Affinity Labeling of a Distinctive Lysyl Residue within the Second Hypervariable Region of γ_2 Chain of Guinea Pig Anti-*p*-Azobenzenearsonate Antibody†

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ABSTRACT: Anti-*p*-azobenzenearsonate (anti-ARS) antibodies, purified from sera of inbred strain 13 guinea pigs, were affinity labeled with *N*-[1-^{14}C]bromoacetylmono(*p*-azobenzenearsonic acid)-L-tyrosine (BAAT). Each mole of anti-ARS antibodies bound 1 mol of BAAT covalently, while only 0.08 mol of BAAT was bound per mole of nonspecific IgG2. The affinity labeling reaction could be inhibited by haptens such as *p*-nitrobenzenearsonate and the N^{α}-acetyl analog of BAAT, preincubated with the antibodies. The proportion of label incorporated into heavy and light chains was estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis to be 2.3:1. Lysine and tyrosine were the only major residues labeled in the whole antibody molecule and their labeled derivatives were recovered in a ratio of 1.6:1, respectively. Cyanogen bromide fragments C-1-n, C-1-a_1, and C-1-a_2 account for residues from N-1 to N-140 of γ_2 chain and include those residue positions which have different amino acids in antibodies of different antigen-binding specificities. Most of these "variable" residue positions occur in three short segments called "hypervariable" regions. The fragments C-1-n, C-1-a_1, and C-1-a_2 were separated from a CNBr digest of affinity-labeled anti-ARS antibodies. About 94% of their total label was estimated to be localized in C-1-a_1 (N-35 to N-83). The peptides from enzymic digests of C-1-a_1, including a radio-labeled tripeptide, were isolated, partially sequenced, and aligned. The anti-ARS antibody, like anti-Dnp antibody, had a C-1-a_1 with a distinctive primary structure of restricted heterogeneity, even within the second "hypervariable" region. The only conspicuously labeled residue in C-1-a_1 occurred at position N-59 and it could be released by automatic sequential degradation. Although antibodies of different antigen-binding specificities have different amino acids at N-59, only a lysyl residue was detected there in anti-ARS antibodies, and radiolabeled CM-lysine was identified at N-59 in affinity-labeled molecules. The highly specific labeling of anti-ARS antibody in a normally "variable" position within a "hypervariable" region suggests that lysine N-59 may be a contact residue in the antigen-binding site. Thus this very lysyl residue, distinctive for anti-ARS antibody, may both contribute to binding specificity for ligands such as BAAT and also be chemically modified by them.

The effects of selective chemical modification of lysyl and arginyl residues have been used to implicate positively charged amino acid residues as contact residues in the combining sites of several rabbit antibodies that bind anionic haptens (Pressman *et al.*, 1970; Grossberg *et al.*, 1973), such as anti-*p*-azobenzenearsonate antibodies (Freedman *et al.*, 1968a,b; Grossberg and Pressman, 1968). A number of investigators (Wofsy *et al.*, 1960; Metzger *et al.*, 1964; Koyama *et al.*, 1968) have used an affinity-labeling reagent, *p*-(arsonic acid)benzenediazonium tetrafluoroborate, to specifically modify rabbit anti-ARS[1] antibodies and presumably to label residues in the combining sites. In these selective modifications, as in other affinity-labeling reactions between diazonium reagents and anti-benzenoid hapten antibodies, tyrosyl derivatives were almost exclusively the products. These modified tyrosyl residues in both H and L chains are thought by some to be a part of "conservative structural features" within antibody active sites (Singer *et al.*, 1967), and may not be related to the antigen-binding specificities of antibodies raised against differently charged immunodominant groups. Except for occasional labeling of a histidine residue, no labeling of other kinds of amino acids has been described by investigators using diazonium affinity labels. Thus it would be informative to use other affinity-labeling reagents, with the potential to label not only tyrosine but also other residues in or near the antigen-binding site, to modify anti-azobenzenearsonate antibodies.

Affinity labels other than the diazonium reagents have been synthesized and used to modify antibodies. For instance, photochemically activated reagents, such as aryl nitrenes (Fleet *et al.*, 1969), which can insert into a C–H, N–H, or S–H bond of any amino acid residue, have shown promise as labels of contact residues of all sorts. However, the nitrene derivatives have been difficult to identify and hence to localize, especially when the primary structure of the modified protein is unknown. Another group of affinity-labeling reagents for antibodies is the bromoacetyl (halomethyl ketone) reagents which have been successfully used to affinity label lysyl as well as tyrosyl residues of myeloma proteins which bind Dnp-ligands and anti-Dnp antibodies (Haimovich *et al.*, 1970; Strausbauch *et al.*, 1971; Givol *et al.*, 1971). The modified residues give

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[1] Abbreviations used are: Ab, antibody; ARS, *p*-azobenzenearsonate; TMA, *p*-azobenzenetrimethylammonium; Dnp, 2,4-dinitrophenyl; MNBDF, *m*-nitrobenzenediazonium fluoroborate; BADL, N^{α}-bromoacetyl-N^{ϵ}-Dnp-lysine; BAAT, *N*-bromoacetylmono(*p*-azobenzenearsonic acid)-L-tyrosine; AAT, *N*-acetyl(*p*-azobenzenearsonic acid)-L-tyrosine; AT, mono(*p*-azobenzenearsonic acid)-L-tyrosine; *p*-NBA, *p*-nitrobenzenearsonic acid; Boc, *tert*-butyloxycarbonyl; Cbz, carbobenzoxy; CM, carboxymethyl; PhNCS, phenylthiohydantoin; CNBr, cyanogen bromide; PBS, phosphate buffered saline; C_H regions, constant regions of heavy chains which show no differences in amino acid sequences among antibodies of different specificities of the same isotype and allotype.

rise to carboxymethylamino acids after acid hydrolysis and are stable and easily identifiable (Weinstein *et al.*, 1969; Lawson *et al.*, 1968). Other amino acid residues which have been known to react with the halomethyl ketone type of site-directed reagents are histidine (Ong *et al.*, 1965) and serine (Lawson *et al.*, 1968) at the active site of trypsin, methionine in chymotrypsin (Lawson and Shramm, 1965), aspartic acid in pepsin (Erlanger *et al.*, 1965, 1966), and glutamic acid in triose phosphate isomerase (Hartman, 1970), elastase (Visser *et al.*, 1971), and carboxypeptidase A (Haas and Neurath, 1971). Strausbauch *et al.* (1971) have successfully used homologs of bromoacetyl reagents of different sizes to probe the active site of myelomas which bind Dnp-ligands. Thus the use of site-directed bromoacetyl reagents to label residues other than tyrosine seemed promising.

To accurately place a modified residue within the primary sequence has always been a difficult task when working with naturally raised antibodies. When small affinity-labeled peptides from antibody molecules are sought, the modified peptides in most instances show considerable heterogeneity in terms of amino acid sequence so that no peptide with a single sequence can be obtained in good yield and be identified. The few assignments of positions of affinity-labeled residues are mostly for anti-dinitrophenyl antibodies and include Franek's (1971) work on porcine λ chain, within which he placed the labeled tyrosyl residues at N-33 and N-93, that of Press *et al.* (1971), who isolated small modified peptides and placed the labeled residues at N-97and N-98 of rabbit γ chain, and our own (Cebra *et al.*, 1971; Ray and Cebra, 1972) indicating that modified tyrosyl residues occurred at N-32/33 and N-60 of guinea pig γ_2 chain. Most other data available concerning the placement of affinity label at a particular residue position in the primary structure of immunoglobulins come from site-directed modification of myeloma proteins (Haimovich *et al.*, 1972; Chesebro and Metzger, 1972; Goetzl and Metzger, 1970). Of the several objections to using a myeloma protein for studying the combining sites of antibody, the most serious one is based on the ambiguity regarding its specificity. Several of the myelomas with ligand-binding activity cross-react with a variety of seemingly unrelated haptens. Unless antibodies with similar primary structures to these myelomas can be raised against the same haptens used to synthesize affinity labels, the functional specificity of these model antibody proteins will remain in doubt.

We have chosen to examine antibodies deliberately raised in inbred guinea pigs to selected antigenic determinants. Previous results from our laboratories have shown that positions in the heavy chain derived from "normal" inbred guinea pig IgG2 having alternative residues occur only in its N-terminal quarter (V_H region) and mainly in three short "hypervariable" segments, while the same positions in at least the first two "hypervariable" regions show restricted heterogeneity or homogeneity in γ_2 chain from anti-Dnp antibody (Cebra *et al.*, 1971). Ray and Cebra (1972) have shown that [^{14}C]-*m*-nitrobenzenediazonium tetrafluoroborate specifically affinity labels only residues within the amino-terminal quarter of anti-Dnp antibody heavy chain obtained from strain 13 guinea pigs. Both to determine whether antibodies of another specificity also have a distinctive primary structure of restricted heterogeneity within "hypervariable" regions and to assess whether residues implicated in determining binding specificity could be affinity labeled, we have analyzed guinea pig anti-ARS antibodies modified with a bromoacetyl reagent reactive with a variety of amino acid residues. We have prepared and used an active-site directed reagent, BAAT, which has a substantial degree of freedom of rotation around many carbon and nitrogen atoms and which may assume many configurations in solution. We have isolated a major labeled peptide from γ_2 chain of anti-ARS antibodies, identified the position of the affinity-labeled lysyl residue in their V_H region and determined a distinctive primary structure of restricted heterogeneity for their second "hypervariable" region.

