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Amino Acid Differences between Highly Cross-Reactive Antibodies*

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ABSTRACT: Antibodies to *p*-azophenylarsonate and its closest chemical analog, *p*-azophenylphosphonate, were shown to be highly cross-reactive. In competitive binding experiments with either antibody, the heterologous hapten was almost as effective as the homologous hapten in displacing the reference ligand. The similarity in binding properties was paralleled by the similarity in the amino acid compositions of the two antibodies. The amino acid contents of the respective heavy chains differed by a single tyrosine residue, while the contents of the light chains were indistinguishable. These results with highly cross-reactive antibodies provided further support that the multiple amino acid differences previously observed for noncross-reactive antibodies are related to their immunological reactivity.

Characteristic differences in amino acid composition have been found for five rabbit antibodies, anti-phenylarsonate, anti-phenylsulfonate, anti-benzoate, anti-phenyltrimethylammonium, and anti-phenyl β-lactoside (reviewed in Koshland, 1966). These compositional differences were shown to be independent of the charge on the antigenic determinant, the simultaneous production of other antibodies, genetic markers located in the variable sequences of the heavy chain (Koshland, 1967), and the class of immunoglobulin isolated (Koshland *et al.*, 1969). Furthermore, the amino acid differences could not be satisfactorily explained by selective synthesis in subclasses of the variable regions since a separate subclass would be required for each antibody. It appeared, therefore, that the observed differences were associated with the immunological specificities of the respective antibodies.

There was no cross-reaction in ligand binding among the five antibodies studied (Landsteiner, 1945; Kreiter and Pressman, 1964), although three, the anti-phenylarsonate, the anti-phenylsulfonate, and the anti-benzoate, were directed against haptens with similarities in structure and charge. Moreover, the compositional differences were found to be as large among the anti-phenylarsonate, anti-phenylsulfonate, and anti-benzoate antibodies as among the antibodies to the chemically dissimilar haptens. To determine both the extent and kinds of compositional differences associated with related specificities, these studies have been extended in the present work to highly cross-reactive antibodies. Phenylarsonate and its closest chemical analog, phenylphosphonate, were chosen as the antigenic determinants because Kreiter and Pressman (1964) have shown that the phenylphosphonate hapten was a strong inhibitor of the precipitation of anti-phenylarsonate antibody by its homologous antigen, and the phenylarsonate hapten was an equally effective inhibitor in the anti-phosphonate system.

Since it is impossible to separate anti-phenylarsonate antibody from anti-phenylphosphonate antibody, the usual technique of minimizing animal variation by preparing the two antibodies in the same animal could not be utilized. As the best alternative, one set of rabbits was immunized with the azoantigens of phenylarsonate and phenyl β-lactoside, while a comparable set was immunized with the azoantigens of phenylphosphonate and the same phenyl β-lactoside antibody. Thus, the anti-phenyl β-lactoside antibody served as a control for any differences in the two sets of animals.

Methods

Preparation of Haptens. The *p*-nitrophenylphosphonic acid was synthesized according to the method of Doak and Freedman (1951); 0.08 mole of *p*-nitrophenyldiazonium fluoroborate, suspended in 100 ml of ethyl acetate, was allowed to react with 0.08 mole of phosphorous trichloride in the presence of 1.6 g of cuprous bromide catalyst. After the volatile by-products of the reaction were removed by steam distillation, any diphenylphosphinic acid produced was crystallized out by concentrating the distilled reaction mixture to 80 ml and incubating overnight in the cold. The

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hemi-sodium salt of *p*-nitrophenylphosphonic acid was obtained from the remaining filtrate by the addition of sufficient 20% sodium hydroxide to bring the pH to 8 and then sufficient concentrated HCl to precipitate the salt. The precipitate was washed several times with ether and dried at reduced pressure.

