1971). This procedure is proving practical for isolation of those fragments associated with antigen-binding specificity from small amounts of specific antibodies.

Finally, perhaps the most immediately valuable feature of the alignment of the CNBr fragments is that it permits the rigorous placement of those fragments having a variable primary structure. These three fragments, C-1-n, C-1-$a_1$, and C-1-$a_2$, account for the N-terminal $\simeq$140 residues. In the accompanying paper (Ray and Cebra, 1972) we have shown that these three fragments are those which are specifically affinity labeled when anti-DNP antibody is reacted with *m*-nitrobenzenediazonium tetrafluoroborate. The data presented here permit the affinity label to be rigorously localized in the N-terminal quarter of heavy chain.

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# Localization of Affinity-Labeled Residues in the Primary Structure of Anti-Dinitrophenyl Antibody Raised in Strain 13 Guinea Pigs†

Alex Ray‡ and John J. Cebra*

ABSTRACT: Several preparations of anti-dinitrophenyl antibody raised in strain 13 guinea pigs were modified with the affinity label, *m*-nitrobenzenediazonium-$^{14}C$ fluoroborate. The molar ratio of modified residues in heavy and light chains was about 8:1, respectively. Fragments accounting for the entire length of the heavy chain ($\gamma_2$ chain) were isolated from CNBr digests of both whole antibody and separated $\gamma_2$ chain. The specifically modified residues were clearly localized to the three fragments C-1-n, C-1-$a_1$, and C-1-$a_2$, comprising the amino-terminal 140 residues of $\gamma_2$ chain. Each of these three fragments from "normal" $\gamma_2$ chain contains a segment with a variable primary structure. The predominant modified residue in affinity-labeled antibody was tyrosine. The positions of the azotyrosine residues appear to be within or just without those segments found to be variable in "normal" $\gamma_2$ chain. The fragments C-1-n and C-1-$a_2$ are linked in the parent molecule by a disulfide bond between half-cystine residues N-22 and N-96 and thus their variable segments would be close in the intact antibody molecule.

Two approaches have been used to locate the antigen binding sites in immunoglobulin molecules and, concomitantly, to deduce the primary structural basis of antibody specificity.

One method is based on the known dependence of protein conformation on amino acid sequence (Epstein *et al.*, 1963) and the corollary that the heterogeneity of antigen binding sites within an immunoglobulin population must be reflected by positions having alternative amino acid residues. Hence, comparison of the sequences of a set of homogeneous myeloma proteins or the determination of the primary structure of one polymorphic form of "normal" immunoglobulin from inbred animals would be expected to define regions of variable primary structure which presumably lie within or determine the conformation of the combining site. By comparison of sequences of human myeloma proteins, variable regions ($V_H$ and $V_L$) have been localized to the amino-terminal half of light chain (Titani *et al.*, 1965; Hilschmann and Craig, 1965) and the amino-terminal quarter of heavy chain (Press and Hogg, 1969; Cunningham *et al.*, 1969; Wikler *et al.*, 1969). Positions in heavy chain having alternative residues have likewise been found only in its N-terminal quarter when

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it derived from normal rabbit IgG (Mole *et al.*, 1971) or normal inbred guinea pig IgG(2) (Birshtein and Cebra, 1971; Cebra *et al.*, 1971). Furthermore, many residue positions showing amino acid interchanges in heavy chain of these normal IgGs appear to be concentrated into three short segments (Birshtein and Cebra, 1971; Mole *et al.*, 1971; Cebra *et al.*, 1971) while most of the region corresponding to $V_H$ of myeloma proteins appears to have a single sequence. However, human myeloma heavy chains can be grouped so that most of the interchanges within a $V_H$ subgroup are also found in segments from positions N-31 to -35, N-52 to -65, N-85 to -91, and N-101 to -119 (Cunningham *et al.*, 1969; Capra, 1971), three of which closely correspond to those variable segments from normal $\gamma_2$ chain. Residue positions within these variable segments of normal and myeloma heavy chains may determine antibody specificity.

A second and complementary procedure for identifying those parts of the antibody molecule which form the antigen binding sites is to covalently label them. The modified intact antibody is subsequently degraded to determine the positions of attached label. Use of a labeling reagent which is structurally homologous to the antigenic determinant and is reactive with a particular antibody, called an affinity label, was introduced by Wofsy *et al.* (1962). The affinity label, *m*-nitrobenzenediazonium fluoroborate, modified only tyrosine residues of rabbit anti-dinitrophenyl antibodies although it could chemically react with both tyrosine and histidine residues of normal immunoglobulin (Good *et al.*, 1967). This same reagent has been successfully used as an active site label for a mouse myeloma protein having anti-dinitrophenyl specificity (Goetzl and Metzger, 1970) and for pig anti-dinitrophenyl antibodies (Franék, 1971). The major points of attachment of this affinity label to these molecules were identified as the tyrosines at positions N-33 and N-93 of porcine $\lambda$ chains and N-34 of the murine myeloma light chain.

We have used a $^{14}C$ preparation of this same affinity label, MNBDF, to specifically modify anti-dinitrophenyl antibodies raised in inbred guinea pigs. The vast majority of attached label was found to be associated with their heavy chains. Most of the modified residues were found in three CNBr fragments of the heavy chains. These three fragments, when derived from normal IgG2, each contain a segment with variable primary structure (Birshtein and Cebra, 1971; Cebra *et al.*, 1971, D. Benjamin, unpublished) and were found to comprise the region corresponding to $V_H$ of myeloma proteins (Benjamin *et al.*, 1972).

