Affinity Labeling of Residues within Hv2 of Guinea Pig Anti-Azobenzenearsonate Antibodies of Different Isotypes and from Different Strains†

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ABSTRACT: Anti-*p*-azobenzenearsonate (ARS) antibodies of IgG1 and IgG2 isotypes produced in inbred strain 13 and strain 2 guinea pigs were affinity labeled with *N*-(bromoacetyl)-3-[(*p*-arsonophenyl)azo]-L-tyrosine (BAAT) or *N*-(bromoacetyl)-*p*-arsanilic acid (BAA). BAAT was shown to modify approximately 50% of the binding sites specifically and BAA approximately 30%. Both reagents preferentially modified residues in the heavy (H) chain to the extent that it contained over 80% of the affinity label associated with the native molecule. At least 80% of label borne by the variable domain of the H chain (V_H) was found in the second hypervariable region (Hv2). BAAT labeled all anti-ARS antibodies exclusively at position N-59, which contains a lysyl residue. BAA labeled predominantly tyrosine at N-57 and, to a lesser extent, lysine-59 and tyrosine-50. Comparison of Hv2 sequences in anti-ARS and in antibodies reactive with other haptens has shown that tyrosine at N-50 and N-57 as well as lysine at N-59 is distinctive of antibodies with anti-ARS specificity, thus implying their involvement in antigen binding. The predominant sequence of Hv2 was identical in anti-ARS IgG1 and IgG2 molecules induced in either inbred guinea pig strain following either carrier priming or conventional immunization. Although limited variability does occur among the various populations of anti-ARS antibodies in certain residue positions in Hv2, no significant differences either in the binding affinities or in the indexes of heterogeneity were seen among the various kinds of anti-ARS antibodies.

The lysyl residue at N-59 in the γ_2 chain of anti-ARS antibodies from strain 13 guinea pigs was found to be highly susceptible to affinity labeling with the chemically reactive ligand *N*-(bromoacetyl)-3-[(*p*-arsonophenyl)azo]-L-tyrosine (BAAT) (Koo & Cebra, 1974). It was concluded that this particular lysyl residue, characteristic of anti-ARS antibody, may contribute to binding specificity for ligands such as BAAT and also be chemically modified by them. This finding and those of others (Freedman et al., 1968; Joniau et al., 1970; Grossberg et al., 1973) illustrate the occurrence of positively charged residues in some antibodies induced against anionic haptens in regions of the molecule where their modification leads to inactivation of the antigen-binding site.

Analyses of immunoglobulins following affinity labeling have illustrated that the use of a homologous series of specific ligands may allow a more comprehensive assessment of residues which might be involved in conferring antigen binding specificity. Haimovich et al. (1972), using various bromoacetyl derivatives of dinitrophenyl (Dnp) ligands, labeled either tyrosine-34 in the first hypervariable region of the light chain or lysine-54 in the second hypervariable region of the heavy chain of a myeloma protein, MOPC-315 with anti-Dnp activity. Subsequently, Becker et al. (1974), using *N*-(bromoacetyl)-*p*-arsanilic acid (BAA) and [(*p*-(*N*-(bromoacetyl)-amino)phenyl)azo]benzenearsonic acid (BAPA), affinity labeled different kinds of residues in goat anti-*p*-azobenzenearsonate (ARS) antibodies. Analysis of intact affinity-labeled molecules showed that BAA modified only tyrosyl residues and labeled the antibody to a greater extent than did BAPA, which modified either lysyl or tyrosyl residues with equal incidence.

Some reports have shown that affinity reagents may label particular subpopulations of antibody elicited against the same hapten. Pressman and his colleagues (Koyama et al., 1968) have reported significant differences in the extent and pattern of labeling among populations of anti-ARS antibodies from individual rabbits with a diazonium derivative of arsonic acid. Studies on anti-Dnp antibodies from different inbred strains of mice have suggested that differences in the distribution of label [N^{α}-(bromoacetyl)-N^{ϵ}-Dnp-lysine] between tyrosyl and lysyl residues as well as the proportion of combining sites modified may be strain related.

This report presents our initial findings on the similarity in primary structure of the second hypervariable region (Hv2) of anti-ARS antibodies of different isotypes which were induced by using different immunizing regimens in guinea pigs of two strains differing in genes controlling immune responsiveness. In addition, we report the positions and identities of residues within Hv2 which were selectively modified by a pair of site-directed bromoacetyl ligands (BAAT and BAA).

Experimental Procedures

Animals. Wright inbred strain 13 and strain 2 guinea pigs raised in our own breeding colonies were used as donors of all antibody preparations analyzed.

Preparation of Hapten–Protein Conjugate. Diazotized *p*-arsanilic acid (Tabachnick & Sobotka, 1959) was coupled to giant keyhole limpet hemocyanin (KLH; Schwarz/Mann) as previously described (Koo & Cebra, 1974); 16 groups of *p*-azobenzenearsonate (ARS) were conjugated to each KLH subunit, molecular weight 75 000 (Carpenter & Van Holde, 1973).

Immunization Procedures. Conventional immunization of guinea pigs was accomplished with 400 μg of ARS-KLH emulsified in complete Freund's adjuvant and distributed equally in all four footpads. After 17 days, animals were skin

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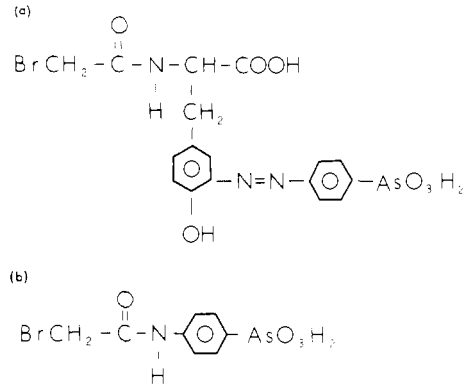

FIGURE 1: Structure of the homologous pair of bromoacetyl affinity labels used: (a) *N*-(bromoacetyl)-3-[(*p*-arsonophenyl)azo]-L-tyrosine, BAAT; (b) *N*-(bromoacetyl)-*p*-arsanilic acid, BAA.

tested with ARS-bovine γ-globulin (ARS-BGG) at four intradermal sites (10 μg/site). Animals were bled by cardiac puncture 3 days later and every third day thereafter for 2 weeks, and then bled out (Birshtein & Cebra, 1971). The sera were pooled.

Carrier priming of guinea pigs with KLH was similar to procedures already described (Koo & Cebra, 1974; Liu et al., 1974). Animals were injected intradermally at six sites with a total of 100 μg of KLH dissolved in sodium phosphate buffered saline (pH 7.4). Two weeks later, the carrier-primed animals were subjected to the conventional immunization procedure outlined above.