To convert the hemi-sodium salt into the acid, the salt was dissolved in a minimum of water, the pH adjusted to 6, and the solution put through a column of Dowex 50W-X8 in the H^+ form, using distilled water as the effluent. The yellow eluate containing the acid was found free of sodium by the platinum wire flame test. The eluate was taken to dryness and the product recrystallized several times from a minimum volume of boiling ethyl acetate. The yellowish-tan crystals had a melting point of 195–198°.

p-Aminophenylphosphonic acid was prepared from the *p*-nitro compound with the use of the Parr hydrogenator. A solution containing 37 ml of water and 65 g of palladium on alumina catalyst/g of *p*-nitrophenylphosphonic acid was adjusted to pH 7 and hydrogenated for 2 hr at 45 psi. The catalyst was filtered off and the *p*-amino compound precipitated by adjusting the pH to 3.0–3.5. *Anal.* Calcd: C, 41.6; H, 4.66; N, 8.1; P, 17.9. Found: C, 42.08; H, 4.77; N, 8.14; P, 17.90.

The *p*-aminophenylarsonic acid was purchased from Eastman-Kodak and recrystallized several times before use. The *p*-aminophenyl β-lactoside was purchased from Cyclo Chemical Corp.

For the equilibrium dialysis experiments *p*-aminophenylphosphonic acid and *p*-aminophenylarsonic acid were reacted with [1-^{14}C]acetic anhydride (New England Nuclear) by the method of Ehrlich and Bertheim (1907). The acetylated products were recrystallized several times from water before use.

Preparation of Antibodies. The immunizing antigens were synthesized by coupling at pH 9.0 the diazonium salt of each hapten, *p*-aminophenylarsonic acid, *p*-aminophenylphosphonic acid, and *p*-aminophenyl β-lactoside, to bovine γ-globulin. The amounts of diazotized hapten added were twice the molar concentration of tyrosine present in the protein carrier. After dialysis to remove excess reagents, equal quantities of the phenyl β-lactoside azoantigen and the phenylarsonate azoantigen were coprecipitated with alum. A second mixture was similarly prepared with equal quantities of the phenyl β-lactoside azoantigen and the phenylphosphonate azoantigen. One or the other mixture was injected intravenously over a period of 4 weeks into New Zealand white rabbits of the genotype $a^1a^1b^4b^4$. Each animal received a total of 120 mg of antigen and was bled out 5 days after the last infection.

The isolation of purified arsonic[1] and lac[1] IgG antibodies has been described fully (Koshland *et al.*, 1962, 1964) and the procedure developed for arsonic antibody was applied without change to the isolation of phosphonic IgG antibody. The yields of purified antibody from individual rabbits ranged from 0.2 to 0.6 mg per ml of serum. No significant differences were detected in the amounts of each antibody produced; the average yields for arsonic, phosphonic, and lac antibodies were 0.33 ± 0.081, 0.26 ± 0.025, and 0.33 ± 0.039 mg per ml of serum.

Preparation of Antibody Subunits. To obtain pure preparations of both heavy and light chains, the disulfide bonds in arsonic and phosphonic antibodies were completely reduced and alkylated. The reduction was carried out with a 0.2–0.5% solution of the antibody containing 1 M 2-mercaptoethanol, 10 M urea, and 0.1 M Tris buffer (pH 8.0). The mercaptoethanol was redistilled before use, and the urea was recrystallized from hot 95% ethanol. The reduction was allowed to proceed for 24 hr at room temperature and then dry recrystallized iodoacetamide in 5% excess of the mercaptoethanol concentration was added. During the addition of the iodoacetamide, the pH of the solution was maintained between 7.8 and 8.2 with 5 N NaOH. The alkylated polypeptide chains were freed of the products of the reaction by dialysis for 48 hr against five 4-l. changes of distilled water and then were lyophilized.

The chains were separated by gel filtration on Sephadex G-200 equilibrated with 0.05 M sodium decyl sulfate in 0.01 M phosphate buffer (pH 8.0). The dried alkylated material was dissolved in the column buffer to a concentration of 5–10 mg/ml and applied to a 1.4 × 100 cm column with a flow rate of 6–7 ml/hr. The protein content of the effluent 2-ml fractions was monitored by readings in a Beckman spectrophotometer at 278 mμ.