Materials and Methods

Preparation of Antigens. The immunizing antigen was prepared by coupling 100 mg of giant keyhole limpet hemocyanin (Schwarz/Mann) in 40 ml of 0.1 M $NaHCO_3$ (pH 8.1) with 60 ml of 0.02 M diazotized *p*-arsanilic acid. The diazotized arsanilic acid was prepared according to Tabachnick and Sobotka (1959). The coupling reaction was carried out at 0–5° by a slow addition of diazotized arsanilic acid solution to the hemocyanin solution, the pH of which was maintained between 8.5 and 9.0 with 5 M NaOH. The reaction mixture was stirred overnight at 0–5°. The ARS-hemocyanin solution was then exhaustively dialyzed against phosphate buffered saline (PBS) at 5°.

A similar procedure was also used for the preparation of ARS–bovine γ globulin.

Preparation of Normal IgG2 and Purified Anti-ARS Antibodies. The nonspecific IgG2 used in affinity-labeling experiments was obtained from anti-lysozyme sera depleted of anti-lysozyme antibodies by specific precipitation. This serum was obtained from Wright strain 13 guinea pigs from our own colony, immunized with lysozyme (Sigma Chemical Co.) in complete Freunds adjuvant. The method for the purification of nonspecific IgG2 was similar to that already reported (Birshtein and Cebra, 1971). Anti-ARS sera were raised in animals immunized with ARS-hemocyanin in complete Freunds adjuvant. Details on immunization and bleeding of the animals are similar to those already reported (Birshtein and Cebra, 1971). The anti-ARS antibodies were separated from the other serum components by precipitation with ARS-bovine γ globulin. The specific precipitate was collected by centrifugation, washed three times with cold (0–5°) PBS, and redissolved by the addition of a cold solution of 0.25 M *p*-nitrobenzenearsonic acid (pH 7). About 10 ml of *p*-nitrobenzenearsonate (Eastman Organic Chemicals) solution was used to dissolve the specific precipitate obtained from 650 ml of serum. The immune precipitates usually completely dissolved, but if any insoluble material persisted, it was removed by centrifugation at 0–5°. The clear supernatant was applied to a column of DEAE-Sephadex, A25 (2 × 54 cm), which had been equilibrated with 0.02 M sodium phosphate buffer (pH 7.2). Following applications of the dissolved precipitate, about 5 ml of 0.25 M *p*-NBA (pH 7) was added to the column. The antibodies were then eluted with 0.02 M sodium phosphate buffer (pH 7.2). While both ARS-bovine γ globulin and *p*-NBA remained adsorbed to the column, the colorless anti-ARS antibodies were eluted as a single protein component after the collection of one column void volume. By this method about 1 mg of purified antibody was obtained per milliliter of starting antiserum.

Synthesis of Mono(p-azobenzenearsonic acid)-L-tyrosine (AT). Mono(*p*-azobenzenearsonic acid)-*N*-*t*-Boc-L-tyrosine was prepared by a slow addition of the diazotized arsanilic acid (7.1 mmol in 133 ml of 0.17 N HCl) to a cold solution of an equimolar amount of *N*-*t*-Boc-L-tyrosine (Sigma Chemical Co.) in 100 ml of 0.02 M sodium borate buffer, maintained at about pH 9 during the entire reaction (Tabachnick and Sob-

otka, 1959). A dark brown colored precipitate was obtained as the solution was titrated to about pH 2 with 6 N HCl. The precipitate was dried, redissolved in absolute ethanol, and recrystallized out of the ethanol solution by adding petroleum ether (bp 30–60°) to the solution. The brown precipitate was collected and redissolved in trifluoroacetic acid to remove the α-NH_2-protective group. The AT thus obtained was precipitated out of the solution by addition of anhydrous ether. The brown AT precipitate was dissolved in a small volume of 0.18 N NH_4OH and applied to a column of DEAE-cellulose (Schleicher and Schuell, Inc.) which had been equilibrated and was eluted with 0.09 N NH_4OH, 0.05 M in NaCl. Fractions containing the first orange-red component to be eluted were pooled and freeze-dried. AT was recrystallized twice from hot water. Only one component, yellow and ninhydrin positive, was detected by thin-layer chromatography on silica gel (Eastman Kodak Co.) or polyamide (Cheng Chin Trading Co., Taipei, Taiwan), with either ethanol–glacial acetic acid–H_2O (5:2:3) or 2-propanol–NH_3–H_2O (7:1:2) solvent systems. The product decomposed slowly and changed color from orange-red to red then to brown at temperatures above 200°. Before elementary analysis, AT was dried over P_2O_5. *Anal.* (by Galbraith Laboratories, Inc., Knoxville, Tenn) Calcd for $C_{15}H_{16}O_6N_3As$: C, 44.03; H, 3.94; N, 10.27. Found: C, 43.80; H, 4.03; N, 10.14. The absorption spectrum and E_{max} of AT were determined on the Cary Model 14 double beam recording spectrophotometer by dissolving the pure compound in PBS to make up a solution of about 0.02 M. The values of E_{325} = 20,500 M^{-1} at pH 7.3 and 20,800 M^{-1} at pH 6.2 which were determined are in agreement with those reported for mono(*p*-azobenzenearsonic acid) chloroacetyltyrosine (E_{325} = 21,600–22,200 M^{-1} at pH 6.2; Tabachnick and Sobotka (1959)).

Synthesis of N-Bromoacetylmono(p-azobenzenearsonic acid)-L-tyrosine (BAAT). BAAT was prepared by acylating AT (204 mg) with 8 equiv of bromoacetyl bromide (808 mg) in alkaline solution (Fischer and Lipschitz, 1915). The reaction mixture was allowed to stand at room temperature for about 20 min before it was titrated to pH 2 with 6 M HCl. The orange precipitate which was formed as the solution was being acidified contained both AT and BAAT. BAAT was extracted from the precipitate with ethyl acetate–methanol (4:1) by trituration. The orange-red solution was filtered and then flash-evaporated to a small volume before it was left overnight at 0°. The orange-red crystals formed overnight were then collected and recrystallized from hot water. The product appeared pure as judged by thin-layer chromatography in ethanol–glacial acetic acid–H_2O (50:20:30) and by its sharp melting point of 164–165°. Before its elementary analysis was carried out, BAAT was dried over P_2O_5. *Anal.* Calcd for $C_{17}H_{17}O_7N_3BrAs$: C, 38.51; H, 3.23; N, 7.92. Found: C, 38.57; H, 3.34; N, 7.90.

Synthesis of N-Acetyl(p-azobenzenearsonic acid)-L-tyrosine (AAT). AAT was prepared by coupling *N*-acetyl-L-tyrosine (Cyclo) with diazotized arsanilic acid as described by Tabachnick and Sobotka (1959). The dark brown AAT was recrystallized twice by titrating an alkaline solution of AAT to pH 3 with a HCl solution. After AAT was collected and dried, it was again dissolved in absolute ethanol. Petroleum ether (30–60°) was added to the ethanol solution until precipitation occurred. The product was judged to be at least 90% pure by thin-layer chromatography or polyamide sheets using ethanol–glacial acetic acid–H_2O (50:20:30); its R_F value relative to AT is 0.54. The only contaminant detected was an unidentified light brown substance (with R_F relative to AT, 0.34).

Synthesis of N-[1-^{14}C]Bromoacetylmono(p-azobenzenearsonic acid)-L-tyrosine. The ^{14}C-labeled BAAT was synthesized by acylating AT with [1-^{14}C]bromoacetyl-*N*-hydroxysuccinimide ester. The ester was prepared by a modification of the procedure of Weinstein *et al.* (1969). [1-^{14}C]Bromoacetic acid (6.9 mg; 5 Ci/mol, New England Nuclear) was mixed with *N*-hydroxysuccinimide (6 mg; 65 μmol) and dicyclohexylcarbodiimide (14 mg; 70 μmol) in 2 mol of dioxane. The mixture was vigorously stirred with a spatula to promote good precipitation of dicyclohexylurea. After 3 hr of incubation at room temperature, the precipitate was removed by filtration. A 21-mg portion of AT (50 μmol) in 2–3 ml of dioxane and 0.7 ml of water adjusted to about pH 9 with triethylamine was added to the radioactive ester prepared above. After the mixture was incubated at room temperature for 1 hr, two portions of 50 μmol each of nonradioactive bromoacetylsuccinimide ester in dioxane were added at 1-hr intervals. A pH of 8.5–9 was always maintained by the additions of triethylamine solution. After one additional hour of incubation, the pH of the solution was brought to between 2 and 3 with acetic acid and it was freeze-dried. The residue was redissolved in 4 ml of absolute ethanol and petroleum ether (bp 30–60°) was added until the red oil had completely settled out. The supernatant was discarded. The red oil was dissolved in 5 ml of ethyl acetate–methanol–2 N aqueous acetic acid (100:25:10), and this solution was applied to a column of Sephadex LH-20 (2 × 66 cm) which had been equilibrated in the same solvent at 5°. After the column was eluted with about two column volumes of solvent, almost all of the AT remained on the column, while [^{14}C]BAAT was the only major eluted component with an absorption maximum at 325 mμ.