Purification of Anti-ARS Antibodies. Specific anti-ARS antibodies were precipitated from pooled immune sera by using ARS-BGG as reported previously (Koo & Cebra, 1974; Liu et al., 1974). Immune complexes were dissociated with 0.2 M *p*-nitrobenzenearsonic acid. ARS-BGG was removed by passing the dissolved precipitate through a column (4.5 × 15 cm) of DEAE-cellulose (Schleicher & Schuell) equilibrated in a solution of phosphate–saline (0.3 M sodium chloride in 0.01 M sodium phosphate, pH 7.4). The purified antibody solution was dialyzed against 0.01 M sodium phosphate buffer (pH 8.0) for 72 h to remove remaining free hapten. The dialyzed protein sample was then chromatographed on a column (4.5 × 15 cm) of DE-52 cellulose (Whatman) equilibrated in the same buffer. After the recovery of the IgG2 fraction in the 0.01 M sodium phosphate eluate, the bound IgG1 isotype was eluted from the column with 0.08 M sodium phosphate buffer (pH 5.9).

Synthesis of Radiolabeled Ligands. *N*-([1-^{14}C]Bromoacetyl)-3-[(*p*-arsonophenyl)azo]-L-tyrosine (BAAT; Figure 1a) was synthesized by acylating 3-[(*p*-arsonophenyl)azo]-L-tyrosine (AT) with [1-^{14}C]bromoacetyl-*N*-hydroxysuccinimide ester according to Koo & Cebra (1974). The specific radioactivity of [^{14}C]BAAT was 8500 cpm/nmol. The radioactivity was determined by using a Packard scintillation counter and Instabray scintillation fluid (Yorktown Research).

N-[^{3}H]Acetyl-3′-[(*p*-arsonophenyl)azo]-L-tyrosine (AAT) was prepared by reacting 0.05 mmol of AT dissolved in 5 mL of alkaline water (pH 10.0) with 0.50 mmol of [^{3}H]acetic anhydride as previously described (Ricardo & Cebra, 1977). [^{3}H]AAT was found to be at least 95% pure as assessed by thin-layer chromatography using the solvent mixture of ethanol–glacial acetic acid–water (5:2:3 v/v). The R_f value of [^{3}H]AAT relative to AT was 0.50. The specific radioactivity of [^{3}H]AAT was 22 000 cpm/nmol.

N-([1-^{14}C]Bromoacetyl)-*p*-arsanilic acid (BAA; Figure 1b) was synthesized according to the method of Ehrlich & Bertheim (1907) employing the modifications of Becker et al. (1974). In brief, *p*-arsanilic acid was acylated with [1-^{14}C]-bromoacetic anhydride, and the product was precipitated with cold 1 M HCl, filtered, washed with ethanol and dried. BAA was purified by recrystallizing twice from boiling water. The derivative was pure by thin-layer chromatography using the solvent mixture of butanol–glacial acetic acid–water (4:1:2 v/v). The R_f values of *p*-arsanilic acid and BAA were 0.54 and 0.66, respectively. The specific radioactivity of [^{14}C]BAA was 2300 cpm/nmol.

N-[^{3}H]Acetyl-*p*-arsanilic acid (AA) was prepared in the same manner as BAA, with [^{3}H]acetic anhydride in place of bromoacetic anhydride. The specific radioactivity of [^{3}H]AA was 12 000 cpm/nmol.

Synthesis of Radiolabeled N-Carboxymethyl-L-lysine (CM-Lys) and O-Carboxymethyl-L-tyrosine (CM-Tyr). The method for preparing CM-Lys and CM-Tyr appeared in an earlier report (Koo & Cebra, 1974). Poly(L-lysine) was radioalkylated with [1-^{14}C]iodoacetic acid and then hydrolyzed. The hydrolysis product, CM-Lys, was purified by high-voltage electrophoresis on paper. The specific radioactivity of the product was 3000 cpm/nmol.

N-Carbobenzyloxy-L-tyrosine was reacted with [1-^{14}C]-bromoacetic acid for 16 h at ambient temperature. The product, *N*-Cbz-*O*-CM-L-tyrosine, was treated with hydrobromic acid to remove the *N*-Cbz group. The CM-Tyr was purified by high-voltage electrophoresis on paper. The specific radioactivity of CM-Tyr was 2500 cpm/nmol.

Equilibrium Dialysis. Binding affinities of anti-ARS antibodies were measured in microdialysis chambers according to the microtechniques of Voss & Eisen (1971) and Reese & Cebra (1975). One cell of each chamber recieved 50 μL of [^{3H}]AAT or [^{3}H]AA [(1–4) × 10^{-8} M] dissolved in phosphate-buffered saline, and the other cell received 50 μL of anti-ARS antibodies in phosphate-buffered saline [(1–6) × 10^{-9} M]. After the mixture was dialyzed for 36 h at 4 °C, 25-μL aliquots were sampled from both cells of each chamber for radioactive counting. The data were corrected for nonspecific binding by using the values obtained with normal guinea pig IgG1 and IgG2.

Kinetics and Specificity of Affinity-Labeling Reactions. Kinetic measurements were performed according to Weinstein et al. (1969). Anti-ARS or normal guinea pig IgG1 or IgG2 antibodies (1 nmol/mL in 0.10 M sodium bicarbonate, pH 9.0) were incubated with [^{14}C]BAAT or [^{14}C]BAA (6 nmol/mL) for 72 h at 37 °C with constant mixture (15 mL) at intervals after the addition of the affinity-labeling reagent (see Figure 3). Labeling was terminated by the addition of 0.5 mL of 75% (w/w) trichloroacetic acid (Cl_3AcOH), followed by incubation overnight at 4 °C. Precipitates that formed were retained on 0.22-μm Millipore filters (prewashed) in 0.2 M *p*-arsanilic acid (pH 9.0). Filters containing the precipitates were washed twice with 5 mL of 5% Cl_3AcOH (w/w), washed twice with 5 mL of ethanol, and dried at 37 °C in scintillation vials. Membranes were suspended in 5 mL of Instabray scintillation fluid, and the solutions were then counted. The number of moles of affinity label bound per mole of antibody was calculated, and the specificity of labeling was determined by comparing the amount of label bound to specific and nonspecific antibodies.