Equilibrium Dialysis Measurements. The immunological activity of the arsonic and phosphonic antibodies was assayed by equilibrium dialysis using the [^{14}C]acetamido derivatives of the haptens. The dialysis were carried out in small Lucite cylinders divided into two compartments by the insertion of a dialysis membrane. In one chamber was inserted, by syringe, 1 ml of the antibody solution and into the other 1 ml containing the appropriate hapten or haptens. All solutions were made up in 0.2 M glycine buffer (pH 8.5) and 0.10 M NaCl. After 44-hr rotation in the cold to reach equilibrium, duplicate 0.1-ml aliquots from each compartment were mixed with 4 ml of scintillation fluid and the radioactivity was determined in a Beckman Model LS-200 liquid spectrometer to better than 5%. The scintillation fluid was prepared by dissolving 4 g of Omnifluor (New England Nuclear) in 1 l. of toluene and mixing 9.5 parts of the toluene with 0.5 part of Bio-Solv solubilizer BBS-3 (Beckman).

In all experiments the antibody concentration was maintained at $1 \pm 0.05 \times 10^{-5}$ M as determined by the absorbancy at 278 mμ and an extinction coefficient, $E_0^{1\%}$, of 1.45 (Koshland *et al.*, 1962). For the direct binding measurements, the concentrations of the radioactive acetamido haptens ranged from 1×10^{-5} to 1×10^{-4} M. For the measurements of cross-reactivity, a single concentration of 3.0×10^{-5} M radioactive hapten was used of which approximately one-half was bound to the antibody. The inhibition of this binding was determined by the addition of unlabeled *p*-nitro derivatives of phenylarsonate and phenylphosphonate at concentrations of 1×10^{-5} to 6×10^{-5} M in the homologous system and 0.2×10^{-4} to 1.5×10^{-4} M in the heterologous system. These ranges were selected to give values around 50% inhibition.

Amino Acid Analyses. Samples of the antibodies and their heavy and light chains were prepared for analysis by precipita-

[1] The antibody to *p*-azophenylarsonate will be referred to subsequently as arsonic antibody; the antibody to *p*-azophenylphosphonate as phosphonic antibody; and the antibody to *p*-azophenyl β-lactoside as lac antibody.

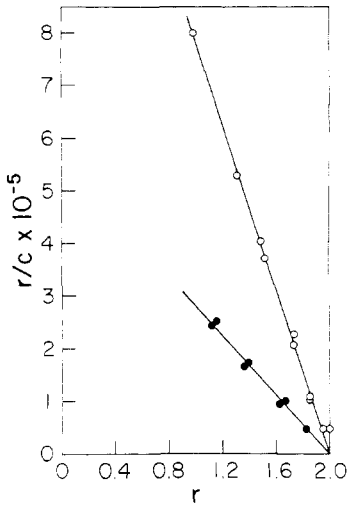

FIGURE 1: Binding properties of arsonic and phosphonic antibodies. (○) Binding of *p*-acetamidophenylarsonic acid by arsonic antibody. (●) Binding of *p*-acetamidophenylphosphonic acid by phosphonic antibody.

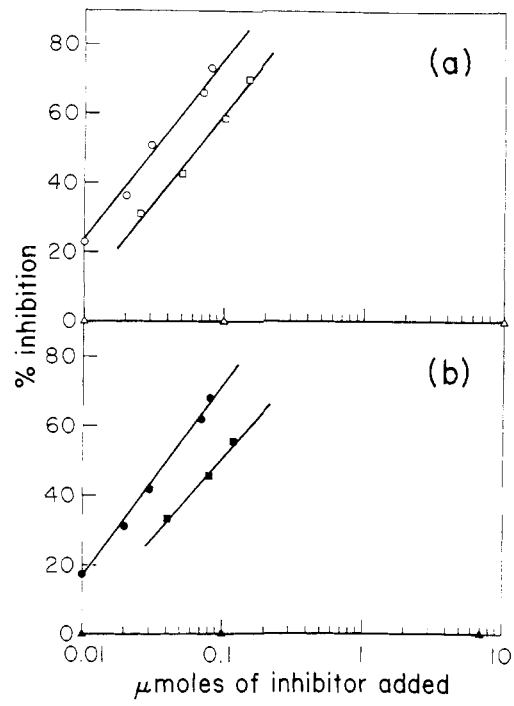

FIGURE 2: Competitive inhibition studies with arsonic antibody and phosphonic antibody. (a) Inhibition of binding of *p*-acetoamidophenylarsonic acid to arsonic antibody by *p*-nitrophenylarsonic acid (○), *p*-nitrophenylphosphonic acid (□), and by *p*-nitrobenzoic acid (△). (b) Inhibition of binding of *p*-acetoamidophenylphosphonic acid to phosphonic antibody by *p*-nitrophenylphosphonic acid (●), by *p*-nitrophenylarsonic acid (■), and by *p*-nitrophenylsulfonic acid (▲).

tion with trichloroacetic acid so that its final concentration was 7–8%. The precipitates were washed with 3 ml of cold absolute ethanol and dried *in vacuo*. The amino acid analyses were performed on the Beckman-Spinco automatic amino acid analyzer, Model 120C, after hydrolysis under reduced pressure in 6 N HCl for 20 hr at 110°. A standard mixture of amino acids and an antibody hydrolysate were analyzed alternately.