Characterization and Radiochemical Purity of [^{14}C]BAAT. Among several thin layer chromatographic systems used to analyze [^{14}C]BAAT thin-layer sheets developed in ethanol–glacial acetic acid–water (5:2:3) at room temperature gave the best separation of all the compounds of interest (R_F values relative to AT for tyrosine, *p*-arsanilic acid, Boc-AT, and BAAT are respectively 1.1, 0.91, 0.73, and 0.63). The [^{14}C]BAAT gave only one yellow migrating spot which was ninhydrin negative and this spot accounted for about 96% of all the radioactivity applied to the thin-layer sheet. [^{14}C]BAAT has the same R_F value as that of nonradioactive BAAT. When it was incubated with 1.5 mg/ml of poly(lysine) in 0.05 M borate buffer, pH 9.2 at 37° for 20 hr, 91% of the radioactivity was covalently incorporated into the polymer. Immediately before [^{14}C]BAAT was used for affinity labeling of antibody, its solvent was removed either by flash evaporation or under a stream of N_2. The [^{14}C]BAAT residue was dissolved in 0.02 M sodium phosphate (pH 7.2) and the absorbance of the solution at 325 mμ was measured. An aliquot was also taken for counting. Counting efficiency was estimated using a set of ^{14}C quench standards (toluene) and the radioactivity of the sample was then appropriately corrected. The [^{14}C]BAAT prepared by the method described had a specific radioactivity of 9050 dpm per nanomole. The [^{14}C]BAAT solution was readjusted to a pH between 8 and 9 with 0.02 M borate buffer (pH 9.2) before it was added to the antibody solution.

Preparation of N^{ϵ}-CM-lysine and O-CM-tyrosine. N^{ϵ}-CM-lysine was obtained by incubating poly(L-lysine) (n = 180, Yeda) with [1-^{14}C]iodoacetic acid (New England Nuclear) at 37° for 24 hr in 0.05 M borate buffer (pH 9.5). The carboxymethylpolylysine was dialyzed against water and then acid hydrolyzed. The hydrolysis product, N^{ϵ}-CM-lysine, was purified by paper electrophoresis in pyridine–acetate buffer (pH 3.6). The electrophoretically neutral, ninhydrin positive component was eluted from the paper and used as standard N^{ϵ}-CM-lysine. The *O*-CM-tyrosine was prepared by reacting *N*-

Cbz-L-tyrosine with bromoacetic acid at pH 10.5. After 12 hr of incubation at room temperature, the solution was acidified to pH 3 with 2 N HCl. The product *N*-Cbz-*O*-CM-L-tyrosine was extracted into ethyl acetate, dried over anhydrous Na_2SO_4, and then flash-evaporated. The residue was dissolved in 4 N HBr in glacial acetic acid. The *O*-CM-L-tyrosine was precipitated upon addition of anhydrous ether. The product was further purified by paper electrophoresis in pyridine–acetate buffer (pH 3.6). *O*-CM-tyrosine, which moved as a cation at pH 3.5, gave a brownish yellow color with the tyrosine stain (Easley, 1965) while L-tyrosine gave a rose color.

Specificity and Kinetic Measurements for the Reaction between [^{14}C]BAAT and Anti-ARS Antibodies. The purified antibody (10.2 nmol, 0.15 mg/ml) was incubated with [^{14}C]-BAAT (51 nmol, specific activity = 9050 dpm per nanomole) in 10.2 ml of 0.02 M borate buffer (pH 7.0). with or without a protecting hapten. To assess hapten protection of antibody against labeling by BAAT, AAT was preincubated with the antibody at 37° for 30 min at 5000-fold molar excess before adding [^{14}C]BAAT. A control for nonspecific labeling was obtained by reacting [^{14}C]BAAT and nonspecific IgG2 under the same conditions. The solutions used for labeling of antibody were incubated in parallel at 37° and at suitable intervals after addition of the affinity label, 1.0-ml portions were withdrawn and transferred to test tubes containing 0.2 ml of 90% trichloroacetic acid (w/w). After thorough mixing and chilling at 5° overnight, the precipitates were collected on 0.22 μ Millipore filters and washed with five 5-ml aliquots of 5% Cl_3CCOOH. The filters were dried and counted in Kinard's solvent (Kinard, 1957) by a Beckman liquid scintillation counter. The moles of affinity label bound per mole of antibody were calculated from the data.

Preparative Affinity Labeling of Purified Anti-ARS Antibody. Anti-ARS antibody (280 mg) in 0.02 M phosphate buffer (pH 7.2) was diluted to 0.15 mg/ml with 0.02 M borate buffer (pH 9.0). A few drops of toluene was added to the solution which was brought to 37° by a constant-temperature water bath. The [^{14}C]BAAT (2.5 equiv relative to antibody site, 9.35 μmol) in 50 ml of 0.02 M borate buffer (pH 9) was very well mixed into the antibody solution. The reaction mixture was incubated in a 37° water bath for 40 hr with constant gentle agitation. At the end of incubation, the reaction was stopped by adding about 5 μmol of phenol. The solution was brought to pH 7.2 with 2 N HCl before it was concentrated by an Amicon ultrafiltration cell at 5° to a protein concentration of about 1.5 mg/ml. The concentrated solution was dialyzed against two 4-l. changes of water (pH 7–8) for 2 days at 5°, and was then freeze-dried.

Sodium Dodecyl Sulfate Gel Electrophoresis. Analytical separation of heavy and light chains by polyacrylamide gel electrophoresis was carried out on 5% gels in 0.1% sodium dodecyl sulfate (Weber and Osborn, 1969). After electrophoresis, the gels were stained with 0.01% Coomassie Brilliant Blue, or the gels were sliced into about 70 thin slices and each slice was incubated with 0.5 ml NCS solubilizer (Amersham-Searle) before they were counted in 10 ml of toluene scintillation fluid.

Localization of Affinity-Labeled Residues in γ_2 Chain by Automated Sequential Degradation. CNBr peptide C-1-a_1 (N-35 to N-83, 1 μmol) was freeze-dried and applied into the reaction cup of a Beckman Automatic Sequencer as previously described (Birshtein and Cebra, 1971). One-fiftieth amount of each butyl chloride extract, delivered by the Sequencer during sequential degradation, was taken and counted in 10 ml of Kinard's scintillant. The remaining residues in each extract were converted to the PhNCS–amino acid derivatives for identification by gas–liquid chromatography. The method for HI regeneration of free amino acids from PhNCS–amino acids was essentially that of Smithies *et al.* (1971).

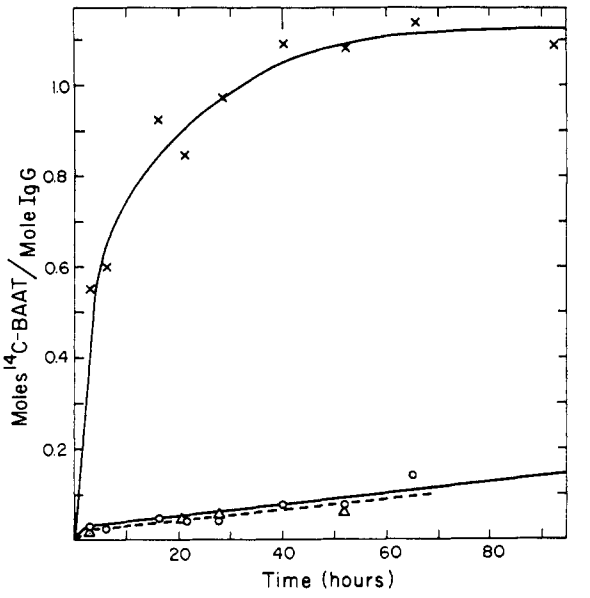

FIGURE 1: Kinetics of affinity labeling of anti-ARS antibody (1.0×10^{-6} M) with BAAT (5.0×10^{-6} M) in 0.02 M sodium borate buffer (pH 9.0) in the absence (×) or the presence (○) of a blocking hapten, AAT (5×10^{-3} M). The dashed line represents a control reaction between "nonspecific" IgG2 with BAAT at similar concentrations.

Other Methods. Procedures for CNBr cleavage of the whole intact IgG molecule and the isolation of CNBr fragments, C-1-n, C-1-a_1, and C-1-a_2, have been described elsewhere (Birshtein and Cebra, 1971; Ray and Cebra, 1972) as have those used for amino acid analysis (Birshtein *et al.*, 1971), amino acid sequence determination by manual methods (Turner and Cebra, 1971), and procedures for determining specific radioactivities of affinity-labeled whole antibody molecules and peptides from the heavy chain (Ray and Cebra, 1972). Radioscanning of strips of chromatographic paper was carried out with a Packard radiochromatogram scanner equipped with a recording rate meter. The standard CM-tyrosine spot was first located by ninhydrin staining and then made radioactive by a radioactive material. The protein concentrations of the unmodified antibody preparations were determined using an $E_1^{1\%}{}_{cm}$ of 14.0 at 280 nm. The molecular weight of guinea pig IgG2 was taken to be 150,000.

