate	40
Benzoate	102
Benzenesulfonate	100

0.30 kcal as compared with the binding of *p*-nitrobenzeneophosphonic acid. A single *meta*-chloro group had no significant effect and even two *meta*-chloro groups did not appreciably decrease the binding energy. The larger nitro group in the *meta* position, although showing a greater effect in reducing the binding than the chloro group, still exhibited only a minor energy contribution of 0.23 kcal. The smaller amino group in the *meta* position reduced the binding to about the same extent as did the nitro group. The *para*-(*p*-hydroxyphenylazo) group increased the binding energy by 1.1 kcal in accord with earlier experiments with comparable systems (Pressman *et al.*, 1944; Pauling and Pressman, 1945). Other *para* substituents, nitro and chloro, increased the binding energy by less than 0.20 kcal.[1] It is interesting that the *para*-amino group contributed more energy (0.27 kcal) to the binding than did the *para*-nitro group, in contrast to the usual observation with other antihapten systems that the nitro group, in the position of attachment of the hapten to the protein of the injected antigen, contributes appreciably more energy than the amino group (Pressman *et al.*, 1944, on anti-*p*-azobenzoate; Pauling and Pressman, 1945; on anti-*p*-azobenzenearsonate).

The effect of substituents on the binding of benzenearsonate by anti-P_p antibody is identical to their effect on the binding of benzenephosphonate after taking into account the degree of ionization shown in Table IV. The anti-P_p antibody present in the pool of anti-P_p sera used here had previously been found to consist essentially of antibody formed in response to the doubly charged phosphonate group and reacting only with the doubly charged phosphonate and arsonate ions.[2] The apparent relative binding constants in Table I were based on the total amount of arsonate present. These values of K_0' (rel) were corrected for the degree of ionization in Table V, where the relative binding constants listed have been calculated on the basis of the concentrations of doubly charged ions only. On this basis the doubly charged benzenearsonate combined just as strongly as the doubly charged benzenephosphonate. The *m*- and *p*-nitrobenzenearsonates yielded relative binding constants that were almost the same as were found for the corresponding benzenephosphonates. *o*-Nitrobenzenephosphonate was not available for comparison, but the small effect of the *ortho*-nitro group in benzenearsonate (Table V) is in agreement with the relatively small effects of other *ortho* substituents on the binding of benzenephosphonate.

Anti-R_p Antibody.—The relative binding constants obtained for the reaction of anti-R_p antiserum with various benzenearsonates (Table II) that have been studied previously agree well with the earlier data[3] that indicate that the combining site of the anti-R_p antibody fits fairly closely about benzenearsonate. Of particular significance is the fact that both P_p and R_p type antibody, nitro, and chloro[4] substituents in *ortho* and *para* positions affected the binding energy of benzenephosphonate qualitatively in the

TABLE IV

DEGREES OF IONIZATION OF BENZENEPHOSPHONATES AND BENZENEARSONATES AT pH 8

Substituent	Per Cent of Doubly Charged Ion at pH 8: Benzenephosphonate[a]	Benzenearsonate[b]
Unsubstituted	90	25
o-Chloro-	91	—
m-Chloro-	96	—
p-Chloro-	95	36
o-Nitro-	95	22
m-Nitro-	98	61
p-Nitro-	98	61
o-Amino-	—	18
m-Amino-	87	19
p-Amino-	75	11
p-(*p*-Hydroxyphenylazo)-	98	56

[a] Calculated from ionization constants taken from Jaffe *et al.* (1953). [b] Calculated from ionization constants taken from Pressman and Brown (1943).

TABLE V

COMPARISON OF BINDING OF NITRO-SUBSTITUTED BENZENEARSONATES AND BENZENEPHOSPHONATES BY ANTI-P_p ANTIBODY. CORRECTION FOR PARTIAL IONIZATION OF HAPTENS

	K_0' (rel): Uncorrected	Corrected[a]
Benzenephosphonic acid		
Unsubstituted	1.00	1.00
m-Nitro-	0.66	0.58
p-Nitro-	1.23	1.09
Benzenearsonic Acid		
Unsubstituted	0.31	1.15
o-Nitro-	0.20[b]	0.91[b]
m-Nitro-	0.29[b]	0.45[b]
p-Nitro-	0.69, 0.50[b]	1.00, 0.73[b]

[a] Calculated assuming that only the doubly charged ion is being bound. [b] Different set of measurements where benzenephosphonate was not run. The values for the arsonates are all adjusted to a standard value of 1.00 for benzenephosphonate using the measured ratios of 1.00/0.31 (uncorrected) and 1.00/1.15 (corrected) for benzenephosphonate/benzenearsonate, respectively.

[1] The contribution of the *para*-nitro group was even smaller than shown in Table I and was apparently zero within experimental error when the K_0' (rel) had been corrected for partial ionization of the haptens, assuming that only the doubly charged ions were being bound (see later discussion and Table V).