The results were normalized to a leucine content of 89 for the intact antibodies, 33 for the heavy chains, and 11 for the light chains. These values were based on previous measurements (Koshland *et al.*, 1966) of the average moles of leucine per mole of arsonic and lac antibodies and their respective subunits, and were assumed to apply also to the phosphonic preparations. The assumption appeared justified since after normalization the sum of the residues present in phosphonic heavy and light chains and in the intact molecule was identical with those of the equivalent arsonic preparations.

Results

Direct Binding Measurements. The binding properties of the arsonic and phosphonic IgG antibodies are compared in Figure 1. The equilibrium dialysis results have been presented in the form of a Scatchard plot, *r/c vs. r*, in which *r* represents the number of hapten molecules bound per molecule of antibody and *c* represents the concentration of free hapten at equilibrium. The fact that both curves extrapolated to an *r* value of two at infinite hapten concentration showed that the antibody preparations were pure within the limits of detection of the dialysis method. Moreover, the linear relationship of the experimental points indicated that the combining sites of both antibodies were homogeneous with respect to their affinities for the homologous *p*-acetamido haptens. The association constants differed by a factor of three, 2.8×10^5 and 7.8×10^5 M^{-1} for phosphonic and arsonic antibody, respectively. These affinities were typical in magnitude and range for anti-azohapten antibodies obtained after a short course of immunization with an alum-precipitated antigen (Karush, 1956; Grossberg *et al.*, 1962).

Competitive Binding Measurements. The overlap in the specificities of arsonic and phosphonic antibodies is illustrated by the inhibition data given in Figure 2 and the derived association constants given in Table I. The extent of cross-reaction of phenylphosphonate and phenylarsonate with the combining site of arsonic antibody was very appreciable. The amount of the heterologous *p*-nitrophenylphosphonate required for 50% inhibition was only twice that required by the homologous *p*-nitrophenylarsonate (Figure 2a), and there was a correspondingly small change in the association constants, 3.48×10^5 M^{-1} for the heterologous hapten compared with 7.17×10^5 M^{-1} for the homologous hapten (Table I). A nearly equivalent amount of cross-reaction was observed for the combining site of phosphonic antibody (Figure 2b); 50% inhibition was achieved with a concentration of arsonate hapten 2.4 times that of the phosphonate. These results were in marked contrast to the effects obtained with other negatively charged haptens. At as high concentrations as 10^{-2} M, neither phenylsulfonate nor benzoate inhibited the binding of arsonic or phosphonic antibodies and their homologous haptens.

The determinations of the 50% end points and the association constants were based on the total arsonate and phosphonate present without correction for differences in their degree of ionization. Previous studies (Kreiter and Pressman, 1963) have indicated that two populations of antibodies

TABLE I: Comparison of Antibody Binding by Structurally Related Molecules.

Antibody	Hapten	Inhibitor	K_0[a]	K_I[a]
Arsonic	$AcNHC_6H_4AsO_3$	None	7.78	
		$NO_2C_6H_4AsO_3$		7.17[b]
		$NO_2C_6H_4PO_3$		3.48[b]
Phosphonic	$AcNHC_6H_4PO_3$	None	2.78	
		$NO_2C_6H_4PO_3$		2.53[b]
		$NO_2C_6H_4AsO_3$		0.866[b]
Lac[c]	Lac dye	None	1.34	
		$NO_2C_6H_4$-β-lactoside		0.675
		$CH_3C_6H_4$-β-lactoside		0.202
		CH_3-β-D-galactoside		0.00075
TNP[d] (early)	2,4,6-tri-$NO_2C_6H_2NH_2$-caproate	None	690	
	2,4-diNO_2C_6-H_3NH_2-caproate	None	27	
TNP[d] (late)	2,4-,6-tri-$NO_2C_6H_2NH_2$-caproate	None	3470	
	2,4-di-$NO_2C_6H_3NH_2$-caproate	None	263	

[a] K_0 and K_I are given as moles/l. $\times 10^{-5}$. [b] See appendix for calculation of K_I. [c] Data from Karush (1957). [d] Data from Little and Eisen (1966).