Results

Specificity and Kinetics of the Affinity-Labeling Reaction. The results of the study of the rate of reaction of BAAT with anti-ARS antibodies in the presence and absence of a blocking hapten and with nonspecific IgG2 are shown in Figure 1. There is a very rapid initial reaction of BAAT with the specific antibodies as compared to its reaction with nonspecific immunoglobulin. After about 40 hr of reaction, the apparent overall rates of the reactions with the two proteins are about the same. The initial rapid rate of reaction with the antibody probably reflects the specific affinity labeling of the antibody active sites, since it can be completely eliminated by the prior addition of AAT, a chemically nonreactive analog of BAAT, to the anti-ARS solution.

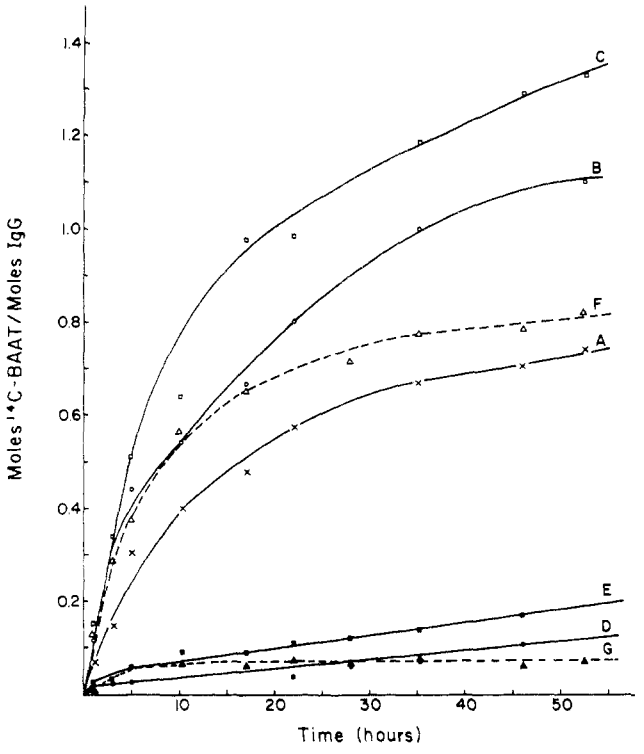

FIGURE 2: Effect of varying antibody and BAAT concentrations on the affinity-labeling reaction. Anti-ARS antibody (0.0015 mM) was incubated with (A) 0.003 mM, (B) 0.006 mM, and (c) 0.012 mM [^{14}C]BAAT in 0.02 M sodium borate buffer (pH 9.0). The controls were "nonspecific" IgG (0.0015 mM) incubated with (D) 0.006 mM, and (E) 0.012 mM [^{14}C]BAAT. Curves F and G are respectively anti-ARS antibody and "nonspecific" IgG2 (0.0033 mM each) incubated with [^{14}C]BAAT (0.0066 mM) under identical conditions and in parallel with the above experiments. All reaction mixtures were incubated at 37° and aliquots were withdrawn at selected time intervals. The affinity-labeling reaction was stopped by Cl_3CCOOH and the Cl_3CCOOH precipitates were filtered, washed, and counted as described in the text.

In order to demonstrate the specificity of anti-ARS antibodies for BAAT, another active-site blocking experiment was carried out. Anti-ARS antibodies (1 nmol) and [^{14}C]BAAT (5 nmol) in 1.0 ml of 0.02 M sodium borate buffer (pH 9) were incubated in the absence and presence of four different concentrations of *p*-NBA, another competitive blocking hapten. After 20 hr of incubation at 37°, the protein was precipitated and its radioactivity was counted. Table I records that the affinity labeling reaction can be partially or completely blocked depending on the concentrations of *p*-NBA present. This "protection" of anti-ARS antibodies is interpreted as indicating that *p*-NBA, and presumably also AAT, blocks the ligand-binding sites of antibody, rendering them unavailable for reaction with any active-site directed reagent, such as BAAT.

Effect of Varying Antibody and BAAT Concentrations on the Affinity-Labeling Reaction. The kinetics of the affinity-labeling reaction as a function of antibody or BAAT concentrations are depicted in Figure 2. The reaction between BAAT and anti-ARS antibody is in general rather slow in achieving maximum labeling of sites. Twofold increases in molar ratio of BAAT to protein (2:1 to 8:1) have brought about increases in the initial reaction rate and extent of covalent labeling of the antibodies, but did not shorten the time required for reaching maximum labeling (Figure 2). This observation contrasts with the affinity-labeling reaction of anti-Dnp antibody with BADL described by Strausbauch *et al.* (1971). The rate and extent of covalent labeling of anti-Dnp antibodies appear to be independent of the concentration of the labeling reagent.

TABLE I: Effect of *p*-Nitrobenzenearsonate on the Affinity-Labeling Reaction.

Type of IgG (1.0 nmol/ml)	μmol of *p*-NBA Added per ml	Mol of [^{14}C]-BAAT Incorporated per mole of IgG[a]
ARS-Ab	0	0.35
ARS-Ab	0.001	0.094
ARS-Ab	0.010	0.025
ARS-Ab	0.040	0.012
ARS-Ab	0.100	0.010
Nonspecific IgG	0	0.019

[a] 5 nmol of [^{14}C]BAAT was added per ml and the extent of reaction was assessed after 20 hr.

A twofold molar increase in anti-ARS antibody concentration while the antibody-BAAT molar ratio was kept constant (Figure 2, A and F) also brought about an increase in reaction rate and appeared to shorten the time required for attaining a given extent of protein modification. When still higher antibody concentrations were tried, protein precipitation occurred during incubation at temperatures above room temperatures.

Distribution of Affinity Label between the Heavy and Light Chains and the Identity of the Residues Modified. The results of the kinetic experiments (Figure 1) helped to define the optimal conditions for the preparative affinity-labeling reaction. We used the same concentrations of BAAT and anti-ARS antibodies employed for the analysis of the time course of modification, shown in Figure 1, and a 40-hr reaction time at 37° for the preparative labeling of antibody. Under these conditions, as shown in Figure 1, about 1.05 mol of label was covalently bound to each mole of antibody, while only 0.08 mol was bound to either nonspecific IgG2 or to anti-ARS antibody preincubated with the *N*-acetyl analog of BAAT. A separate determination of specific radioactivity of the labeled antibodies obtained from the preparative reaction mixture was carried out. The affinity-labeled antibody was totally reduced and alkylated with iodoacetic acid (Birshtein *et al.*, 1971) and then dialyzed against distilled water to ensure complete removal of noncovalently bound BAAT. It was then acid hydrolyzed, and its amino acid composition and radioactivity were determined. By this method, about 1.04 mol of label was estimated to be covalently linked to each mole of antibodies.

Separation of heavy and light chains from totally reduced and alkylated antibody was achieved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and these gels were sliced and assayed for distribution of radiolabel. The ratio of label on the H:L chains was 2.3:1. In an attempt to identify the labeled residues in the whole affinity labeled antibody molecule, the water-dialyzed, affinity labeled whole molecule was acid hydrolyzed to obtain free modified residues which were then separated and identified by paper electrophoresis in pyridine–acetate buffer (pH 3.6). The only predominantly labeled residues identifiable by the radio-scanning method were CM-lysine and CM-tyrosine which occurred in a ratio of 1.6:1, respectively. Since CM-lysine was the only predominantly labeled residue in H chain (see below), most of the CM-tyrosine in the molecule must have come from the L chain.

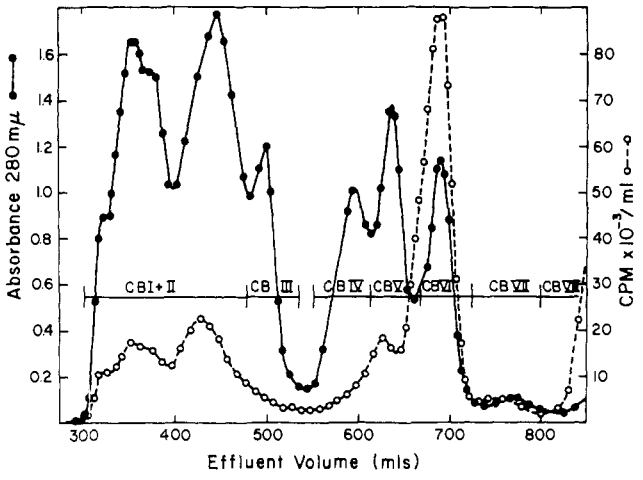

FIGURE 3: Elution diagram of separation of CNBr fragments from 2 μmol of affinity-labeled anti-ARS antibody on a Sephadex G-100 column (2 × 225 cm) in 8 M urea and 0.1 M formic acid. Pool CBVI is C-1-a_1 which contains a large amount of radiolabel.