## Materials and Methods

*Immunizations and Antisera.* Wright strain 13 guinea pigs from our own colony were used exclusively to provide normal IgG2 and antibodies. The immunogen, Dnp[1]-limpet hemocyanin, was prepared according to Eisen (1964) and the cross-reacting antigen, Tnp-human serum albumin, used to prepare pure anti-Dnp antibody, was made according to the procedure of Little and Eisen (1966), as previously reported for both (Birshtein and Cebra, 1971). The immunization regime with Dnp-hemocyanin and the bleeding period were as detailed before (Birshtein and Cebra, 1971). About 20 ml of serum was obtained from each animal and each preparation of antibody was from the pooled serum derived from 15 to 20 guinea pigs.

*Purified Antibody and IgG2.* The anti-Dnp antibody was purified by the procedure of Eisen *et al.* (1967) which makes use of Tnp-serum albumin to precipitate antibody, dinitrophenol to dissolve and dissociate the antigen–antibody complexes, and double-layered columns of Dowex 1 and DEAE-cellulose to remove antigen and hapten from the antibody. Ordinarily anti-Dnp antibody was precipitated by antigen from a solution of immunoglobulin (20 mg/ml) consisting of that fraction precipitated from immune serum at 37% saturation with ammonium sulfate (Birshtein and Cebra, 1971). The preliminary experiments assessing the specificity of the MNBDF reagent were carried out on antibody purified from guinea pig IgG2. The IgG2 was prepared from serum or the immunoglobulin fraction precipitated at 37% saturation with ammonium sulfate by the chromatographic method already described (Birshtein *et al.*, 1971a).

*Preparation of Affinity Label.* The *m*-nitrobenzenediazonium-$^{14}C$ tetrafluoroborate was prepared by diazotization of *m*-nitroaniline-$^{14}C$ (4.51 Ci/mole, Mallinckrodt) following the method of Traylor and Singer (1967). The *m*-nitroaniline (40 mg) was dissolved in 34% fluoroboric acid (0.45 ml) and brought to 0° in an ice water bath. After dropwise addition of sodium nitrite (24 mg in 0.11 ml), the reaction mixture was stirred for 1 hr. The white precipitate was collected by filtration and washed successively with chilled (0°) 25% fluoroboric acid, absolute ethanol, and anhydrous ether. The washed precipitate was dissolved in acetone (25°) and recrystallized by the addition of anhydrous ether. The product was recovered in a yield of 57%. It was stored as the solid salt *in vacuo* at 5° in an opaque glass vial. Immediately preceding use the reagent was dissolved in 0.01 M HCl. The specific radioactivity, determined for concentrations of MNBDF ranging from 1.6 mM to 1.6 $\mu$M in 0.01 M HCl, was 4.58 mCi/mmole. A second preparation, made using precursor *m*-nitroaniline-$^{14}C$ diluted tenfold with nonradioactive carrier, was obtained in a yield of 43% and a specific radioactivity of 0.48 Ci/mole.

*Preparation and Spectrum of m-Nitrobenzene-$^{14}C$-azotyrosine.* The copolymer of L-glutamic acid and L-tyrosine (Miles-Yeda, Glu:Tyr, 9:1) was dissolved in water by titration with NaOH to pH 9.3 to give a final concentration in terms of tyrosine residues of 0.015 mmole/ml. An equal volume of a solution of MNBDF-$^{14}C$ (0.015 mmole/ml, 0.48 Ci/mole) in 0.01 N HCl was added at 5°, and the reaction was continued at that temperature for 4 hr. The reaction mixture was dialyzed for 72 hr against 0.01 M sodium phosphate (pH 8.0) and then distilled water. A stock solution of the derivatized copolymer was prepared to contain about 5 mg/ml. The concentration of azotyrosine derivative in this stock was 1.2 mM as determined from the light absorbance at 325 and 490 m$\mu$ of a dilution in 0.2 M NaOH (Traylor and Singer, 1967). This is exactly the stock concentration calculated from its radioactivity, and in the following experiments the molar proportion of affinity label derivatized to IgG or its fragments was determined by measurement of radioactivity. The copolymer had 31% of its tyrosine residues modified, and its absorption spectrum, determined on a Cary recording spectrophotometer, was used as a qualitative standard for tyrosine derivatization of antibody and its fragments.

*Specificity of the Reaction of MNBDF-$^{14}C$ with Anti-Dnp Antibodies.* The anti-Dnp antibody (4 mg/ml), purified from IgG(2), was incubated in 0.2 M sodium acetate (pH 5.0) with or without protecting haptens. The blocking haptens $\epsilon$-Dnp-lysine and $\epsilon$-Dnp-aminocaproate were preincubated with the antibody for 1 hr at room temperature at 100- and 19-fold

[1] Abbreviations used are: Dnp, dinitrophenyl; Tnp, trinitrophenyl; MNBDF, *m*-nitrobenzenediazonium fluoroborate; Amp, $N^{\epsilon}$-4-azido-2-nitrophenyl.

molar excess, respectively. The mixtures were then brought to 0° in an ice water bath and a threefold molar excess of MNBDF-$^{14}C$, relative to antibody, was added as an 8 mM solution in 0.01 N HCl. At intervals after addition of the affinity label, portions of the reaction mixture (0.2 ml) were pipetted into 10-ml centrifuge tubes, each containing 0.05 M phenol (0.1 ml). The protein from each sample was then precipitated and washed free of excess reagents after the procedure of Wofsy *et al.* (1962). The washed precipitates were dissolved in sodium hydroxide (0.15 M), and the moles of affinity label bound per mole of antibody were determined.