TABLE II: Comparison of Amino Acid Compositions[a] of Cross-Reactive Arsonic and Phosphonic Antibodies and Noncross-Reactive Lac Antibody.

Amino Acid	Lac Antibody A[b]	Lac Antibody B[c]	Δ (Lac − Arsonic)	Arsonic Antibody	Phosphonic Antibody	Δ (Phosphonic − Arsonic)
Lys	69.5 ± 0.73[d]	70.1 ± 0.56[d]		69.9 ± 0.16[d]	69.7 ± 0.31[d]	
His	16.7 ± 0.39	17.0 ± 0.19		16.4 ± 0.30	16.4 ± 0.12	
Arg	45.0 ± 0.59	45.8 ± 0.42		45.3 ± 0.60	45.5 ± 0.11	
Asp	113 ± 1.5	113 ± 0.87	+6	107 ± 1.0	108 ± 0.58	
Thr	171 ± 1.8	171 ± 0.97		170 ± 1.2	170 ± 0.66	
Ser	151 ± 1.9	150 ± 0.99	−6	157 ± 1.8	155 ± 0.73	
Glu	124 ± 0.58	123 ± 0.45		124 ± 0.58	123 ± 0.33	
Pro	110 ± 0.70	111 ± 0.41		111 ± 0.58	110 ± 0.32	
Gly	111 ± 1.0	110 ± 0.45		111 ± 0.41	110 ± 0.41	
Ala	76.9 ± 0.15	77.5 ± 0.64	−2	79.3 ± 0.27	79.1 ± 0.28	
Val	132 ± 1.2	131 ± 0.63	+2	130 ± 0.47	129 ± 0.48	
Met	13.4 ± 0.66	13.4 ± 0.43		13.4 ± 0.74	13.5 ± 0.11	
Ilu	46.0 ± 0.53	46.1 ± 0.46		47.2 ± 0.57	46.9 ± 0.27	
Leu	89	89		89	89	
Tyr	50.6 ± 0.35	50.3 ± 0.32	−6	56.1 ± 0.44	55.0 ± 0.36	
Phe	45.0 ± 0.44	45.2 ± 0.34		44.6 ± 0.17	44.5 ± 0.19	

[a] After 20-hr hydrolysis. The threonine, serine, tyrosine, and valine values represent 95.5, 90.1, 99.0, and 95.0% recoveries, respectively, as determined from additional data on 72-hr hydrolysates. [b] Lac A antibodies produced in the same animal with arsonic antibody. [c] Lac B antibodies produced in the same animal with phosphonic antibody. [d] Standard error of the mean.

are formed in response to the *p*-azophenylarsonate hapten, one specific for the singly charged ion, the major species at physiological pH, and the other specific for the doubly charged ion. However, the arsonic antibody preparations used in our studies were found to have quite different properties. In direct binding measurements the affinity did not change when the pH was varied from 7.5 to 8.5, although the ratio of the two ionic forms of the *p*-acetamidophenylarsonate hapten shifted from approximately 9:1 to approximately 1:1 over that pH range. Furthermore, the combining sites were homogeneous in their reaction with either the homologous or the heterologous haptens, as indicated by the linear and parallel slopes in Figure 2a. On the basis of these data, it was concluded that the combining sites of the arsonic

TABLE III: Comparison of Amino Acid Compositions[a] of Heavy and Light Chains from Cross-Reactive Arsonic and Phosphonic Antibodies.