For further assessment of the specificity of the affinity-labeling reaction, cold AAT or AA (6 μmol/mL) was incubated with anti-ARS antibodies at 37 °C for 4 h prior to addition of either [^{14}C]BAAT or [^{14}C]BAA. The number of moles of [^{14}C]BAAT or [^{14}C]BAA bound per mole of antibody was calculated and the degree of specific blocking determined by

comparing the amount of label bound to unprotected and protected anti-ARS antibodies.

Preparation of Affinity-Labeled Anti-ARS Antibody. Purified anti-ARS antibody (2 μmol) was diluted to 1 nmol/mL with 0.1 M sodium bicarbonate buffer (pH 9.0). Caprylic acid (7×10^{-4} M) was added to inhibit bacterial growth in the antibody solution which was kept at 37 °C. The affinity label [^{14}C]BAAT or [^{14}C]BAA (12 μmol) in 100 mL of 0.1 M bicarbonate buffer was then added sllowly to the antibody solution with gentle stirring. The reaction mixture was incubated at 37 °C for 72 h. After incubation, the reaction was terminated by adding phenol (4 μmol) and adjusting the pH to 7.0 with 2 M HCl. The solution was then concentrated by ultrafiltration (Amicon 402 unit equipped with a PM-10 membrane) to 10 nmol/mL (final volume 200 mL). The concentrated antibody solution was dialyzed against 6 L of 0.05 M formic acid for 3 days at 4 °C and freeze-dried.

Isolation of Heavy and Light Chains. Lyophilized affinity-labeled antibody (20 mg) was dissolved in 2 mL of 7 M guanidine hydrochloride (Gdn-HCl) and 0.1 M in Tris-HCl buffer (pH 8.0). The protein samples were then treated with dithiothreitol (0.05 M) for 2 h at ambient temperature under N_2 with continuous slow stirring. For termination of the reaction, iodoacetamide dissolved in the same buffer was added to a final concentration of 0.11 M. The pH was maintained between 7 and 8 with Tris during the 1-h alkylation period. The reduced and alkylated protein samples were dialyzed overnight at room temperature against freshly deionized 8 M urea made 1 M in glacial acetic acid. Heavy (H) and light (L) chains were separated on a column of Bio-Gel P-150 (2.4 × 95 cm) equilibrated with the same buffer. The fractionated H and L chains (1 mg/mL each) were dialyzed against phosphate-buffered saline for 4 days at 4 °C. The specific radioactivities of affinity-labeled whole antibody molecules, L chains, H chains, and CNBr fragments derived from the H chains were determined as previously described (Ray & Cebra, 1972).

Isolation of Heavy-Chain Variable Region CNBr Fragments. CNBr cleavage of the intact IgG1 and IgG2 molecules and the isolation of CNBr fragments C-1-n, C-1-a_1, and C-1-a_2, containing Hv1, Hv2, and Hv3, respectively, were performed as previously described (Birshtein & Cebra, 1971; Ray & Cebra, 1972; Tracey et al., 1976).

In brief, CNBr digests of affinity-labeled IgG1 or IgG2 (2 μmol) were fractionated on columns of Sephadex G-100 (2.2 × 240 cm) equilibrated in freshly deionized 8 M urea, 0.1 M in formic acid. The labeled C-1-a_1 fragment was recovered in this separation (Figure 4). Fragments C-1-n and C-1-a_2 (10 mg/mL), which remained associated by intrachain disulfide linkage, were reduced with 0.05 M dithiothreitol in 7 M Gdn-HCl and 0.1 M Tris-HCl (pH 8.0). The reaction was performed at ambient temperature under N_2 for 1 h. Iodoacetamide, calculated to make the reaction mixture 0.1 M, was dissolved in 0.1 M Tris. This solution was added dropwise to the reaction mixture, which was stirred constantly under N_2. The pH was adjusted to 8.0, and the alkylation was allowed to proceed for 30 min. Afterward, the reaction mixture was applied to a Sephadex G-50 column (2.2 × 240 cm) equilibrated with 8 M urea, 0.1 M in formic acid. The C-1-a_2 fragment eluted before the C-1-n fragment.

Automated Sequential Degradation of C-1-a_1. Several preparations of C-1-a_1 fragments (1.0 μmol) were subjected to automatic Edman degradation by using a Beckman Model 890-B sequencer. The peptide program (111374, Beckman Instruments) which employs dimethylallylamine as the solvent buffer was used in all experiments. One-twentieth of each butyl chloride extract containing a thiazolinone derivative was counted in a Packard scintillation counter. The remainder of each thiazoline sample was converted to the phenylthiohydantoin (PTH) derivative as described previously (Birshtein & Cebra, 1971; Tracey & Cebra, 1974). The PTH derivatives were identified by gas chromatography on a Beckman GC-45 instrument and by thin-layer chromatography on polyamide sheets according to Summers et al. (1973). Amino acids regenerated from PTH derivatives by hydrolysis with hydriodic acid (Smithies et al., 1971) or HCl (Van Orden & Carpenter, 1964) were analyzed on a Technicon TSM amino acid autoanalyzer.

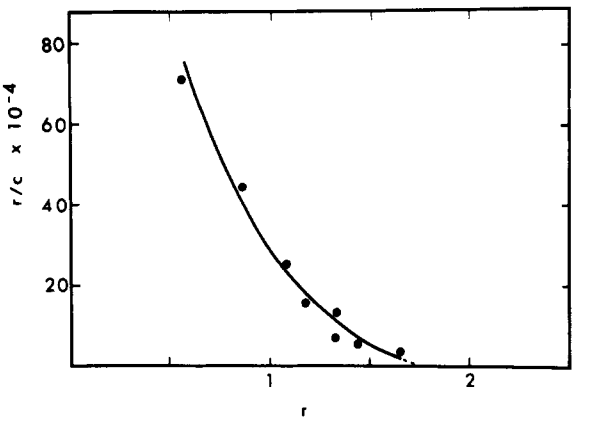

FIGURE 2: Scatchard plot of the binding data for IgG2 anti-ARS antibodies obtained by equilibrium dialysis against *N*-acetyl-3-[(*p*-arsonophenyl)azo]-L-tyrosine, AAT. The symbol *r* represents the moles of hapten bound per mole of antibody; *c* represents the free hapten concentration.

Distribution of Affinity Label between Lysyl and Tyrosyl Residues. The acid-hydrolyzed affinity-labeled residues derived from C-1-a_1 fragments together with the standards CM-Lys and CM-Tyr were electrophoresed on Whatman No. 1 paper in pyridine–acetate buffer (pH 3.6) for 45 min at 3 kV. After electrophoresis, either the chromatogram was dried and exposed to Kodak X-ray film (BB54) for 2 weeks or the chromatographic strips were radioscanned by using a Packard radiochromatogram scanner (Model 7200) equipped with a recording ratemeter (Model 385).