*Specific Radioactivities.* The moles of MNBDF-$^{14}C$ bound per mole of antibody, heavy chain, light chain, and CNBr fragment of heavy chain were determined using 0.1–0.2 ml of solutions of these in 5 ml of Kinard's solvent (Kinard, 1957) and a Nuclear-Chicago liquid scintillation counter. Counting efficiencies were estimated using a set of $^{14}C$ quench standards (toluene), and the radioactivity of each sample was then appropriately corrected. The concentration of intact IgG was determined from light absorbance (0.15 M NaOH) using $\epsilon_{280}^{1\%} = 14$, and for heavy (H) and light (L) chains (1 M propionic acid) using $\epsilon_{280}^{1\%} = 13$ and 11, respectively. The concentrations of the CNBr fragments were determined directly by amino acid analysis of measured portions.

*Preparative Affinity Labeling of IgG2 Containing Anti-Dnp Antibody.* Labeling was carried out following the method of Good *et al.* (1967) with modifications. An immunoglobulin solution (2 mg/ml), made up from protein precipitated at 37% saturation with ammonium sulfate from anti-Dnp antiserum containing 1.0–1.5 mg/ml of specific antibody, was prepared in 0.2 M sodium acetate buffer (pH 5.0). This solution of immunoglobulin (7.4 μmoles) was mixed with 0.2 molar equivalent of MNBDF-$^{14}C$ per mole of IgG, added as a 0.074 mM solution in 0.01 M HCl, at 0°. The reaction mixture was stirred for 1 hr at 0° and then the reaction was terminated by the addition of a 500-fold molar excess of phenol, added as a 0.048 M solution, over MNBDF. The protein was freed of reagents by passage through a column of Sephadex G-25 (coarse, 4 × 52 cm) equilibrated in pH 5.0 sodium acetate buffer. Determinations of specific radioactivity indicated that 0.062 mole of affinity label was bound per mole of immunoglobulin. A threefold molar excess of ε-Dnp-lysine over MNBDF, present in a small portion of the same reaction mixture, decreased the extent of labeling by 70%. Most of the affinity-labeled immunoglobulin (6.7 μmoles) was used to prepare IgG2 (3.9 μmoles) by the procedure of Birshtein *et al.* (1971a). This preparation of IgG2 containing modified antibody is referred to as "Ab1."

*Preparative Affinity Labeling of Purified Anti-Dnp Antibody.* One preparation of antibody (456 mg) was isolated from anti-Dnp antiserum (304 ml). Light absorption measurements at 358 mμ indicated that this antibody retained, after purification, sufficient dinitrophenol to block 11% of its antigen binding sites. This antibody was labeled under conditions similar to those used for the immunoglobulin which contained antibody, except that a threefold molar excess of MNBDF-$^{14}C$ was added, as a 1.8 mM solution in 0.01 M HCl, to the antibody (2.7 μmoles) at a concentration of 4 mg/ml, and the reaction was terminated after 50 min by the addition of 100-fold molar excess of phenol over MNBDF. The modified antibody, when freed of reagents by gel filtration on Sephadex G-25, contained 0.56 mole of label per mole of protein. The presence of a 94-fold molar excess of ε-Dnp-lysine over MNBDF in a portion of the same reaction mixture decreased the extent of labeling by 77%. The unprotected, modified antibody is referred to as "Ab2."

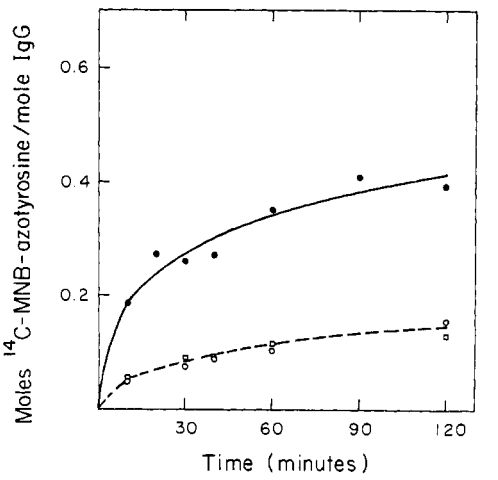


FIGURE 1: Inhibition of affinity labeling of anti-Dnp antibody by reversibly bound hapten. Antibody (4 mg/ml) was allowed to react with a 3-fold molar excess of MNBDF-$^{14}C$ in the absence of hapten (●), in the presence of a 100-fold molar excess of ε-Dnp-lysine relative to affinity label (○), and in the presence of a 19-fold molar excess of ε-Dnp-aminocaproate (□). Extent of modification was determined at the times indicated from specific radioactivities of the precipitated protein.

A second preparation of antibody (3.0 μmoles) was purified from another pool of anti-Dnp antiserum (395 ml) and contained enough residual dinitrophenol to block 18% of its antigen binding sites. Reaction of this antibody with MNBDF-$^{14}C$, using the same conditions as for Ab2, resulted in 0.46 mole of label being bound per mole of protein. Protection of a portion of this reaction mixture with ε-Dnp-lysine decreased the affinity labeling by 78%. The modified, unprotected antibody is referred to as "Ab3."

*Modification of Normal IgG2 with MNBDF-$^{14}C$.* A preparation of IgG2 (57 mg) from serum taken from guinea pigs which had not been immunized was allowed to react with MNBDF-$^{14}C$ under the same conditions used for Ab2 and Ab3. The reaction was terminated as for Ab2 and the protein recovered after gel filtration contained 0.13 mole of label per mole. The presence of a 100-fold molar excess of ε-Dnp-lysine over MNBDF in a portion of the reaction mixture did not diminish the extent of protein modification.