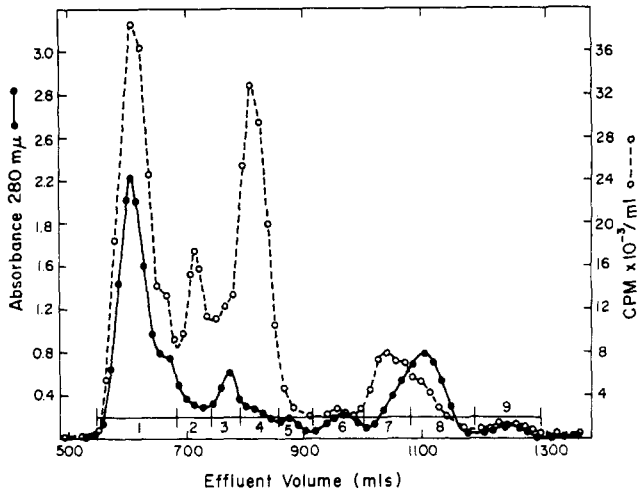

FIGURE 4: Elution diagram of the separation of CNBr fragments in pool CBI + II, which had been totally reduced and alkylated. The Sephadex G-50 (fine, 3 × 183 cm) column was equilibrated and eluted with 8 M urea in 0.1 M formic acid.

Distribution of Affinity Label on the Fragments from a CNBr Digest of γ_2 Chain. Affinity-labeled anti-ARS antibody (280 mg) was cleaved by CNBr and the fragments were separated by passing the sample through a column of Sephadex G-100 (2 × 225 cm), equilibrated with 8 M urea, 0.1 M in formic acid. The elution profile from such a column is shown in Figure 3. Most of the modified residues were found in pools CBI + II and CBVI. Pool CBVIII contained some free affinity label. The most striking feature of this elution profile is the localization of radioactivity in pool CBVI, which is known to contain the fragment C-1-a_1 almost exclusively (Birshtein and Cebra, 1971). Pool CBI + II was desalted and freeze-dried. All disulfide bonds of peptides in this fraction were totally reduced and then alkylated with iodoacetic acid before the peptides were applied to a column of Sephadex G-50 (fine, 3 × 183 cm), equilibrated with 8M urea, 0.1 M in formic acid. The elution profile is shown in Figure 4. The peptide fragments in pools 6 and 9, which have little radiolabel, have amino acid compositions characteristic of C-1-a_2 and C-1-n, respectively. Table II lists the amino acid compositions and mole per cent labeling of those three CNBr fragments which include the three "hypervariable" segments of γ_2 chain (Birshtein and Cebra, 1971; Ray and Cebra, 1972). From the results it is clear that C-1-a_1 is

TABLE II: Amino Acid Compositions and Specific Radioactivities of CNBr Fragments Accounting for the V_H Region of γ_2 Chain.[a]

	C-1-n		C-1-a_1		C-1-a_2	
	IgG2	Ab	IgG2	Ab	IgG2	Ab
Lys	0.7	0.5	3.1	2.8[b]	2.1	1.0
His					0.6	0.1
Arg	1.0	1.1	3.0	2.5	3.0	2.0
CMCys	0.7	1.0			1.5	1.8
Asp	1.4	0.9	4.9	4.9	4.6	5.5
Thr	1.6	2.1	3.6	3.0	5.0	5.4
Ser	4.3	5.2	4.4	5.8	5.9	6.5
Glu	4.2	4.7	3.4	4.1	2.7	2.8
Pro	1.4	1.1	2.0	1.7	3.5	3.7
Gly	5.1	4.8	5.5	4.4	4.2	4.5
Ala	1.8	1.4	2.9	2.6	5.3	5.1
Val	3.2	3.3	2.9	2.5	4.7	4.3
Ile			3.0	3.5	1.6	1.2
Leu	3.9	3.7	3.6	3.5	3.5	3.8
Tyr	1.3	1.0	2.5	3.6	2.9	2.5
Phe	1.8	2.0	1.6	1.3	1.9	2.5
Hsr	1.0	1.1	1.3	1.4	0.9	1.3
Moles of label/mole of fragment	0.0031 ± 0.0004		0.315 ± 0.015		0.017 ± 0.002	

[a] The results for normal IgG2 were taken from the average values of Ray and Cebra (1972). The compositions of the antibody fragments are averages of two different analyses normalized to contain 33 residues for C-1-n (exclusive of the CMCys residue), 47 residues for C-1-a_1 (exclusive of two tryptophan residues), and 52 residues for C-1-a_2 (exclusive of two CMCys). [b] Not including modified lysine (CM-lysine).

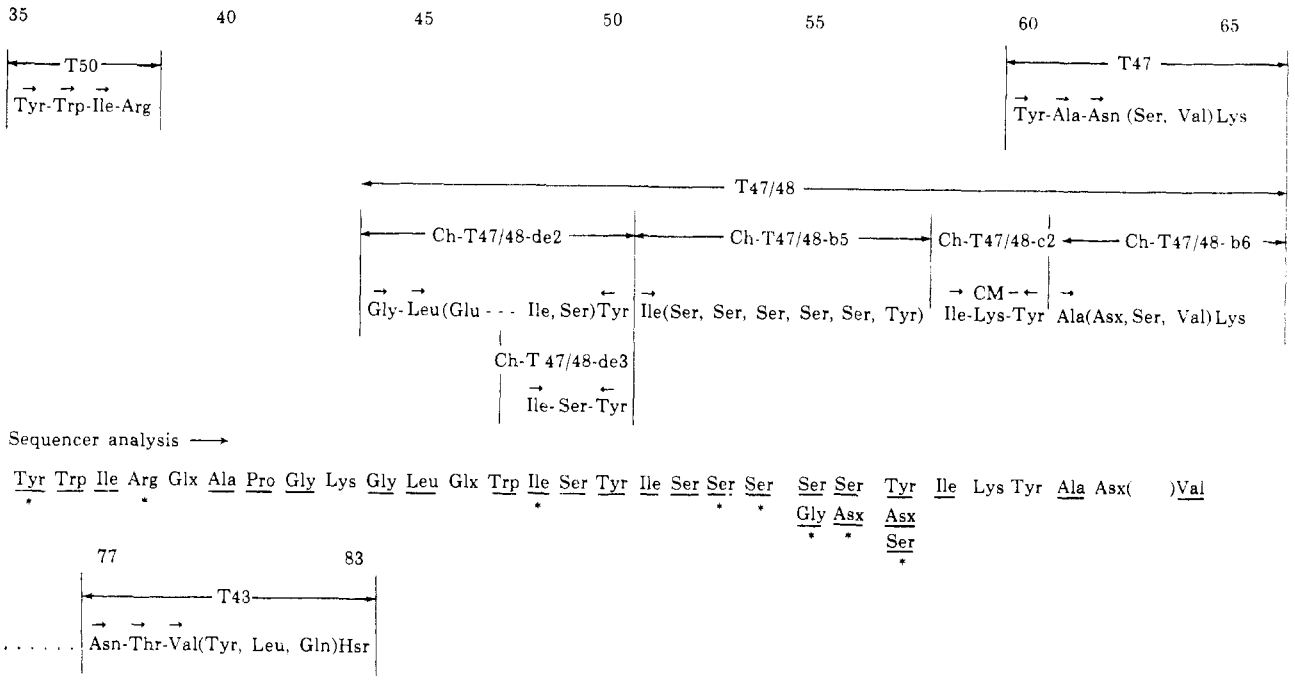

FIGURE 5: A summary of the procedures and resulting data used to determine the primary structure of C-1-a_1. Exact compositions of peptides are given in Table III: (→) removal of a residue by the Edman degradation procedure; (←) identification of a residue by treatment with carboxypeptidase A. The subtractive Edman procedure was used to identify the residue removed in each manual step. The PhNCS derivative obtained from each manual step was also identified by gas chromatography. During automated sequential degradation, the PhNCS derivative of each residue was identified as described in the text. The sequencer data were obtained by HI regeneration of the free amino acid followed by amino acid analysis. An underlined residue indicates the amino acid was also identified by gas chromatography, (*) positions in which minor alternate residues occur, and their mole per cent relative to the major residues are shown as follows: Lys-35 (8%), Lys-38 (8%), Lys-48 (10%), His-53 (8%), Asx-54 (9%), Gly-55 (23%), Asx-56 (24%), Ser-57 (33%), Asx-57 (11%).

by far the most highly labeled fragment and it contained about 94% of the total label in the NH_2-terminal 140 residues of γ_2 chain. Since one expects little or no label to be found in C_H regions of H chain, C-1-a_1 in principle should contain as much as 94% of the total label in the entire H chain. The low specific activities of fragments from C_H regions are evident from the elution profiles in Figures 3 and 4. The principal fragments in pools CBIV, CBV, CBVII (Figure 3), and pool 8 (Figure 4) are C-4, C-3, C-5, and C-1-b, respectively, and very little radiolabel is associated with them.

Localization of Affinity-Labeled Residues in C-1-a_1. The affinity-labeled residues in C-1-a_1 have been localized by two independent methods. The first method utilized the Beckman automatic sequencer to sequentially cleave off amino acid residues from the amino-terminal end of C-1-a_1, and a sample of each extracted residue was monitored for radioactivity. The second method involved isolation and identification of labeled peptides, the details of which will be described later.