Amino Acid Analyses. All amino acid analyses of C-1-n, C-1-a_1, and C-1-a_2 were performed on a Beckman Model 121 amino acid analyzer. The procedure for hydrolysis has been described previously (Birshtein et al., 1971).

Physical Constants. The molecular weights and extinction coefficients for guinea pig IgG1, IgG2, H chain, and L chain were previously reported (Leslie & Cohen, 1970).

Results

Determination of Binding Affinities by Microequilibrium Dialysis. A representative Scatchard plot obtained for purified anti-ARS IgG2 antibody by microequilibrium dialysis using the hapten [^{3}H]AAT is shown in Figure 2. Extrapolation of the binding curve to the abscissa showed 1.75 binding sites per molecule of antibody. The average intrinsic association constant, K_0, was equal to the reciprocal of the free hapten concentration, *c*, at the point on the curve where one-half of the antibody combining sites were occupied by hapten (i.e., $r = 0.87$). K_0 was determined to be 4×10^5 L/mol. The nonlinearity of the binding curve reflects the heterogeneity of the antibody population with respect to affinity. Similar Scatchard plots were obtained for anti-ARS IgG1 antibodies.

The binding data obtained for the ligand [^{3}H]AAT reacting with IgG1 and IgG_2 antibodies produced in different guinea

Table I: Average Affinities (K_0) and Heterogeneity Indices (a) of Anti-ARS Antibodies Obtained by Equilibrium Dialysis Using the Hapten [^{3}H]AAT

immunization	strain	isotype	K_0 (L/mol)	a
conventional	13	IgG1	5.2×10^5	0.61
		IgG2	4.0×10^5[a]	0.57
carrier primed	13	IgG1	6.0×10^5	0.54
		IgG2	4.5×10^5	0.62
conventional	2	IgG1	3.7×10^5	0.56
		IgG2	4.9×10^5	0.59

[a] Represented in Figure 2.

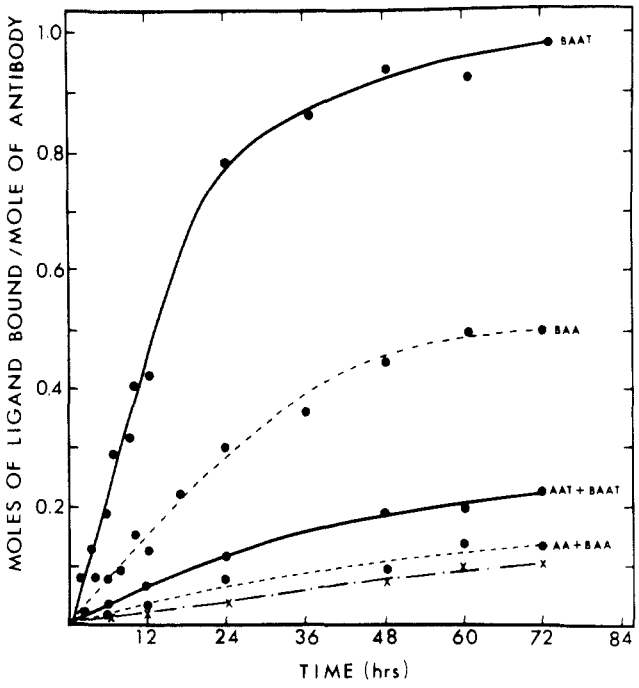

FIGURE 3: A comparison of the kinetics of affinity labeling of anti-ARS IgG2 antibody with BAAT (—) and BAA (- - -) in the presence or absence of blocking haptens, AAT or AA. The molar ratio of affinity label to antibody is 6:1. The line (-·-) represents a control reaction between nonspecific IgG2 with either BAAT or BAA at similar molar ratios.

pig strains and by different immunization regimens are summarized in Table I. The average binding affinity is 4.7×10^5 L/mol, and the average index of heterogeneity a is 0.58. The table shows no significant difference in K_0 or a among the populations of anti-ARS antibodies.

In experiments using the ligand [^{3}H]AA, the average binding affinity for IgG1 or IgG2 anti-ARS antibodies was approximately 5×10^4 L/mol, and the average index of heterogeneity a was 0.53.

Assessment of the Kinetics and Specificity of the Affinity-Labeling Reactions. Purified anti-ARS antibodies were allowed to react with a 6-fold molar excess of [^{14}C]BAAT or [^{14}C]BAA in the presence or absence of the blocking hapten AAT or AA. The kinetics and specificity of labeling of anti-ARS IgG2 immunoglobulin and nonspecific guinea pig IgG2 immunoglobulin are given in Figure 3.

There is a significant difference, both in rate and extent of labeling, between the reactions carried out in the presence or absence of blocking hapten and between specific and nonspecific immunoglobulin. Also, quantitative differences are seen in the kinetics and extent of reactions when the same population of anti-ARS antibodies are labeled with the homologous bromoacetyl ligands.

When [^{14}C]BAAT is used for affinity labeling, the reaction is distinguished by an initial rapid rate for 24 h and a gradual slowing of the rate to a plateau level during the following 48 h. The number of moles of ligand bound per mole of specific antibody approaches 1.0 at 72 h compared to 0.08 for nonspecific antibody. Prior addition of AAT, a chemically nonreactive analogue of [^{14}C]BAAT, completely eliminates the initial rapid reaction such that only 0.2 mol of sites is labeled at 72 h.

Table II: Affinity Labeling of Anti-ARS Antibodies with Homologous Bromoacetyl Ligands

immunization	strain	isotype	ligand	extent of reaction[a]	half-time reaction[b] (h)
conventional	13	IgG1	[^{14}C]BAAT	0.95	11
conventional	13	IgG2	[^{14}C]BAAT	0.98[c]	12
conventional	13	IgG1	[^{14}C]BAA	0.60[d]	17
conventional	13	IgG2	[^{14}C]BAA	0.48[c]	21
carrier primed	13	IgG1	[^{14}C]BAA	0.64	16
carrier primed	13	IgG2	[^{14}C]BAA	0.52	20
carrier primed	13	IgG1	[^{14}C]BAAT	0.99[d]	10
conventional	2	IgG2	[^{14}C]BAA	0.57[d]	19

[a] The extent of the reaction is given as moles of ligand bound per mole of antibody at 72 h. [b] The time required for covalent labeling of half of the sites which finally were labeled at 72 h. [c] Represented in Figure 3. [d] Antibodies whose second hypervariable region (Hv2) was sequenced.