*Other Procedures.* The preparation of $\gamma_2$ chain, its cleavage with CNBr, and the isolation of its fragments have been detailed previously (Birshtein *et al.*, 1971a). The cleavage of the whole IgG molecule with CNBr and the purification of its component fragments followed the procedures given by Birshtein and Cebra (1971). Procedures for amino acid analysis have been described (Birshtein *et al.*, 1971a) as have those used to determine the amino acid sequence of peptides by manual methods (Turner and Cebra, 1971) and using the Beckman automatic sequencer (Birshtein and Cebra, 1971).

## Results

*Specificity of the Modification of Anti-Dnp Antibody by MNBDF-$^{14}C$.* Anti-Dnp antibody was allowed to react with a threefold molar excess of MNBDF -$^{14}C$ for various times in the presence or absence of blocking haptens. Figure 1 indicates the considerable difference, both in rate and extent of modification, between the reactions carried out in the ab-

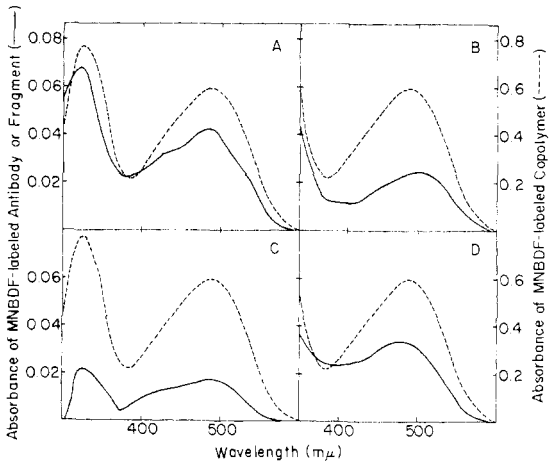


FIGURE 2: Absorption spectra of the copolymer glutamyltyrosine after modification with MNBDF and of affinity-labeled anti-Dnp antibody and its modified fragments. All absorption spectra were determined using 0.2 N NaOH as solvent. For comparison of the quality of spectra measured on a scale of 0.0–1.0 absorbance unit *vs.* measured on the expanded scale of 0.0–0.1, used for modified antibody and its fragments, a spectrum of a dilute solution of MNB-azotyrosine (solid line) is shown in part A. The absorption spectrum of the modified copolymer (dashed lines) is shown for comparison to those of modified antibody and its fragments (solid lines). (A) Spectrum of MNB-azotyrosine-$^{14}C$ isolated from the modified copolymer after consecutive digestion with pepsin and aminopeptidase-M. (B) Difference spectrum of modified Ab3 compared to unlabeled anti-Dnp antibody. A 1.7-mg amount of Ab3 was dissolved in 1.2 ml of 0.2 N NaOH. (C) Difference spectrum of C-1-n from affinity-labeled Ab2 compared with C-1-n from unmodified anti-Dnp antibody. The fragment (0.04 μmole) was dissolved in 1.2 ml of 0.2 N NaOH. (D) Difference spectrum of C-1-$a_2$ isolated from affinity-labeled Ab3 compared to C-1-$a_2$ from unmodified anti-Dnp antibody. The fragment (~0.1 μmole) was dissolved in 1.5 ml of 0.2 N NaOH.

sence of blocking hapten and those run in the presence of either 100-fold molar excess of ε-Dnp-lysine, relative to affinity label, or 19-fold molar excess of ε-Dnp-aminocaproate. By 1 hr, 0.35 mole of label had reacted per mole of "unprotected" antibody, as compared to 0.11 mole per mole of the antibody "protected" by hapten. The ε-Dnp-lysine and ε-Dnp-aminocaproate inhibited labeling to the same extent although there was a fivefold difference in concentrations of the two haptens in their separate reactions solutions. The inhibition of labeling by the Dnp-haptens was 72% after 10 min, 69% after 1 hr, and 63% after 2 hr. Table I shows that hapten inhibition of affinity labeling of the two large preparations of purified antibody, Ab2 and Ab3, which were modified on a preparative scale using the same reaction conditions as for the time course study shown in Figure 1, was 77 and 78% effective, respectively, after 50-min reaction.

The IgG(2) containing about 10–15% anti-Dnp antibody, Ab1, was reacted on a preparative scale with 0.2 mole of MNBDF-$^{14}C$ per mole of IgG(2) for 1 hr. Although it was modified to a considerably lesser extent than was purified antibody, hapten protected this immunoglobulin to the extent that it inhibited 70% of the labeling (Table I).

The IgG2 from nonimmunized guinea pigs was allowed to react with MNBDF-$^{14}C$ under conditions identical with those for modification of purified anti-Dnp antibody. After 10 min of reaction, 0.06 mole of label had reacted per mole of IgG2 and, after 50 min, 0.13 mole had reacted. The presence of a 100-fold molar excess of ε-Dnp-lysine over affinity label failed to inhibit this binding of MNBDF-$^{14}C$ to normal IgG2.

TABLE I: Modification of Antibody (Ab2, Ab3), Immunoglobulin-Containing Antibody (Ab1), and Normal Immunoglobulin (IgG) by Affinity Label.

| Preparation of Immunoglobulin | Moles of Label/Mole of IgG (%) | % Inhibition of Labeling by Hapten | Mole Fraction of Label | |
|---|---|---|---|---|
| | | | On H Chain | On L Chain |
| Ab1 | 6.2 | 70 | 0.89 | 0.11 |
| Ab2 | 56 | 77 | 0.88 | 0.12 |
| Ab3 | 46 | 78 | | |
| IgG | 13 | 0 | 0.61 | 0.39 |

Table I indicates this lack of protection of normal IgG2 from modification by MNBDF-$^{14}C$ after 50-min reaction.