[2] These conclusions do not necessarily mean that there was no antibody formed in response to the singly charged *p*-azobenzenephosphonate, but rather that such an antibody was not detected by the means used, i.e., reaction with singly charged arsonate ion at pH 6. Such an antibody, however, if present, was probably present in only relatively small amounts.

[3] The values of K_0' (rel) previously obtained for benzenephosphonate and for *o*-nitro-, *m*-nitro-, *p*-nitro-, and *p*-(*p*-hydroxyphenylazo)-benzenearsonates were 0.98, 0.60, 1.5, 5.3, and 13, respectively (Pressman and Siegel, 1953; Pressman *et al.*, 1945).

[4] The values of K_0' (rel) found for *o*-, *m*-, and *p*-chlorobenzenearsonates and anti-R_p antiserum were 0.30, 1.3, and 2.2, respectively (Pauling and Pressman, 1945).

same manner as they affected the binding energy of benzenearsonate. However, the effect of substituents on the binding energy of the reaction of benzenearsonate or benzenephosphonate and anti-R_p antibody is seen to be quantitatively different from their effect on the binding energy for the reaction of benzenephosphonate or benzenearsonate with anti-P_p antibody. In the R_p system there was not only a larger reduction in binding by *ortho* groups, than in the P_p system, but it is especially noticeable that *para* groups greatly increased the binding energy while, except for the azophenyl substituent, almost no effect by *para* groups was seen in the P_p system (K_0' [rel] corrected for ionization). A *meta*-nitro group increased the binding energy with anti-R_p antibody while the binding energy was decreased in the case of the anti-P_p antibody (see Table V, corrected K_0' [rel]).

Discussion

Combining Site of Anti-P_p Antibody.—The effect of various substituents on the binding energy of the reaction of benzenephosphonate with anti-P_p antibody indicates that the anti-P_p combining site fits rather loosely about the doubly charged phosphonate hapten. This loose fit is confirmed by lack of substituent effects on the binding energy of benzenearsonate which combines equally well with anti-P_p combining sites. In agreement with a previous observation (Kreiter and Pressman, 1963), the anti-P_p combining sites apparently combine only with the doubly charged arsonate ion, since good correlation of the relative binding constants of benzenearsonates and benzenephosphonates is obtained only if the constants are calculated on the basis of the concentration of the doubly charged ion.

The extent of cross reaction of benzenearsonate and benzenephosphonate with the combining sites of the anti-P_p antibody is very appreciable in spite of the fact that the arsonate group is larger than the phosphonate group.[5] The accommodation of the benzenearsonate by a combining site complementary for the smaller benzenephosphonate therefore indicates that the portion of the combining site complementary to the phosphonate group ($PO_3^=$) is more spacious than appears to be necessary to accommodate the phosphonate group.

Combining Site of the Anti-R_p Antibody.—Although the pool of anti-R_p antiserum used here has been shown previously to contain some antibodies formed in response to the singly charged *p*-azobenzenearsonate and others to the doubly charged hapten, only the contribution of the antibody directed against the doubly charged hapten is apparent in these experiments. The strong binding of benzenephosphonates by the γ-globulin fraction from anti-R_p serum must be due, for the most part, to the binding of doubly charged phosphonate ions, since at *p*H 8 the benzenephosphonates are essentially all in the doubly charged form and constants calculated on the basis of the small amount of singly charged phosphonate ions present are not reasonable because they are about 15–40 times higher for the heterologous haptens than those observed for the homologous hapten. Moreover, in the case of the homologous hapten itself, benzenearsonate, the binding of the doubly charged ion apparently is also largely what is being observed because of the qualitative similarity in the effect of nitro and chloro substituents on the binding of benzenephosphonate and benzenearsonate by anti-R_p antibody.

Any contribution of antibodies directed against the singly charged hapten is difficult to define. Although the relative binding constants of the benzenearsonates are larger than the corresponding constants of the benzenephosphonates and this might be because of interaction of the singly charged arsonate with the corresponding antibody, it might also be owing to the difference in size of the arsonate and phosphonate.[6]

The reason why we appear to be studying anti-R_p antibody that is directed against the doubly charged ion may be that, even though a large proportion of the arsonate hapten in the injected antigen at physiological *p*H is in the singly ionized form, the proportion of antibody formed in response to the singly charged ion present in the pool of serum used is low.[7] Another factor that may be involved is that the total antigen concentrations for optimum precipitation of antibody formed in response to the singly charged and to the doubly charged forms are quite different, and the amount of antigen used was that which brought down the antibody directed against the doubly charged hapten.