	Heavy Chains			Light Chains		
Amino Acid	Arsonic Antibody	Phosphonic Antibody	Δ (Phosphonic − Arsonic)	Arsonic Antibody	Phosphonic Antibody	Δ (Phosphonic − Arsonic)
Lys	25.1 ± 0.14[b]	25.0 ± 0.17[b]		9.3 ± 0.08[b]	9.2 ± 0.06[b]	
His	6.3 ± 0.14	6.5 ± 0.08		1.3 ± 0.03	1.2 ± 0.01	
Arg	19.4 ± 0.19	19.6 ± 0.17		3.0 ± 0.13	3.0 ± 0.04	
Asp	33.4 ± 0.18	32.9 ± 0.13		19.8 ± 0.09	19.8 ± 0.10	
Thr	51.7 ± 0.16	51.4 ± 0.16		31.8 ± 0.25	32.1 ± 0.41	
Ser	54.5 ± 0.30	53.7 ± 0.12		22.5 ± 0.11	22.0 ± 0.22	
Glu	39.3 ± 0.23	38.7 ± 0.09		21.7 ± 0.16	21.4 ± 0.17	
Pro	42.8 ± 0.13	43.1 ± 0.38		12.1 ± 0.17	12.2 ± 0.13	
Gly	34.5 ± 0.22	34.4 ± 0.04		20.4 ± 0.11	20.1 ± 0.04	
Ala	22.8 ± 0.19	22.7 ± 0.14		16.9 ± 0.35	16.6 ± 0.13	
Val	42.4 ± 0.13	42.5 ± 0.22		21.0 ± 0.09	20.8 ± 0.12	
Ilu	15.9 ± 0.17	15.7 ± 0.09		7.4 ± 0.04	7.4 ± 0.13	
Leu	33	33		11	11	
Tyr	17.4 ± 0.13	16.5 ± 0.14	−0.9	10.6 ± 0.10	10.8 ± 0.10	
Phe	15.4 ± 0.19	15.5 ± 0.16		6.7 ± 0.04	6.6 ± 0.13	
CM-Cys	13.3 ± 0.34	13.2 ± 0.67		7.2 ± 0.12	7.4 ± 0.22	

[a] After 20-hr hydrolysis. [b] Standard error of the mean.

antibody used in these experiments did not distinguish between the ionic forms of the haptens, and therefore, the inhibition measurements were independent of the degree of ionization.

In contrast to arsonic antibody, Kreiter and Pressman (1963) found that antibody to the *p*-azophenylphosphonate group was directed only against the double charged species which comprises 89% of the hapten at physiological pH. In our studies there was insufficient phosphonic antibody to measure the binding of the heterologous arsonate hapten as a function of pH and thus determine the reaction between the singly charged arsonate and the combining site of the phosphonic antibody. In order to minimize any effect of the singly charged ion, the inhibition measurements were carried out using the substituted phenylarsonate with the lowest pK_a, *p*-nitrophenylarsonate, and the equivalent phosphonate derivative. At pH 8.5, the *p*-nitrophenylarsonate was 83% in the doubly ionized form compared with the 98% doubly ionized for the *p*-nitrophenylphosphonate. Thus, if the phosphonic combining site excluded the univalent arsonate ion, the concentration of arsonate hapten observed to give 50% inhibition in the phosphonic system (Figure 2b), was too large by 15%. The degree of ionization might explain the observation that the relative amount of arsonate hapten required for 50% inhibition in the phosphonic antibody system was greater than the relative amount of phosphonate hapten required for 50% inhibition in the arsonic antibody system. However, the result could be alternatively explained by the difference in the size of the arsonate and phosphonate groups since in the inorganic salts the As–O bond has been shown to be 0.19 Å longer than the P–O bond (West, 1930; Helmholz and Levin, 1942). If the hydration of the organic salts did not compensate for this difference, the possibility remains that some of the combining sites in phosphonic antibody might not accommodate the larger phenylarsonate group. That either the charge or the size of the arsonate group affected the binding at some of the phosphonic combining sites was evident from the difference in the slopes of the homologous and heterologous reactions (Figure 2b).

Composition Analyses. The amino acid compositions obtained for the lac antibody controls and for the arsonic and phosphonic antibodies are summarized in Table II. The data in columns 1 and 4 represent the average yields from five preparations, each pair of lac and arsonic antibodies having been isolated from a single animal; the data in columns 2 and 5 represent similar averages of six pairs of lac and phosphonic antibodies.