The experiments represented in Figure 1 and Table I, in which hapten was found to block much of the affinity labeling of specific antibody but not of normal IgG2, provide one kind of support for 70–78% of the modified residues of anti-Dnp being associated with antigen binding sites.

*Absorption Spectrum of Anti-Dnp Antibody Modified by Reaction with MNBDF.* Another criterion for specific modification of antibody is the finding, by examination of absorption spectra, that only one particular kind of amino acid has reacted with affinity label (Wofsy *et al.*, 1962). The absorption spectrum of the copolymer of glutamic acid and tyrosine, modified by reaction with MNBDF, is shown in Figure 2A. This figure also shows the spectrum of the derivatized tyrosine isolated from the copolymer, recorded using the same sensitivity needed for the available amounts of modified antibody and its fragments. Figure 2B shows the difference spectrum of affinity-labeled anti-Dnp antibody (Ab3) measured against unmodified antibody. The absorption maximum at 490 mμ is characteristic of the tyrosine derivative and the spectrum indicates that tyrosine is the principal modified residue. Traylor and Singer (1967) have shown that the spectrum of azohistidine, the other derivative likely to form under the conditions used for the reaction of antibody with MNBDF, has an absorption maximum at about 415 mμ and an extinction coefficient 2.2 times that of azotyrosine at 490 mμ.

*Distribution of Affinity Label between the Heavy and Light Chains of Anti-Dnp Antibody and of Normal IgG2.* A clear-cut preferential localization of affinity label on antibody $\gamma_2$ chains is shown in Table I. From 88–89% of the modified residues of specifically labeled purified antibody or IgG2 containing antibody were found in the $\gamma_2$ chains. In modified normal IgG2, only 61% of the labeled residues were found in the heavy chain.

*Distribution of Affinity Label among the Fragments after the Initial Fractionation of CNBr Digests of $\gamma_2$ Chain.* One of the most convincing proofs for the specificity of modification—the labeling of residues in or nearby the MNBDF-binding sites—would be the localization of the label to a limited number of residue positions containing tyrosine. The $\gamma_2$ chains, obtained after reductive cleavage of interchain disulfide bonds of the whole molecule and carboxymethylation of the resulting cysteines, from modified Ab1, Ab2, and normal IgG2 were cleaved with CNBr (Birshtein *et al.*, 1971a). The digests were applied to columns of Sephadex G-100 in 8 M urea, 0.1 M in formic acid, to effect the initial fractionating step in the

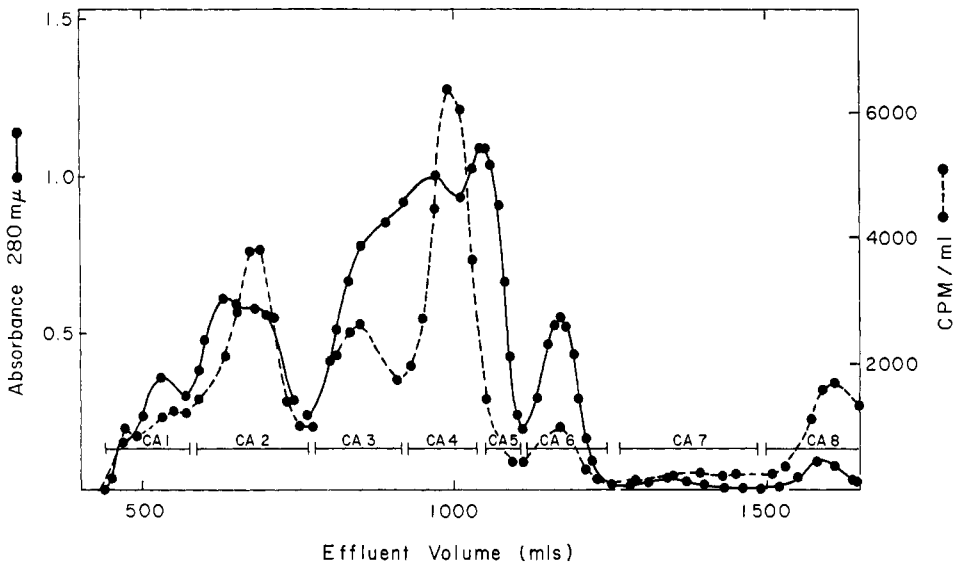


FIGURE 3: Elution profile of the initial separation of CNBr fragments from 7.1 μmoles of $\gamma_2$ chain obtained from Abl. The CNBr digest was applied to a column of Sephadex G-100 (3.5 × 145 cm) equilibrated with 8 M urea, 0.1 M in formic acid, and 10-ml fractions were collected.

separation of the component fragments of $\gamma_2$ chain (Birshtein *et al.*, 1971a). The elution profiles, such as the one shown in Figure 3 for the digest of $\gamma_2$ chain from Ab1, were very like those already reported for the isolation of five CNBr fragments, each having a single amino acid sequence from normal $\gamma_2$ chain (see Figure 2, Birshtein *et al.*, 1971a). However, the component(s) from Ab1 and Ab2 containing the majority of the affinity label, included in fraction CA4, elute from the column between two of these fragments, C-3, having 93 amino acid residues, and C-4, having 83 residues. The labeled component(s) in CA4 did not correspond to any of the five C-terminal fragments of $\gamma_2$ chain. Another fragment of about 55 residues, C-1-$a_2$, was previously isolated from this fraction (Birshtein *et al.*, 1971a). The C-1-$a_2$ is known to contain the easily reduced half-cystine which participates in formation of the disulfide bond between heavy and light chain (Birshtein *et al.*, 1971b; Oliveira and Lamm, 1971) and is only isolated from fraction CA4 after reduction. Thus C-1-$a_2$, joined to another fragment by a disulfide bond to form a component with about 90 residues, was a candidate for the fragment(s) in CA4 modified by affinity label.