Affinity-labeling experiments using the hapten [^{14}C]BAA show a much slower initial reaction rate during the first 24 h when compared to that for [^{14}C]BAAT (Figure 3). However, in the subsequent 48 h, both reagents show a similar leveling off of the reaction rate. Maximum labeling with [^{14}C]BAA is achieved at 72 h when approximately 0.5 mol of affinity label is bound per mol of specific antibody. On the other hand, anti-ARS antibodies protected by the competing ligand AA bind only 0.1 mol of [^{14}C]BAA per mol of specific immunoglobulin. The results shown in Figure 3 are representative for other kinds of anti-ARS antibody populations.

Table II shows comparative affinity labeling data for IgG1 and IgG2 antibodies produced in strain 13 and strain 2 guinea pigs either conventionally immunized or carrier primed. In every case, regardless of the kind of anti-ARS antibodies used in the experiments, the hapten [^{14}C]BAAT modified approximately 50% (1.0 mol of ligand bound/mol of antibody) of the combining sites while the ligand [^{14}C]BAA labeled about 30% (0.6 mol of ligand bound/mol of antibody) of the active sites. The half-time of the labeling reaction (the time required to bind half the label that is finally bound at 72 h) generally is several hours longer for [^{14}C]BAA (16–21 h) than for [^{14}C]BAAT (10–21 h).

Distribution of Affinity Label in Anti-ARS Antibody. Specific IgG1 or IgG2 immunoglobulins (300 mg) were preparatively affinity labeled with either [^{14}C]BAAT or [^{14}C]BAA for 72 h under the same conditions as in the kinetic experiments. To obtain CNBr fragments of the H-chain variable region, affinity-labeled antibodies were subjected to cleavage with CNBr and filtered through columns of Sephadex G-100 in 8 M urea, 0.1 M in formic acid (Figure 4a,b). For both IgG1 and IgG2 antibodies, much of the label was found in the pool known to contain the fragment C-1-a_1 (Birshtein & Cebra, 1971; Tracey et al., 1976). C-1-a_1 contains the second hypervariable region of the heavy chain (Hv2) and accounts for residues N-35 to N-83 of both the γ_1 and γ_2 chains.

The remainder of the radioactivity in Figure 4a,b is found in pools 1 and 2 of each chromatograph. In both instances,

Table III: Distribution of Affinity Label on L and H Chains and CNBr Fragments of H Chain Containing the V_H Region of Anti-ARS IgG1 and IgG2 Antibodies

immunization	strain	isotype	ligand	mole of label per mole of				
				L chain	H chain	C-1-n	C-1-a_1	C-1-a_2
conventional	13	IgG1	[^{14}C]BAA	0.04	0.30	0.02	0.21	0.03
carrier primed	13	IgG1	[^{14}C]BAAT	0.07	0.47	0.03	0.36	0.04
conventional	2	IgG2	[^{14}C]BAA	0.05	0.26	0.01	0.18	0.02

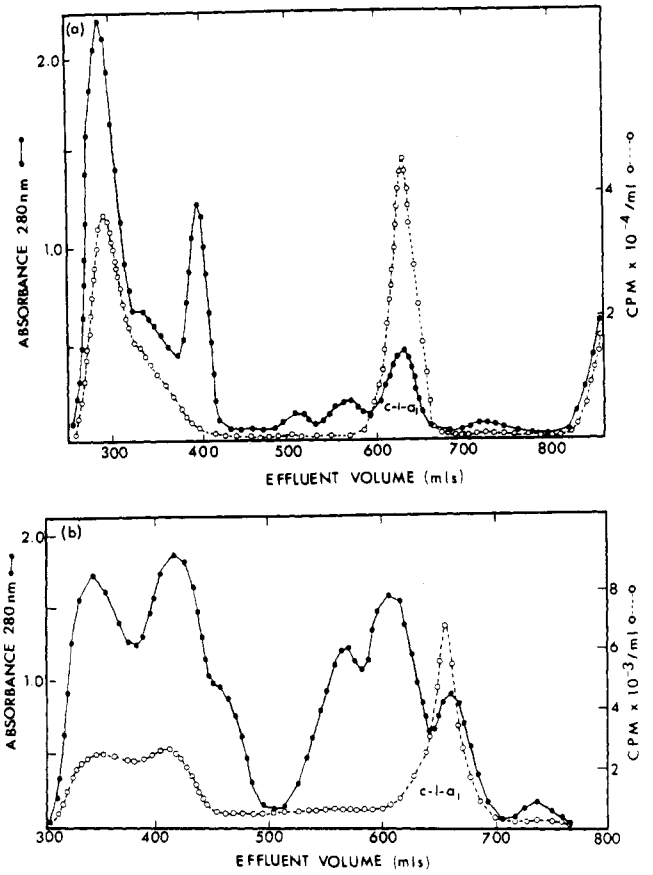

FIGURE 4: Elution profiles of CNBr digests of IgG1 and IgG2 from columns of Sephadex G-100 (2.0 × 240 cm) in 8 M urea, 0.1 M in formic acid. The radiolabeled CNBr fragment C-1-a_1 is released intact in each case. (a) Fractionation of IgG1 labeled with [^{14}C]BAAT. (b) Fractionation of IgG2 labeled with [^{14}C]BAA. The antibodies used in (a) were isolated from carrier-primed strain 13 animals and in (b) from conventionally immunized strain 2 animals.

these pools contain L chains and other H-chain CNBr fragments. C-1-n and C-1-a_2, which contain respectively the first and third H-chain hypervariable regions (Hv1 and Hv3), are isolated from these pools following total reduction and alkylation of the components of the pools. The peptides were fractionated on columns of Sephadex G-50 as has been described (Birshtein & Cebra, 1971; Tracey et al., 1976). C-1-n spans residues N-1 to N-34, and C-1-a_2 spans residues N-84 to approximately N-138. Together with C-1-a_1, these fragments account for the entire H-chain variable region.

The overall differences noted in the elution profiles of IgG1 and IgG2 antibodies (Figures 4a,b) reflect the different positions of methionyl residues in the Fc portion of the molecule and the extra intra-H-chain disulfide bond in IgG1 molecules (Tracey, 1973; Tracey et al., 1976).

The distributions of affinity label between H and L chains and between the H-chain variable region CNBr fragments derived from different isotypes and from different strains are given in Table III. In each case, over 80% of the affinity label was found associated with the H chain and less than 20% with the L chain. Of label found in the H-chain variable region, at least 80% was borne by the C-1-a_1 fragment and the remainder almost evenly distributed between C-1-n and C-1-a_2 fragments. A higher molar fraction of affinity labeling was obtained each time [^{14}C]BAAT than with [^{14}C]BAA.