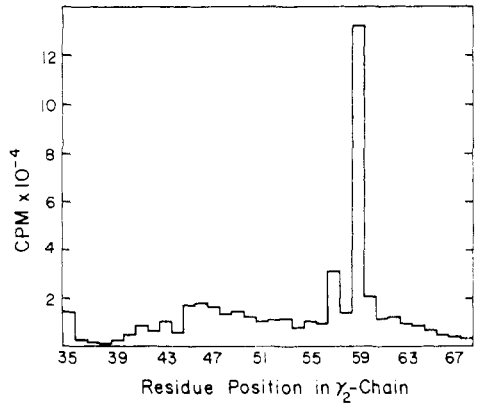

FIGURE 6: A nomogram representing radioactivity detected in the first 34 residues cleaved by automatic sequential degradation of C-1-a_1 (N-35 to N-85) from γ_2 chain of affinity-labeled anti-ARS antibodies.

About 1 μmol of salt-free, dried C-1-a_1 was used for each automatic sequencer run. One-fiftieth amount of thiazolinone derivative of each amino acid residue in the butyl chloride delivered by the sequencer was taken for determination of radioactivity in Kinard's scintillation liquid. The remainder of each sample was converted to the PhNCS–amino acid and identified by gas chromatography as PhNCS–amino acids or as sialylated derivatives. The free amino acids were regenerated by HI in N_2 atmosphere at 127° for 20 hr and were identified on an amino acid analyzer. By this method, we were able to determine the primary structure of γ_2 chain of anti-ARS antibody, beginning at the NH_2 terminus of C-1-a_1 (N-35) through the second "hypervariable" region, and to clearly identify the valyl residue at N-64. The sequence data obtained from such an analysis are presented in Figure 5. The location of affinity labels within N-35 to N-68 is shown in Figure 6. It is clear that residue N-59 is the most conspicuously labeled residue within this region which includes the "variable" positions N-35, and N-48 to N-59. Though there are three tyrosyl residues (N-35, N-50, and N-57) associated with the anti-ARS specificity and two neighboring tyrosyl residues to position N-59 (N-57 and N-60), none of them was highly labeled.

Identification of the Affinity-Labeled Residue in C-1-a_1. From the result of automatic sequential degradation of C-1-a_1 (Figure 6), residue N-59 was found to be the only predominantly labeled one. After HI regeneration to the free amino acid, the only unmodified residue at this position identified by a Technicon liquid-column autoanalyzer was lysine, and the

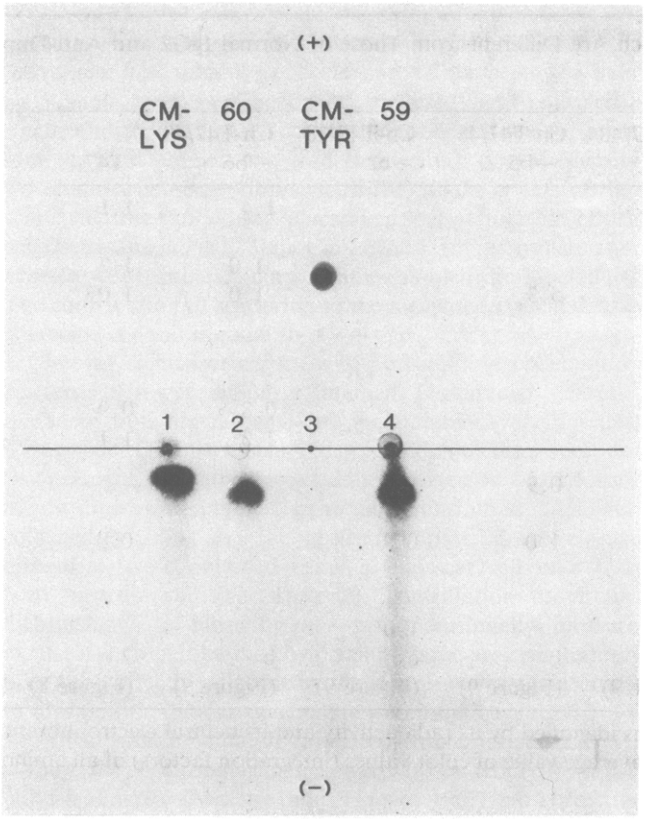

FIGURE 7: Autoradiographic patterns of residues N-59 and N-60 obtained from automatic sequential degradation of C-1-a_1. Free amino acids were regenerated by HI acid hydrolysis before they were electrophoresed on Whatman 3 mm paper in pyridine–acetate buffer (pH 3.6) for 1 hr at 1500 V. The standards shown here are N^{ϵ}-[^{14}C]carboxymethyllysine (CM-Lys) and *O*-carboxymethyltyrosine (CM-Tyr). After electrophoresis, the ninhydrin positive CM-Tyr spot was made radioactive to mark its position by radioautography by spotting with a ^{14}C material before it was exposed to film (Kodak, NS-54T).

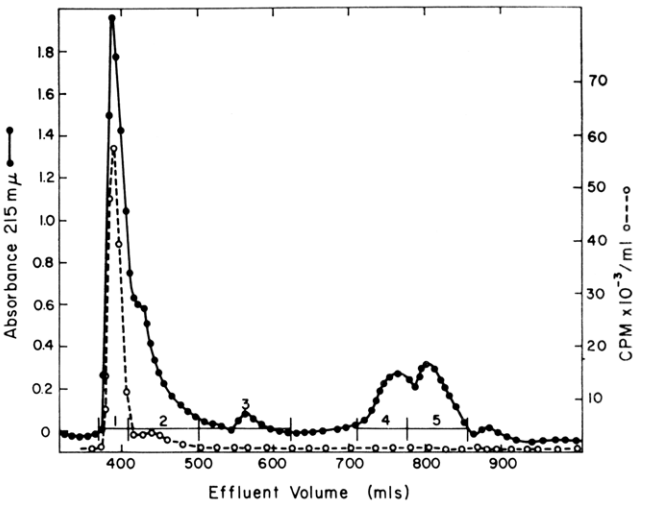

FIGURE 8: Elution diagram of tryptic peptides from 1.0 μmol of C-1-a_1 on Sephadex G-25 (fine, 2 × 247 cm) in 0.05 M NH_4OH.

accompanying modified residue was identified as CM-lysine by electrophoresis along with standard CM-lysine and CM-tyrosine at pH 3.6. Figure 7 shows that residue N-59 has the same electrophoretic mobility as that of CM-lysine, and no trace of CM-tyrosine was detected in either positions N-59 or N-60, although unmodified residue N-60 is clearly identifiable as tyrosine. A small amount of labeled residue at N-60 was also identified as CM-lysine and it presumably represents the carry-over cleavage product of the preceding, highly labeled residue N-59. Other supporting evidence for N-59 being a base would be the isolation of a peptide (residues N-60 to N-65), from a tryptic digest. Such a tryptic peptide, T47, has been isolated and identified from two independent preparations of anti-ARS antibodies (Table III).

Isolation of Peptides Distinctive of the Anti-ARS Specificity, Including a Radiolabeled Peptide, after Digestion of C-1-a_1 from Affinity-Labeled Antibody. About 1 μmol of salt-free affinity-labeled C-1-a_1 was digested with trypsin at pH 8. The hydrolysate was then applied to a column of Sephadex G-25 (fine, 2 × 247 cm) which was equilibrated and eluted with 0.05 M NH_4OH. The elution profile is shown in Figure 8. Almost all of the radioactivity was localized in pool 1, the first peptide fraction to be eluted, suggesting that the labeled tryptic peptide was the largest of all. This finding is consistent with the analysis of C-1-a_1 using the automatic sequencer, which shows that there is no lysine or arginine between residues N-44 and N-58 (Figure 5). Other pools containing tryptic peptides accounting for residues N-35 to N-43 and N-60 to N-83 (Birshtein and Cebra, 1971) have almost no radioactivity.

Pool 1 (Figure 8) was concentrated by flash evaporation and then digested with chymotrypsin (200 μg) at pH 8.1 for 14 hr at 37°. A small amount of hydrolysate was analyzed by paper electrophoresis at pH 3.6. One major and one minor radioactive migrating component was detected. The chymotryptic peptides were separated by a Sephadex G-25 (fine, 2 × 247 cm) column, preequilibrated and eluted with 0.05 M NH_4OH. The profile from such a column also shows there are at least two radioactive components (Figure 9, pools a and c). The peptides in each of six pools (see Figure 9) were further purified by paper electrophoresis at pH 3.6. The amino acid compositions of peptides in C-1-a_1 which are different from those of nonspecific IgG(2) and anti-Dnp antibodies are shown on Table III. These peptides have been placed in the sequence of C-1-a_1 as shown in Figure 5, based on their compositions and on their partial sequences determined by manual degradation.