Additional affinity label is found in fraction CA3 and in fraction CA6, which contains almost exclusively C-1-$a_1$, a fragment with a variable primary structure (Birshtein and Cebra, 1971). Fractions CA1 and CA2 are aggregates of fragments found in other fractions and do not contain any unique fragment not accounted for elsewhere (Birshtein *et al.*, 1971a). The elution profile of the CNBr digest of Ab2 and the distribution of affinity label was the same as shown for

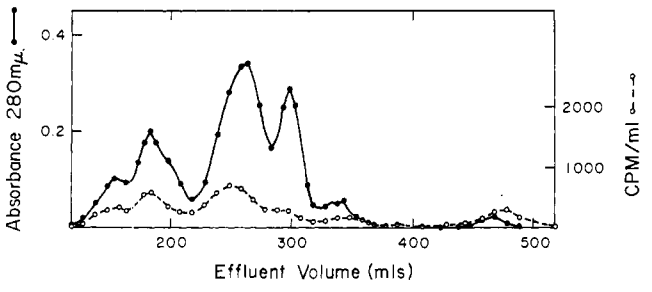


FIGURE 4: Elution profile of the separation of fragments in a CNBr digest of $\gamma_2$ chain (0.5 μmole) from normal IgG(2) modified with MNBDF-$^{14}C$. The digest was applied to a column of Sephadex G-100 (2.6 × 94 cm) equilibrated as described in Figure 3 and 5.0-ml fractions were collected.

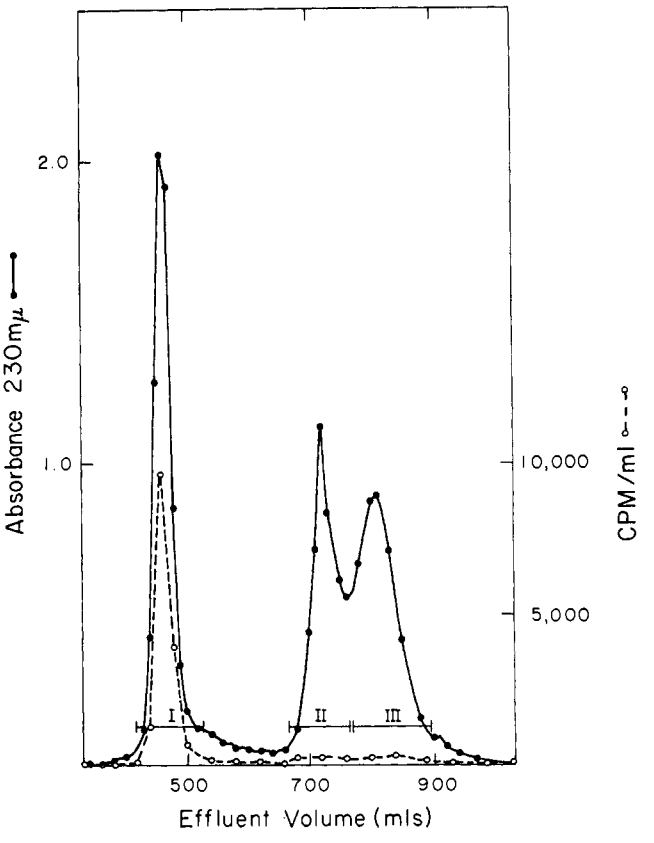


FIGURE 5: Separation of components in pool CA3 (Figure 3) obtained from a CNBr digest of Ab2. The freeze-dried fragments were dissolved in 1.25 M guanidine HCl, 0.05 M in $NH_4HCO_3$, before application to a column of Sephadex G-75 (3 × 180 cm) equilibrated with 0.05 M $NH_4HCO_3$. Fractions of 10 ml were collected.