The compositions of the lac antibodies from the two groups of animals were found to be identical within the limits of detection of the analytical method. The accompanying standard errors of the mean showed the results to be accurate within 2% except for the yields of the labile methionine residues. On the basis of these controls, a direct comparison could be made of the compositions of the arsonic and phosphonic antibodies. Their amino acid contents were very similar, as the data in columns 4 and 5 show. For example, per mole of arsonic and phosphonic antibody, respectively, the moles of lysine were 69.7 and 69.9; the moles of aspartic acid, 108 and 108; and the moles of isoleucine, 46.9 and 47.2. The single indication of a difference occurred in the tyrosine recoveries, 56 in the case of arsonic antibody and 55 in the case of phosphonic antibody. By the application of the student t test, this difference was significant at the 80% confidence level.

Although little or no amino acid changes were observed between the antibodies with closely related specificities, the characteristic differences between antibodies of unrelated specificities were obtained (column 3). Compared with arsonic or phosphonic antibody, the lac antibody contained six additional aspartic acid residues, two additional valines, six less serines, two less alanines, and five or six less tyrosines. These were precisely the differences found in previous comparisons of lac and arsonic antibodies isolated from alb4 rabbits (Koshland *et al.*, 1966, 1969).

To investigate the possibility of a different distribution of amino acids in the subunits of arsonic and phosphonic antibodies, the compositions of their constituent polypeptide chains were examined. The average results from the analyses of four light- and four heavy-chain preparations of each antibody are given in Table III. The amino acid contents of the phosphonic and arsonic light chains were completely indistinguishable from each other (columns 1 and 2). On the other hand, the compositions of the heavy chains could be distinguished on the basis of a single residue difference (columns 3 and 4); the phosphonic heavy chain contained one less tyrosine than the arsonic heavy chain. These data not only confirmed the results from the analyses of the intact antibodies, but also increased the significance of the tyrosine difference to the 99.9% confidence level.

Discussion

The competitive binding studies of the cross-reacting arsonic and phosphonic antibodies showed that their combining sites are very similar. The close relation in their specificities is further emphasized by the comparison in Table I of the association constants obtained for arsonic and phosphonic antibodies with those obtained in similar studies of two other systems, anti-phenyl β-lactoside antibody (Karush, 1957) and anti-trinitrophenyl antibody (Little and Eisen, 1966). In the lac system, a change in the immunodominant group on the hapten, the substitution of methyl β-D-galactoside for methyl β-lactoside, reduces the binding affinity by a factor of 300. In the TNP system, a change in the immunodominant group involving the removal of the six nitro group has a smaller effect, decreasing the binding affinity 15–30-fold. However, in the arsonate system the substitution of phosphorus, or in the phosphonate system the substitution of arsonic, causes only a minor change of two to three in the binding affinity. This small change is the order observed by modifications in other than the immunodominant group. For example, the substitution of a methyl group for the *p*-nitrophenyl group in the position of attachment of the lactoside determinant to the protein carrier reduces the association constant by a factor of three.

The similarity in the binding properties of the cross-reacting arsonic and phosphonic antibodies is paralleled by the similarity in their compositions. The amino acid contents were shown to differ by a single tyrosine residue in the heavy chains. These results provide an important control for the evaluation of compositional differences among anti-hapten antibodies. In previous studies of five noncross-reactive antibodies, the amino acid contents of the heavy chains were found to vary by two to six residues and those of the light chains by four to seven residues. The argument might be made that some of these compositional differences represent substitutions in the variable region which are not associated with antibody specificity. However, the finding in the present work of only a single tyrosine difference between arsonic and phosphonic antibodies argues against such an interpretation and provides strong support that the amino acid differences observed among the noncross-reactive antibodies are related to their immunological reactivity.

The data obtained in studies of the cross-reacting dinitrophenyl and trinitrophenyl antibodies are consistent with the results presented above. The two antibodies appear intermediate in their degree of cross-reaction as determined by binding affinities (Table I) and intermediate in the number of specificity differences, the major change involving two tryptophans in the heavy chain. Furthermore, although the tryptophan content of both antibodies was found to increase with length of immunization, the specificity differences were maintained. At comparable times after immunization, the dinitrophenyl antibody consistently contained more tryptophan than the trinitrophenyl antibody (Little and Eisen, 1968).