Localization and Identification of Affinity-Labeled Residues in the C-1-a_1 Fragment. The affinity-labeled C-1-a_1 fragments (1 μmol) from each of the anti-ARS antibodies were subjected to automatic sequential degradation from the NH_2-terminal end. A portion of the thiazolinone derivative of each amino acid residue in the butyl chloride fraction was taken for determination of radioactivity. The nomogram shown in Figure 5a represents the profile of radioactivity for the first 30 residues of C-1-a_1 labeled with [^{14}C]BAA derived from IgG1 molecules from conventional strain 13 animals. It is apparent that residue N-57 is the principal residue modified by BAA followed by N-59 and N-50. All three residue positions reside in the Hv2 region (N-48 to N-59). When IgG1 antibodies, isolated from carrier-primed strain 13 animals, are radiolabeled with [^{14}C]BAAT, N-59 is the most conspicuously modified residue within Hv2 (Figure 5b). The C-1-a_1 labeled with [^{14}C]BAA but derived from IgG2 molecules produced in conventionally immunized strain 2 guinea pigs yielded a radioactive profile (Figure 5c) similar to that given by C-1-a_1 from IgG1 likewise labeled with BAA (Figure 5a).

The acid-hydrolyzed residues N-50 and N-57 from BAA modified antibodies, when analyzed by high-voltage electrophoresis on paper followed by autoradiography, have the same electrophoretic mobility as the radioactive standard CM-Tyr, while N-59, following hydrolysis, migrates in the vicinity of the radioactive standard CM-Lys (Figure 6a). The autoradiograph in Figure 6a is representative of C-1-a_1 from IgG1 or IgG2 modified with the ligand [^{14}C]BAA. Figure 6b shows the autoradiographic pattern of residues N-50, N-57, and N-59 obtained from C-1-a_1 labeled with [^{14}C]BAAT derived from IgG1 molecules. Only N-59 is modified and migrates electrophoretically with CM-Lys. Radioscanning of chromatographic strips of C-1-a_1 hydrolysate or H-chain hydrolysate also indicates that lysine and tyrosine residues are modified. These results, together with the sequence for anti-ARS C-1-a_1, which shows N-50 and N-57 to be occupied predominantly by tyrosine and N-59 by a lysine (Figure 7b), seem to confirm the identity of residues labeled at these positions.

Sequencing of Affinity-Labeled Hv2 from Different Anti-ARS Isotypes. The primary structures of Hv2 (N-48 to N-59) were determined by automatic Edman degradation of C-1-a_1 fragments. The tyrosyl residues at N-50 and N-57 and the lysyl residue at N-59 are characteristic of anti-ARS specificity when compared to dinitrophenyl (Dnp) and (*p*-azophenyl)-trimethylammonium (TMA) specificities (Figure 7a,b). The Hv2 of anti-ARS antibodies is further distinguished by contiguous serine residues from N-52 to N-56. The primary structures of Hv2 from all the anti-ARS antibodies showed these characteristic amino acids as major residues at the appropriate positions. Alternatives detected at some of these positions and which contribute to the heterogeneity of Hv2 in various anti-ARS antibodies are given in Figure 7b. The

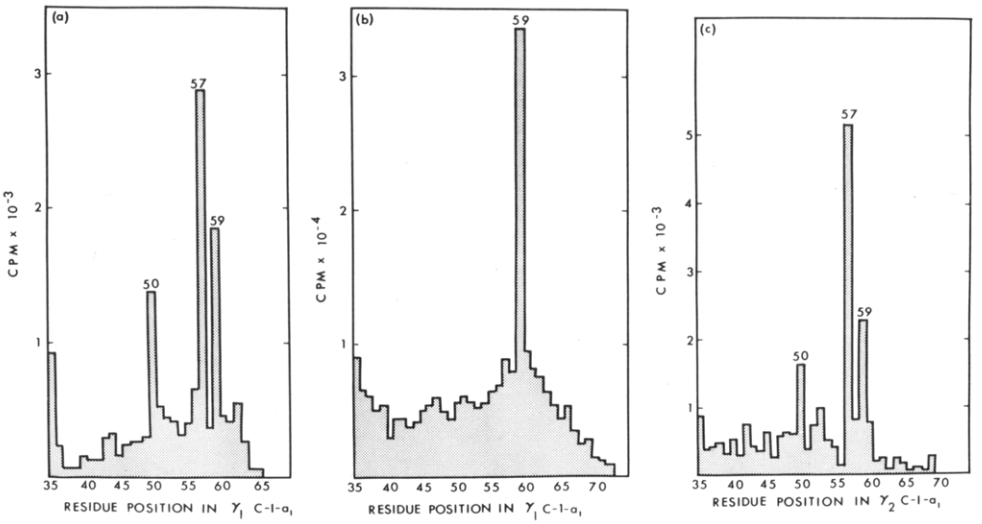

FIGURE 5: Nomograms showing the different radiolabeling patterns detected for the first 30 residues of [^{14}C]BAAT or [^{14}C]BAA modified C-1-a_1 (N-35 to N-83) by automatic sequential degradation of the fragment: (a) C-1-a_1 labeled with [^{14}C]BAA and derived from IgG1; (b) C-1-a_1 labeled with [^{14}C]BAAT and derived from IgG1; (c) C-1-a_1 labeled with [^{14}C]BAA and derived from IgG2. The majority of the radioactivity is within Hv2 (N-48 to N-59). The antibodies used in (a) were obtained from conventionally immunized strain 13 animals and those of (b) and (c) as in Figure 4.

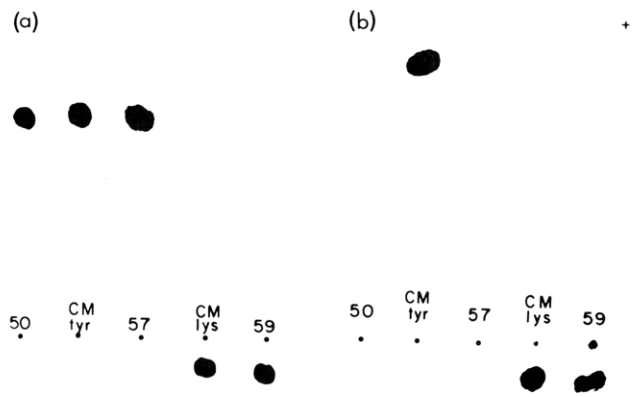

FIGURE 6: Autoradiographic patterns of labeled residues obtained from automatic sequential degradation of C-1-a_1: (a) a representative autoradiographic pattern for labeled residues N-50, N-57, and N-59 obtained for C-1-a_1 derived from either IgG1 or IgG2 antibodies labeled with [^{14}C]BAA; (b) autoradiographic pattern for the same residue positions of C-1-a_1 derived from IgG1 antibody modified with [^{14}C]BAAT. Positions of radiolabeled standards CM-Lys and CM-Tyr are indicated.