Comparison of Combining Sites of Anti-P_p and Anti-R_p Antibodies.—There is no particular difficulty in understanding the combination of the smaller phosphonate ion with the anti-R_p combining site, but how antibody formed in response to the smaller phosphonate ion can accommodate the larger arsonate ion needs explanation. This cross reaction may be caused by a greater state of hydration of the phosphonate ion, enough to compensate for difference in size of the unhydrated ions. Since phosphonate ion is smaller and has a greater charge density, it might be more strongly hydrated. In addition, some of the difference between the specificities observed for anti-P_p and anti-R_p antibodies may also be due to differences in the energies of hydration of benzenephosphonate and benzenearsonate ions or in the configurations of the hydrated ions. In view of the fact that combining sites of either anti-R_p or anti-P_p antibodies (directed against the doubly charged ions) can accommodate either arsonate or phosphonate haptens equally well, the marked difference in specificities between the combining sites of anti-R_p and anti-P_p antibodies was unexpected.

The difference in specificity may be of fundamental significance in concepts of antibody site structure. It may be that the amino acid sequence of the primary peptide chain can fold only in certain ways, and the only possible folding around the phosphonate ion yields a configuration which can accommodate the larger arsonate as well as the smaller phosphonate.

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[5] The P—O bond length in KH_2PO_4 is 1.55°A (West, 1930). The As—O bond length is 1.74°A in KH_2AsO_4 (Helmholz and Levin, 1942).

[6] Interactions of doubly charged arsonate with antibody formed in response to singly charged hapten or singly charged arsonate with antibody formed in response to doubly charged hapten were probably negligible.

[7] This situation could arise if the relative amount of antibody directed against the doubly charged ion were very high in the serum of the two rabbits that contributed the largest proportion to the pool of anti-R_p serum.

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The Polypeptide Chains of Rabbit γ-Globulin and Its Papain-cleaved Fragments*

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It is shown by sedimentation equilibrium measurements that rabbit γ-globulin consists of four polypeptide chains, two with a molecular weight of close to 50,000, and two with a molecular weight of close to 25,000. Similar measurements show that each of the three fragments of γ-globulin, which are obtained by the action of papain, has a molecular weight of close to 50,000. Each fragment consists of two polypeptide chains of molecular weight close to 25,000 apiece. From these data it can be concluded that the action of papain on the parent molecule has been to split the two heavier polypeptide chains into approximately equal halves. All of the foregoing data confirm the model of the gross structure of γ-globulin which has been proposed by Porter (1962).

It has recently been suggested by Porter (1962) that rabbit γ-globulin probably consists of four polypeptide chains, two A chains having a molecular weight of about 50,000 each, and two B chains having a molecular weight of about 25,000 each. A similar structure has been suggested by Edelman and Benacerraf (1962) as one of several possibilities for human γ-globulin. The work presented in this paper was initiated before Porter's and Edelman's results were available. It set out to determine the number of polypeptide chains in rabbit γ-globulin, and their molecular weights, by a method which would not require separation of the chains. The result is an unequivocal demonstration of the correctness of Porter's conclusions.

In addition to determining the nature of the polypeptide chains of rabbit γ-globulin itself, we have made similar studies on the fragments of the molecule which are obtained by the action of papain. Each molecule yields three fragments by this procedure: fragment III, which is crystalline and plays no part in conferring specificity on those γ-globulin molecules which are specific antibodies, and two fragments which are the carriers of antibody specificity. These latter fragments are designated fragments I or II, depending on their ease of elution from a carboxymethyl-cellulose column at *p*H 5.5. We have studied fragments of type I only, but there is every reason to believe that the gross structure of fragments of type II is the same as that of fragments of type I (Palmer *et al.*, 1962).

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MATERIALS AND METHODS

Rabbit γ-globulin has been prepared in this laboratory by a variety of methods. Physical and chemical studies which will be published at a later date indicate that most of these methods yield proteins which do not differ appreciably from each other, except that they possess somewhat different titration curves. The sedimentation velocity data reported in this paper were obtained from several such different preparations.

The quantitative measurement of the molecular weights of the polypeptide chains of γ-globulin (Table II), which constitutes the most important result reported, was carried out on a sample of rabbit γ-globulin prepared by a modification of the procedure of Gorodskaya (1950), using the reagent Rivanol (2-ethoxy-6,9-diamino-acridine lactate) for the initial precipitation of the acidic proteins of rabbit serum. Excess Rivanol was removed by precipitation with approximately 1 M KI, and the crude globulin was then purified by chromatography on DEAE-cellulose (0.05 M potassium phosphate buffer, *p*H 7.0), precipitation with $(NH_4)_2SO_4$, and a second column chromatography, this time on DEAE-Sephadex. The final product was dialyzed against 0.1 M KCl and stored as a lyophilized powder. This protein proved virtually homogeneous by sedimentation velocity, including little or none of the heavy component ($s_{20,w} \sim 10$) which we have found present in most preparations of γ-globulin and of specific antibodies.

The fragments of γ-globulin were prepared by the method of Porter (1959), except that mercaptoethanol was used as reducing agent in place of cysteine. The reaction was stopped by addition of iodoacetamide.

In order to reduce the proteins to their polypeptide chains they were dissolved in 6–7 M guanidine hydrochloride and then allowed to stand at room temperature for at least 12 hours in 0.1 M mercaptoethanol