The question arises why antibodies directed against haptens which appear not too different in charge and structure, such as arsonate and sulfonate, differ by as many amino acid residues as antibodies to chemically dissimilar groups such as arsonate and trimethylphenylammonium. A gradual and increasing number of changes might be expected which would correlate with the gradual and increasing differences in chemical structure of the antigenic determinant. The observed discontinuum in differences can be explained by the existence of families of binding site receptors. Within one family, variations to allow cross-reacting binding sites are achieved by changes in one or a few amino acids. Once a threshold of specificity differences is surpassed, a new family is activated whose specificity site is determined by quite different amino acids.

Appendix

A single equation similar to the two previously described by Karush (1956) was derived using only measurable quantities. It can be derived from the conservation and equilibrium eq 1–5, where A, I, and H refer to antibody, inhibitor, and

$$[A_{t_i}] = [A_{f_i}] + [I_{b_i}] + [H_{b_i}] \quad (1)$$

$$[I_t] = [2I_f] + [I_b] \quad (2)$$

$$[H_t] = [2H_f] + [H_b] \quad (3)$$

$$K_0 = \frac{[H_b]}{[A_f][H_f]} \quad (4)$$

$$K_I = \frac{[I_b]}{[A_f][I_f]} \quad (5)$$

hapten, respectively. Subscripts f and b refer to the concentrations of free and bound ligands, respectively; subscript t refers to the total concentration added and in the case of antibody describes the concentration of binding sites. Subsubscript i refers to concentrations measured in the experiments with hapten and inhibitor which were performed as described in

the text. K_0 was calculated from experiments in the absence of inhibitor. Simplifying these equations gives eq 6 which was used to calculate K_I values.

$$K_I = \frac{2\left[[A_{t_i}] - [H_{b_i}] - \frac{[H_{b_i}]}{K_0[H_{f_i}]}\right]}{\frac{[H_{b_t}]}{K_0[H_{f_t}]}\left[[I_f] - [A_{t_i}] + [H_{b_i}] + \frac{[H_{b_i}]}{K_0[H_{f_i}]}\right]} \quad (6)$$

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Polarography of Cobalamins and Cobinamides*

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ABSTRACT: The polarographic behavior of several cobalamins and cobinamides provides additional evidence indicating that the nature of the ligands bound to cobalt in the upper and lower coordination positions profoundly influences the reactivity of the cobalt atom. In the cobalamin series, the first wave half-potential is determined by the nucleophilicity of the upper ligand. An increase in the nucleophilic character of this ligand causes a shift of the polarographic wave to a higher potential.

If this upper ligand is a strong nucleophile, such as ^-OH, ^-CN, or $^-CH_3$, the polarographic wave represents a reduction by two electrons; on the other hand, the polarogram of aquocobalamin shows two distinct waves, each corresponding to a one-electron reduction. In the cobinamide series, the half-wave potential of the two waves is also determined by the nucleophilicity of both ligands in the upper and lower coordination position. The equilibrium between cyanoaquocobinamide and aquocyanocobinamide is established at a rate which is slow enough that each isomer is reduced separately at the dropping mercury electrode. The polarographic behavior of the cobinamides and the cobalamins indicates that even in the base-off position, the 5,6-dimethylbenzimidazole nucleotide greatly influences the electronic character of the cobalt atom, and thus the differences in reactivity between cobalamins and cobinamides cannot be due solely to steric factors.

Two different types of reactions in which a corrinoid participates as a coenzyme have been described. 5′-Deoxyadenosylcobalamin is required in reactions involving the transfer of hydrogen, while methylcobalamin is involved in methyl group transfer reactions (Hogenkamp, 1968). The evidence accumulated thus far suggests that during some of these transfer reactions, the carbon–cobalt bond of the coenzyme is cleaved heterolytically to an electrophilic organic moiety (5′-deoxyadenosyl cation or methylcarbonium ion) and the powerful nucleophile, cob(I)alamin.[1]

Such a heterolytic cleavage of suitable alkylcobalamins has been reported by Barnett *et al.* (1966) who found that cyanoethylcobalamin and 2(methoxycarbonyl)ethylcobalamin undergo a base-catalyzed E_2 elimination reaction in which the electron pair of the carbon–cobalt bond remains with the

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: cob(I)alamin, reduced aquocobalamin containing monovalent cobalt; cob(II)alamin, reduced aquocobalamin containing divalent cobalt; the same designation is used for the cobinamides; S.C.E, saturated calomel electrode.