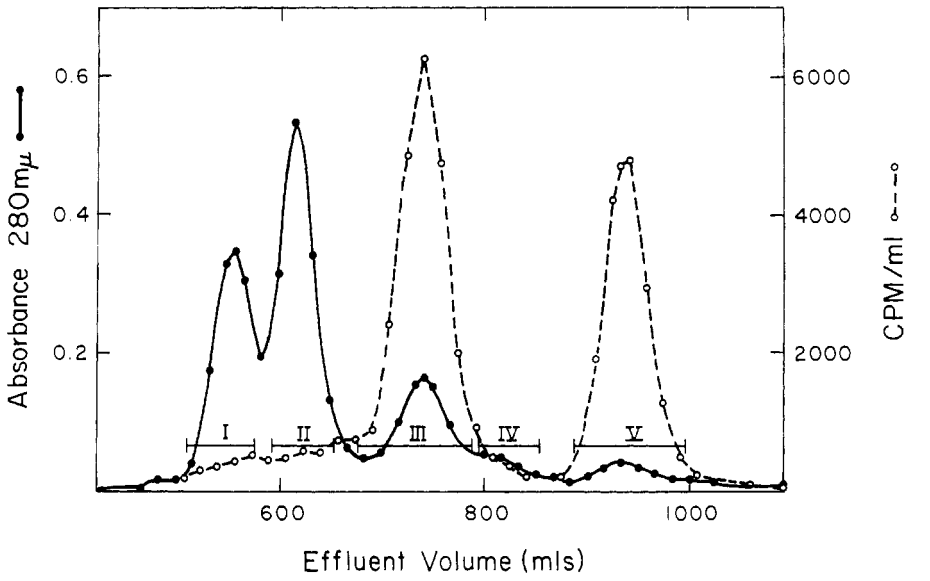


FIGURE 6: Separation of CNBr fragments of $\gamma_2$ chain found in pool CA4 (Figure 3) after reductive cleavage of all disulfide bonds and carboxymethylation of cysteine residues. The fragments in pool CA4 derived from Ab2 were applied to a column of Sephadex G-50 (fine, 3 × 180 cm) equilibrated with 8 M urea, 0.1 M in formic acid. Fractions of 10 ml were collected.

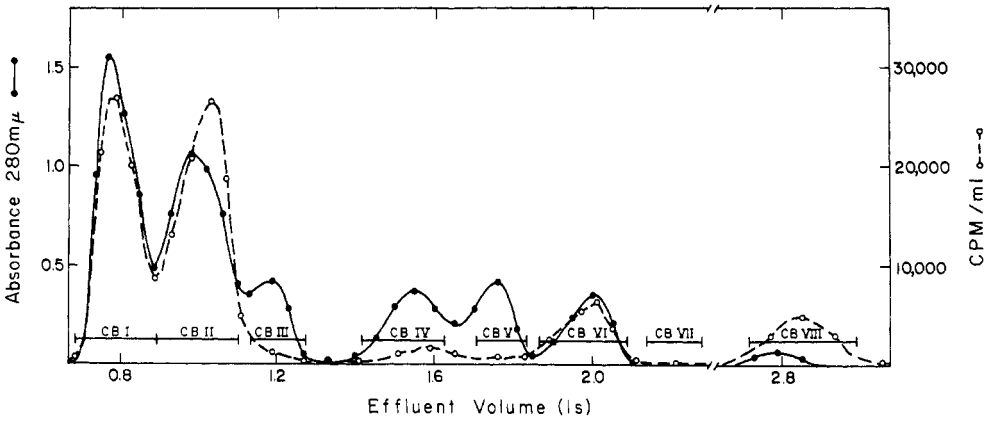


FIGURE 7: The elution profile of the total CNBr digest of Ab3 (3.0 μmoles) obtained by filtration through a column of Sephadex G-100 (3.5 × 210 cm). The column was equilibrated with 8 M urea, 0.1 M in formic acid, and 10-ml fractions were collected.

Ab1 except that this purified antibody gave rise to somewhat more aggregated material. In contrast, the distribution of the affinity label among the fractions of the CNBr digest of $\gamma_2$ chain from normal IgG(2), shown in Figure 4, is fairly uniform relative to absorbance at 280 mμ of the fractions. No concentration of label in fragment(s) eluting between C-3 and C-4 is apparent. These findings are consistent with the modification of particular residues in Ab1 and Ab2 and the more random substitution of amino acid residues by MNBDF in normal IgG2.

*The Isolation of CNBr Fragments Composing the Entire Length of $\gamma_2$ Chain.* Fragments C-1-b, C-1-c, C-3, C-4, and C-5 have been isolated previously from fractions of a CNBr digest of normal $\gamma_2$ chain corresponding to CA3 through CA7 shown in Figure 3 (Birshtein *et al.*, 1971a). These five fragments each consist of a single amino acid sequence (Turner and Cebra, 1971; Birshtein *et al.*, 1971b; D. Tracey and T. Trischmann, unpublished) and have been aligned in the order C-1-b, C-1-c, C-3, C-4, and C-5, with the latter fragment at the C terminus of $\gamma_2$ chain (Benjamin *et al.*, 1972). A sixth fragment, C-1-$a_1$, was recognized as the major component in fraction CA6 (Figure 3) (Birshtein *et al.*, 1971a), and its variable primary structure has been determined (Birshtein and Cebra, 1971). In this present study, the fragments from digests of heavy chain from Ab1 and Ab2 were isolated following the published procedures. For instance, Figure 5 shows the second step in the fractionation of CA3 (Figure 3). Upon refiltering pool CA3 through a column of Sephadex G-75, the C-1-a fraction containing label (pool I) is separated from unlabeled C-3 (pool II) and C-1-b–C-1-c (pool III). In the course of further fractionation of pool CA4 (Figure 3), which contained the major affinity-labeled component(s), we followed the procedure used for the corresponding fraction from normal $\gamma_2$ chain (pool CB3, Birshtein *et al.*, 1971a). The pool CA4 was reduced and carboxymethylated to cleave all remaining disulfide bonds and to derivatize the resulting cysteines. The mixture was then applied to a gel filtration column, and the elution profile shown in Figure 6 was obtained. Pools I and II, which were fragments C-3 and C-4, respectively, contained very little affinity label but two other fragments, isolated in