The Sequence of an Affinity-Labeled Peptide and Identification of Its Modified Residue. Pool C (Figure 9) was concentrated and electrophoresed on paper at pH 3.6 to obtain separation of all the component peptides. The electrophoretically neutral, radioactive peptide (Ch-T47/48-C2) was then eluted with 0.2 N acetic acid. Amino acid compositional analysis of peptide Ch-T47/48-C2 (Table III) showed that about equimolar amounts of isoleucine and tyrosine were present, and that there was also an unidentified amino acid which was eluted just before isoleucine from the long column of the amino acid analyzer. Digestion with carboxypeptidase A for 10 min released only tyrosine from the peptide as the carboxyl-terminal residue. The first manual Edman degradation removed a residue which was identified as isoleucine. The second Edman degradation removed a radioactive residue which, after regeneration to the free amino acid by hydrolysis, was identified as CM-lysine by paper electrophoresis at pH 3.6. Thus the sequence of the peptide is Ile-CMLys-Tyr and the second residue is the modified lysine which was affinity labeled.

Although the majority of affinity label was in pool A (Figure 9) after consecutive tryptic and chymotryptic digestion of C-1-a_1, no modified peptide could be cleanly isolated from this fraction containing the larger digest products. We presume that the modified lysyl residues in this fraction, which was also rich in serine, were associated with a mixture of partial chymo-

TABLE III: Amino Acid Composition of Peptides from C-1-a_1 Which Are Different from Those of Normal IgG2 and Anti-Dnp Antibody.

	T50	T43	Ch-T47/48 −de2	Ch-T47/48 −de3	Ch-T47/48 −b5	Ch-T47/48 −c2	Ch-T47/48 −b6	T47
Lys							1.4	1.1
Arg	1.0							
Asp		1.1					1.0	1.2
Ser		0.6	0.8	1.1	5.3		1.0	1.0
Thr		1.1						
Glu		1.2	0.9					
Gly			1.1					
Ala							0.8	0.9
Val		0.8					1.3	1.1
Ile	1.0		1.0	1.0	0.9	1.0		
Leu		1.1	1.1					
Tyr	1.1	1.0	1.0	1.0	1.0	0.9		0.8
Hsr		1.0						
Trp[a]	+		ND[d]					
CMLys[b]						1.0[c]		
Pool of Origin	5 (Figure 8)	2 (Figure 8)	d + e (Figure 9)	d + e (Figure 9)	b (Figure 9)	c (Figure 9)	b (Figure 9)	2 (Figure 8)

[a] Trp was identified by Ehrlich stain (Easley, 1965). [b] CMLys was identified by its radioactivity and its neutral electrophoretic mobility in pyridine–acetate (pH 3.6) buffer. [c] Calculated from an average value of color values (integration factors) of all amino acids excluding that of proline. [d] ND = not determined.

tryptic digest products, which were recovered due to slow cleavage at residue positions N-47, N-50, N-57, and N-60. Affinity-labeled peptides, larger than Ile-CMLys-Tyr, may also have arisen due to the presence of primary structural variants of C-1-a_1 having amino acid residue alternatives to tyrosine at N-57 and N-60.

Discussion

Our present results indicate that about 70% of all radioactivity incorporated into the whole anti-ARS antibody molecule, modified to contain 1.05 mol of affinity label per mole, was localized in its heavy chain. The 49-residue fragment, C-1-a_1, which contains the second "hypervariable" segment of heavy chain (Birshtein and Cebra, 1971), had a specific radioactivity indicating 0.32 mol of modified amino acid residue per mole of fragment. Thus, since two C-1-a_1 fragments can be derived from each antibody molecule, the modified residues in C-1-a_1 could account for as much as 90% of the affinity label found in whole heavy chain. Assuming that about 8% of the total modification of the molecule is nonsite directed, an assumption supported by the data in Figure 1, we would expect that C-1-a_1 could contain almost all the specifically affinity labeled residues in heavy chain. Such restricted localization of the affinity labels in a fragment of V_H region is consistent with the previous findings (Ray and Cebra, 1972; D. Tracey and J. Cebra, unpublished) that little or no modification of residues outside of V_H occurred during affinity labeling of anti-Dnp antibodies, regardless of whether a diazonium reagent (MNBDF) or a bromoacetyl reagent (BADL) was used. The high specific radioactivity of C-1-a_1 from affinity-labeled anti-ARS antibodies, compared with the low degree of labeling of other CNBr fragments (see pools CBIV, CBV, and CBVII, Figure 3 and pools 6, 8, and 9, Figure 4, which contain mainly fragments C-3, C-4, C-5, C-1-a_2, C-1-b, and C-1-n, respectively), is also consistent with most of those residues modified in heavy chain being localized in this fragment from V_H region.

As judged by two independent methods, automatic sequential degradation and isolation of labeled peptides from C-1-a_1 digested consecutively with trypsin and chymotrypsin, the lysine at N-59 is the most conspicuously modified residue. One would thus question how unusual is the occurrence of a lysyl residue at position N-59 in antibodies of different antigen-binding specificities. Could this lysyl residue be a contact residue inside the active site of anti-ARS antibodies? Conversely, could this lysyl residue be an exposed and highly reactive residue near but outside of the antigen-binding site

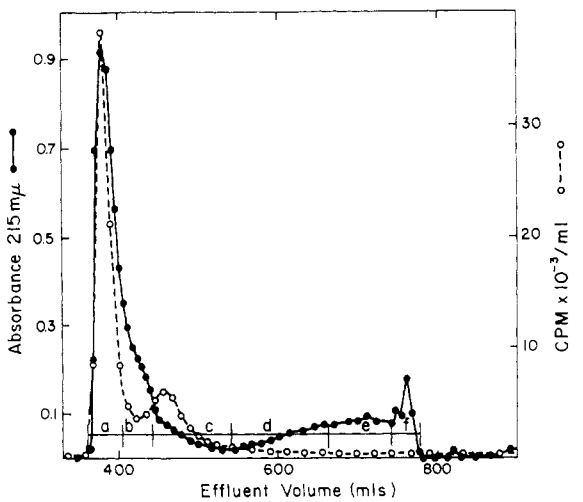

FIGURE 9: Elution diagram of chymotryptic peptides from a digest of pool 1 (Figure 8) in Sephadex G-25 (fine, 2 × 247 cm) column, equilibrated and eluted with 0.05 M NH_4OH. Pool C in the preparation contains the affinity-labeled tripeptide (see text).

which has nothing directly to do with the specificity of the antibody molecule for its ligand? Position N-59 is known to be variable, having a number of alternatives in "normal" γ_2 chain (Birshtein and Cebra, 1971). Our data (Figure 5) show that each of the variable positions in the second hypervariable region (Hv2) in C-1-a_1 from anti-ARS antibody is of restricted heterogeneity or even homogeneous with respect to species of amino acid compared with "normal" C-1-a_1. Any alternative residues (see Figure 5) are usually present in only a small fraction of the molecules and have so far only been detected by automatic sequential degradation. With respect to the restricted heterogeneity of the Hv2 segment, anti-ARS antibodies from strain 13 guinea pigs are like anti-Dnp antibodies raised in inbred animals of the same strain (Cebra *et al.*, 1971; P. Koo, A. Ray, and J. Cebra, unpublished). Primary structural analysis of antibodies from strain 13 guinea pigs specific for a third hapten, the *p*-azobenzenetrimethylammonium determinant, has also revealed a dramatic restriction in heterogeneity in Hv2 compared with that of "normal" C-1-a_1 (J. Cebra, unpublished). The primary structures of the three anti-hapten antibodies of different specificities, in addition to being markedly restricted in heterogeneity, also seem distinctive within Hv2, as shown in Figure 10. Since the particular amino acid present in a residue position in Hv2 seems characteristic of antibody of a particular ligand binding specificity, it seems possible that the unique residues of anti-ARS antibodies, such as lysine N-59, may be present in the combining site and also that they may help to determine the binding specificity by serving as contact residues. Previously, the second hypervariable segment of heavy chain, Hv2, has been implicated in formation of a ligand-binding site by affinity labeling of lysyl residue N-54 of MOPC-315, a Dnp-binding mouse myeloma protein (Haimovich *et al.*, 1972). Our findings are the first to definitively indicate affinity labeling of a residue in Hv2 of antibody deliberately raised to and reactive with the same haptenic determinant used to synthesize the affinity label. Furthermore, the residue in anti-ARS antibodies that is modified, lysine N-59, is unique among antibodies of three different ligand-binding specificities. If this lysine N-59 is generally correlated with antibodies with binding specificity for *p*-azobenzenearsonate, then it probably has some sort of specific role, for instance, contribution of a positive charge for specific interaction with the negative arsonate moiety of the hapten. Such a role has been suggested for basic residues in the binding of negative ligands by antibodies based on chemical modification studies (Pressman *et al.*, 1970; Grossberg *et al.*, 1973).

It is not possible at present to exclude there being more than one basic amino acid residue correlated with *p*-azobenzenearsonate binding specificity and perhaps involved as a contact residue in the ARS-antibody combining site, since the primary structure of light chain and that portion of fragment C-1-a_2 containing the third hypervariable region (N-99 to N-119) have not been elucidated. Nevertheless, lysine N-59 could certainly be one of the residues which provides some of the binding energy by charge interactions with the hapten. The next obvious question is, could the reactive bromoacetyl group of BAAT label the same residue which is in contact with the immunodominant arsonic acid group? In order to answer this question, one must consider two main factors: (1) whether the antibody combining site is big enough to accommodate the entire BAAT molecule, and (2) whether it is likely that BAAT assumes a folded configuration in the site, so that the immunodominant group and the reactive group can be in close contact with the same amino acid residue of antibody.