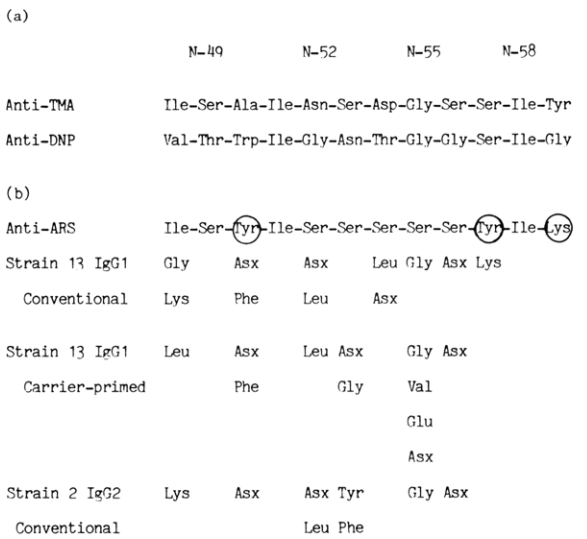

FIGURE 7: Primary structure of anti-ARS C-1-a_1 from N-48 to N-59, the Hv2 region: (a) a comparison of the prototype sequence of Hv2 for antibodies elicited against TMA and Dnp haptens (Cebra et al., 1974); (b) the prototype sequence of Hv2 for all anti-ARS antibodies sequenced and the variants detected at their respective positions for those antibodies examined. The affinity-labeled residues are circled.

degree of variability detected at any given position was generally about 15% per alternate residue. Some of these variants at a given position are shared by the two isotypes while it appears that others are not. Although no alternate residue was detected for lysine at N-59, substitutions for tyrosine at N-50 and N-57 were found (Figure 7b). Position N-48 contains alternatives to isoleucine while at N-51 and N-58 no substitutions were detected for the isoleucines.

Amino Acid Composition of C-1-a_1 Derived from Different Isotypes. Table IV shows the amino acid compositions of C-1-a_1 derived from IgG1 and IgG2 molecules produced in carrier-primed strain 13 animals and conventionally immunized strain 13 and strain 2 animals. The amino acid analyses of these four C-1-a_1 fragments are similar and show the high tyrosine content characteristic of this fragment from anti-ARS antibodies (Koo & Cebra, 1974). The small compositional differences noticed probably reflect the heterogeneity in Hv2 since the framework regions (residue positions outside Hv2; N-36 to N-47 and N-60 to N-83) appear identical for these C-1-a_1 fragments (data not shown).

Discussion

The immune response of animals to the simplest of antigenic determinants gives rise to antibody molecules which, although specific to the antigen, are heterogeneous in amino acid sequence. Underlying this structural variability is a corresponding heterogeneity of antibody-producing cells, for a single immunocyte generally gives rise to a single molecular species of antibody molecule (Cebra et al., 1966; Haber, 1968; Nordin et al., 1970). The ability of inbred strain 13 guinea pigs to produce highly restricted antihapten antibody (Cebra et al., 1974; Cebra, 1977) suggests that a regulatory mechanism(s) may be functioning to diminish the number of responding clones. Therefore, we have examined a variety of anti-ARS antibody populations derived from two different inbred guinea pig strains to assess structural variation in the Hv2 region, which appears to form part of the combining site as judged by analysis of antibodies modified with an affinity-labeling

Table IV: Amino Acid Compositions of C-1-a_1 Fragments Derived from Anti-ARS IgG1 and IgG2 Antibodies Containing the Hv2 Region of the H Chain[a]

	strain 13			strain 2
	conventional	carrier primed		conventional
	IgG1	IgG1	IgG2	IgG2
Lys	3.1	3.4	3.1	3.2
His	0.2	0.1	0.3	0.2
Arg	3.1	3.0	2.9	3.0
CM-Cys				
Asp	4.9	4.7	4.6	4.3
Thr	3.2	2.8	2.9	2.9
Ser	5.6	5.8	5.7	6.0
Glu	3.7	3.4	3.3	3.6
Pro	2.0	1.6	1.7	1.8
Gly	3.9	4.4	4.3	4.1
Ala	2.8	2.4	2.5	2.6
Val	2.6	2.3	2.4	2.7
Ile	3.5	4.0	3.5	3.6
Leu	2.3	2.4	2.7	2.5
Tyr	3.8	3.6	3.7	3.9
Phe	1.2	1.2	1.3	1.4
Hsr	0.9	1.3	1.2	1.3
Trp[b]	+	+	+	+

[a] Compositions are reported as moles of amino acid per mole of peptide. The compositions were based on three analyses of each of several different preparations and were normalized to 47 residues, exclusive of two Trp. Values are from 18-h hydrolysis time. [b] Trp was identified by Ehrlich stain (Easley, 1965).

reagent (Koo & Cebra, 1974). Such a comparative examination may provide some insight into the degree of clonal persistance or replacement.

Regardless of their origins, guinea pig IgG1 and IgG2 anti-ARS antibodies were found to have similar binding affinities, similar amino acid compositions for the section of their V_H analyzed (Table IV), and similar primary structures for their Hv2 complementarity region. All of the guinea pig anti-ARS antibodies had a K_0 of approximately 4.7×10^5 L/mol for the ligand AAT (Figure 2; Table I). This value is in agreement with previously reported ones for other anti-ARS antibodies derived from different species (Davis et al., 1969; Becker et al., 1974; Kapsalis et al., 1976). The average index of heterogeneity (a) for the various populations of anti-ARS antibodies is approximately 0.58. This compares to approximately 0.75 for anti-Dnp antibodies which have a K_0 of approximately 1×10^7 L/mol (Reese & Cebra, 1975). This difference, though small, indicates that induced populations of anti-ARS antibodies in other species are less restricted in variability than guinea pig anti-DNP antibodies. Sequence analysis of the Hv2 region from anti-Dnp antibodies supports this notion (Cebra et al., 1974; Trischmann, 1975). The anti-ARS variability is at least partially associated with the Hv2 region (Figure 7) and is such that both the average K_0 and a of both IgG1 and IgG2 antibodies produced in different strains were very similar (Table I). Thus, both strains of guinea pigs make heterogeneous populations of antibodies, but the mixture of subpopulations of V_H/V_L domains in each may be very much alike. The restricted variability and similarity of Hv2 found in anti-ARS antibodies from either isotype complement earlier reports which have shown that the Hv1 region of phosphorylcholine-binding mouse myeloma proteins are nearly identical (Barstad et al., 1974) and that guinea pig IgG1 and IgG2 anti-Dnp antibodies have similar binding sites based on association constants for ligands, localization of residues modified by an affinity label, and primary structural analysis of Hv1 and Hv2 regions (Tracey, 1973; Trischmann, 1975). Likewise, Trissi & Kolb (1975) reported that IgG1 and IgG2 antibodies from individual mice raised against antigenic determinant oligo(D-alanine) are likely to have similar combining sites, as judged from measurements of affinity for ligand and reaction rate constants.