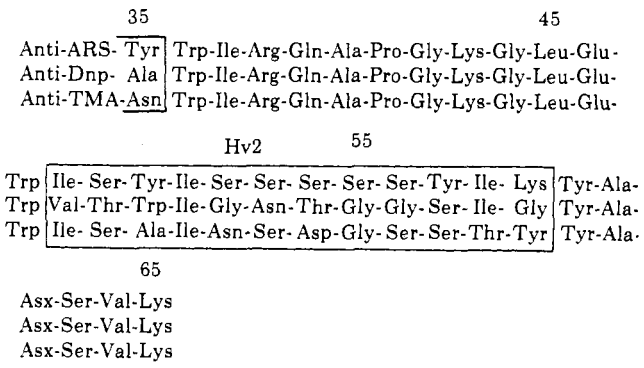

FIGURE 10: A comparison of amino acid sequences of the first 31 residues from the NH_2 terminus of C-1-a_1, obtained from antibodies raised against ARS, Dnp, and TMA haptens (P. Koo, A. Ray, and J. Cebra, unpublished).

By inhibition studies, Kabat has shown that the antibody combining site may be quite large. The human anti-dextran antibody site can accommodate a hexamer of glucose. Isomaltohexaose tested in the study has dimensions of about 34 × 12 × 7 Å in its extended form (Kabat, 1962, 1968). Though the hexasaccharide may fold over into some more compact form in the site, it is nevertheless bigger than BAAT, which has dimensions of about 14 × 5 × 2 Å units in its fully extended configuration. Thus there is a distinct possibility that at least a fraction of anti-ARS antibody combining sites is large enough to accommodate the entire BAAT molecule, particularly since the immunogen, ARS-hemocyanin, contains many determinant groups as big as BAAT (*e.g.*, *p*-monoazobenzenearsonate-tyrosyl groups and *p*-bisazobenzenearsonate-tyrosyl groups).

BAAT has a substantial degree of freedom to assume many conformations in solution. There is a relatively free rotation about each nonbenzenoid carbon and nitrogen atom of tyrosine and the bromoacetyl group, so that in its coiled conformation the chemically reactive group can be brought much closer to the immunodominant group than in its extended form. Haimovich *et al.* (1972) suggested that their affinity label (BADE) may be in its folded configuration in the active site, since two affinity labels BADE and MNBDF, of two different extended sizes, labeled the same residue in the light chain of MOPC 315 protein (Goetzl and Metzger, 1970). BAAT may also assume a folded conformation in the active site. Besides the above-mentioned possible conformations, the substituted benzene rings on both sides of the azo bond can be in either anti (trans) or syn (cis) configurations (Hartley, 1937, 1938; Cook and Jones, 1939). The anti isomer is generally the prevalent form, but it can convert freely to syn isomer in solution when it is exposed to light. In the case of BAAT (Figure 11), once it is converted to the syn configuration, the syn isomer could be stabilized by hydrogen bondings between oxygen atoms of arsonate group with oxygen atoms of bromoacetyl group and carboxyl group of tyrosyl moiety. In such cases, the molecule is in the folded configuration and the bromoacetyl group is just next to the arsonate group. Analogous H bondings also could occur in the immunogen, ARS-hemocyanin; at the physiological pH only 25% of arsonic groups are in doubly ionized form (pK_a = 7.9). When BAAT is in the anti configuration the arsonic group can be brought within about 3–4 Å from the methylene carbon atom of the bromoacetyl group (Figure 11). It thus becomes clear that when BAAT is in the syn configurations, lysine N-59 could be within distances from both the immunodominant and

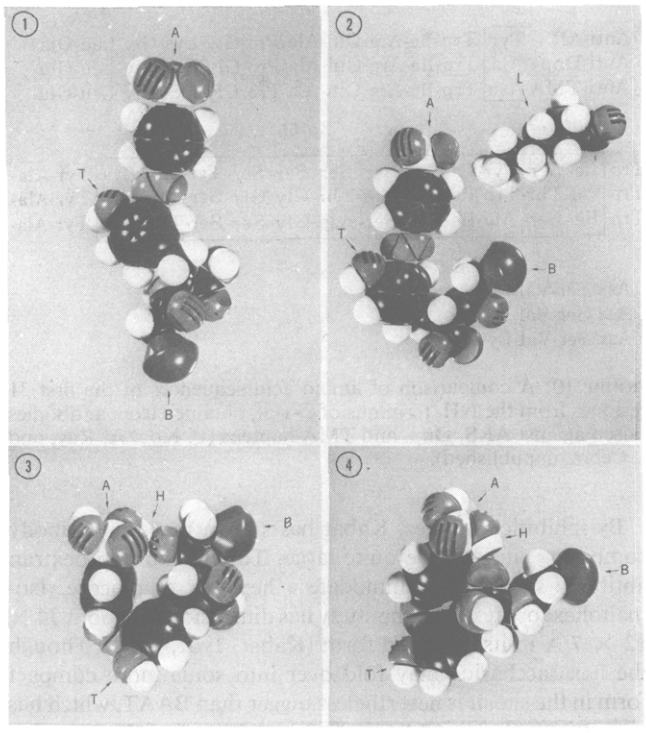

FIGURE 11: CPK space filling models showing four possible configurations of BAAT: (1) fully extended anti-BAAT; (2) coiled anti-BAAT and a lysyl side chain from the protein nearby; (3) syn-BAAT, in which a linear H bond may exist between an arsonic oxygen and carbonyl oxygen of bromoacetyl group at pH's BAAT was prepared; (4) syn-BAAT, in which a less stable nonlinear H bond may exist between an arsonic oxygen and a carboxyl oxygen. A = arsonic group; B = bromoacetyl group; H = hydrogen bond; L = lysyl side chain of antibody; T = phenolic group of tyrosine.

reactive groups favorable for stepwise specific binding and nucleophilic displacement reaction to occur, whereas BAAT in the less folded anti configuration may not react with the same basic residue(s) which bind noncovalently with the arsonic group unless there is a change of orientation of anti-BAAT once it is in the site to favor labeling of lysine N-59. Nothing is known about the photochemical equilibrium of the syn and anti form of BAAT in solution between pH 2 and 9, or under prolonged light exposure such as when the antigen is prepared for immunization or during the affinity-labeling reaction. Also there is no information as to whether antibodies may be formed to all possible configurations of the modified tyrosine determinant or to only one or a few of the more stable configurations.

In examining the amino acid compositions and comparing the primary structures of C-1-a_1 peptides from normal IgG2 and three specific antibodies, namely, anti-Dnp (Cebra *et al.*, 1971), anti-TMA (J. Cebra, unpublished), and anti-ARS antibodies, one will notice there are more hydroxyamino acids (tyrosine and serine) in anti-ARS antibodies than antibodies to the other two specificities (see Figure 10). Also, both anti-ARS and anti-TMA antibodies have more hydroxyamino acids than do anti-Dnp antibodies. From the physicochemical properties of the haptens one could postulate that the hydroxyamino acids could help to create a hydrophilic environment or a subsite around lysine N-59 and provide some of the binding energy for the specific interactions between antibody and a hydrophilic ligand, such as one with an arsonate or trimethylammonium group.

There is another aspect to the finding that there are more tyrosyl residues in anti-ARS antibodies than antibodies to the other two specificities. By modification studies, Pressman's group (Grossberg *et al.*, 1962; Grossberg and Pressman, 1960) has shown that rabbit anti-ARS antibody was more sensitive to loss of binding activity by iodination than was anti-TMA antibody. These findings lend support to our contention that hypervariable regions determine binding specificity in that at least one of these unique tyrosines in a hypervariable region may contribute to anti-ARS specificity and may reside within the active site. Although those tyrosyl residues (N-35, N-50, N-57) seem so far to be peculiar to anti-ARS antibody, they were not extensively affinity labeled even though tyrosine N-57 and tyrosine N-60 are in close proximity to the highly labeled lysine N-59. Such unusual stereospecificity of the affinity-labeling reaction has also been demonstrated for mouse myeloma protein MPOC-315, affinity labeled with BADL reagent (Haimovich *et al.*, 1972). Once the hapten is in the antibody site, it is believed to be held in a particular configuration and to be highly immobile. The amino acids which make up the site are also believed to be fixed in rigid position when the hapten is bound noncovalently in the active site. The singularity of labeling lysine N-59 may be another indication that not only the immunodominant arsonate group but also the bromoacetyl group may enter into and be rigidly held within the site.

Evidence has been accumulated in recent years that antibodies raised to positive haptens (*e.g.*, TMA) contain negatively charged residue (*e.g.*, aspartic acid), while antibodies raised to the negative haptens contain lysine and arginine in the site (Pressman *et al.*, 1970). Also antibodies raised to a bivalent hapten, such as 5-azoisophthalate, are believed to have both lysine and arginine in the same site (Grossberg *et al.*, 1973). Our next task is to demonstrate whether lysine N-59 is the only basic residue within a hypervariable region that is unique to anti-ARS antibody compared with anti-Dnp and anti-TMA antibodies and to determine whether nonsite directed chemical modification of this lysyl group leads to inactivation of the antibody.

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