A comparison of the ligand structures (Figure 1) suggests that BAAT, with dimensions of approximately $14 \times 5 \times 2$ Å, may have greater intrinsic rotational freedom along the axis between the immunospecific group (arsonate) and the reactive bromoacetyl moiety than BAA, with approximate dimensions of $6 \times 5 \times 2$ Å. Because the dimensions of BAAT approach that reported for the ligand binding groove of Fab' New (Poljak et al., 1973), which is partially lined by Hv2, the possibility exists that at equilibrium BAAT molecules in the unfolded configuration are sterically restricted from entering the groove while molecules in the folded configurations can enter the groove more easily. As postulated by Koo & Cebra (1974), affinity labeling with BAAT may occur preferentially when the molecule is in the folded (cis) configuration—a spatial orientation which brings the arsonate and bromoacetyl groups into close proximity. Such a configuration could be a spatial requirement necessary for BAAT to display its high degree of specificity for modification of lysine N-59 and may influence the kinetics of the labeling reaction. On the other hand, BAA has fewer nonbenzenoid carbons and therefore less rotational freedom than BAAT. This restriction in flexibility may limit the rate of reaction with lysine N-59 should this residue be a "contact" one vis-à-vis the arsonate group. This attempt to correlate variations in the structural parameters of the ligands to differences in their pattern of modification of residues within Hv2 are conjectural since the preferred ligand configuration for binding at equilibrium is not known and considerable differences do exist in the overall shape, size, and general chemical nature of hapten binding sites (Amzel & Poljak, 1979). However, the use of a pair of affinity labels, BAA and BAAT, to compare the populations of binding sites in a variety of anti-ARS antibodies does seem to permit some reasonable inferences: (1) The 50% of all anti-ARS antibodies modified by BAAT and the 30% modified by BAA all seem to interact with and orient the arsonate group similarly such that most residues that are chemically substituted occur in Hv2. (2) The BAAT which reacts with the 50% of sites in both IgG1 and IgG2 antibodies seems to display restricted mobility once it enters the binding site since lysine-59 is almost exclusively modified in all cases despite the occurrence of several reactive tyrosyl residues nearby. (3) The 30% of molecules reactive with BAA found in both isotypes from all sources seems to be comprised of a mixture of binding sites in which their relative distributions, in the various anti-ARS antibody populations analyzed, appear to be similar. This contention is based on the finding that Hv2 of IgG1 (Figure 5a) and IgG2 (Figure 5c) anti-ARS antibodies, obtained from conventionally immunized strain 13 and strain 2 guinea pigs, respectively, display similar labeling patterns when modified with BAA (Tyr-57 > Lys-59 > Tyr-50). The inference also depends on the observation that a particular one of a homologous series of affinity labels usually only modifies one particular residue position or none at all when reacting with a homogeneous immunoglobulin. It should be noted that in at least one instance—the reaction of the myeloma immunoglobulin MOPC 315 with N^{α}-(bromoacetyl)-N^{δ}-(dinitrophenyl)-L-ornithine—modification at either of two residue positions in a homogeneous population of binding sites by the same affinity label has been observed (Givol et al., 1971).

The identification of modified residues at more than one position after reaction of anti-ARS antibodies with BAA may

reflect the heterogeneity in the arsonate antibody population confirmed by the nonlinearity of the binding curve (Figure 2) and primary structural analysis of Hv2 (Figure 7b). Alternatively, the multiplicity of labeling with BAA may imply that the orientation of this ligand near the Hv2 of the arsonate combining site is less restricted than that of BAAT. Support for this possibility comes from the finding that anti-ARS antibodies bind BAA with a lower affinity than BAAT and that BAA is a smaller molecule with a different geometrical relationship between its bromoacetyl reactive group and the arsonate affinity group compared to BAAT. Either or both of these possibilities seem tenable in explaining the multipoint modifications by BAA. With respect to those molecules reactive with either or both BAA and BAAT, it is concluded that the populations of combining sites in all preparations of anti-ARS are very similar and probably represent the products of clones of cells expressing one or another of the same relatively small groups of structural V genes.

When there is considerable difference in K_0 between site-directed affinity reagents, as is apparent in this study, the possibility of nonequivalence of the two binding processes (BAAT vs. BAA) should be expected. Since Lys-59 is invariant (Figure 7b), exclusive labeling of this residue by BAAT does not infer that only a single major combining site has been modified. The tyrosine residues (N-50 and N-57) modified by BAA recently have been detected also in the Hv2 of the murine anti-ARS combining site (Capra & Nisonoff, 1979). Since Tyr-57 is the most reactive tyrosine in the Hv2 of guinea pig anti-ARS antibodies, probably due to its spatial proximity to Lys-59, the tripeptide Tyr-Ile-Lys (N-57 to N-59) in this part of the complementarity-determining segment of the H chain may be an important arsonate binding subsite. Although neither affinity label is an ideal reagent, the use of pair labeling, in this investigation, has contributed to our understanding about the relationship between the molecular structure and binding properties of the arsonate combining site. In addition, the results have illustrated the inherent complex geometry involved in hapten ligation to antibodies.

Complementary primary structural studies on the first and third hypervariable regions of the heavy chain as well as on the V_H framework are in progress for the various anti-ARS antibody populations. Upon completion of these studies, assessment of the predominant sequence for the entire heavy-chain variable region (V_H) will be possible. Such studies are essential in the determination of any structural differences which may have occurred which were suggestive of expression of different V_H region genes due either to differing genetic background of the two guinea pig strains or to selective stimulation of particular B-lymphocyte clones by carrier priming.

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