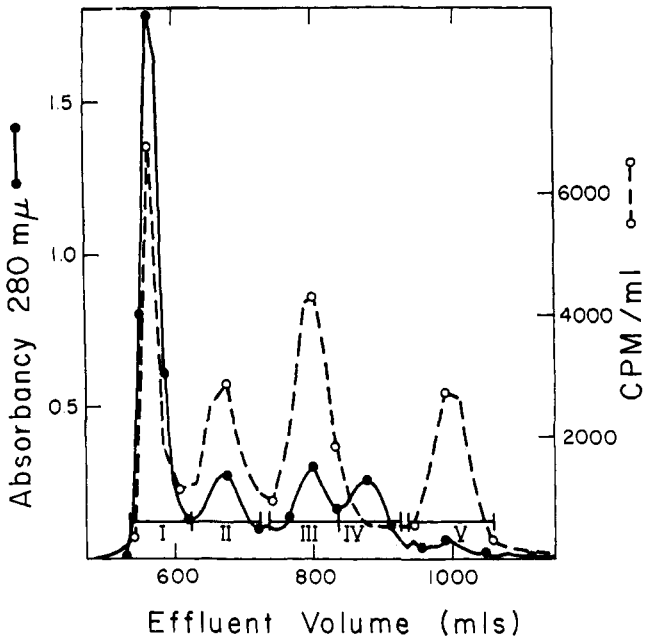


FIGURE 8: Separation of affinity-labeled fragments from pool CBII (Figure 7) obtained from Ab3. The disulfide bonds present in the components of CBII were reductively cleaved and cysteine residues were carboxymethylated. The mixture was applied to a column of Sephadex G-50 (fine, 3 × 180 cm) equilibrated with 8 M urea, 0.1 M in formic acid, and fractions of 10 ml were collected.

near equimolar amounts relative to each other, shared most of the label. These modified fragments, obtained both from Ab1 and Ab2, were different from the six CNBr fragments already characterized from normal $\gamma_2$ chain. One of them, pool III (Figure 6), was C-1-$a_2$, a fragment found in low yield in a digest of normal $\gamma_2$ chain (Birshtein *et al.*, 1971a) and the other, found in pool V (Figure 6), was called C-1-n. These two additional fragments, C-1-n and C-1-$a_2$, along with the other six which had been characterized, have been found to account for the entirety of the $\gamma_2$ chain (Benjamin *et al.*, 1972).

*Isolation of Fragments from CNBr Digest of Intact Anti-Dnp Antibody.* One could contend that those fragments with most of the affinity label isolated from the $\gamma_2$ chain of Ab1 and Ab2 might contain small amounts of different "passenger" fragments which actually bore the label, while the identification of the fragment depended largely on the compositional analysis of the major component. The isolation of C-1-n and C-1-$a_2$ from $\gamma_2$ chain digests depends on their being cleaved from the chain while remaining joined to each other by a former intrachain disulfide bond. In a second step these small fragments are released from each other and separated from the much larger C-3 and C-4. Recently, an alternate procedure for obtaining most of the CNBr fragments from $\gamma_2$ chain after only one or two fractionation steps has been described (Birshtein and Cebra, 1971). This digest is performed on whole molecules and the separation of C-1-n and C-1-$a_2$ is based on their covalent linkage to light chain during the first gel filtration step. To determine whether C-1-n and C-1-$a_2$ isolated in this alternate way still were found to be major affinity-labeled fragments, Ab3 was digested directly with CNBr. Figure 7 shows the elution profile of the digest after passage through a column of Sephadex G-100. Most of the modified residues were found in pools CBI, CBII, and CBVI. Pool CBVIII contained salt present in the freeze-dried CNBr digest and a small amount of free affinity label.

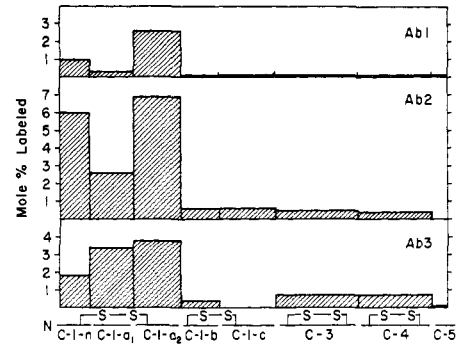


FIGURE 9: The localization of affinity label on CNBr fragments accounting for the entirety of the $\gamma_2$ chain. The extent of labeling is given as moles of modified residue per mole of fragment, in per cent. The alignment, length, and disulfide bonding of the fragments are represented schematically along the abscissa. Fragment C-1-c was not purified from Ab3.

Fragments C-3, C-4, C-1-$a_1$, and C-5 were recovered directly by desalting pools CBIV, CBV, CBVI, and CBVII, respectively. Pool CBI is the result of aggregation of fragments found elsewhere. Pool CBII was reduced to cleave all disulfide bonds and the resulting cysteine residues were carboxymethylated. The mixture was then applied to a column of Sephadex G-50 and the fragments C-1-$a_2$, C-1-b, and C-1-n were isolated cleanly from pools III, IV, and V, respectively (Figure 8).

*Amino Acid Compositions and Mole % Labeling of the CNBr Fragments from Ab1, Ab2, and Ab3.* Table II lists the amino acid analyses and specific modification of the five CNBr fragments accounting for the C-terminal 303 residues of the $\gamma_2$ chains from the various affinity-labeled antibody preparations. The column of integral numbers of residues for each fragment is based on its amino acid sequence.

The table shows that the modification of these particular fragments is very slight, and the schematic diagram in Figure 9 portrays the low level of affinity labeling in these fragments relative to those three from the N-terminal end of $\gamma_2$ chain. In the schematic shown in Figure 9, the order and length of the fragments are represented on the abscissa. The order of the CNBr fragments from the N-terminal end of $\gamma_2$ chain is C-1-n, C-1-$a_1$, and C-1-$a_2$ (Benjamin *et al.*, 1972). The analyses of the three N-terminal fragments from the antibody preparations are given in Table III. They are compared with average analyses, based on five preparations each, from the corresponding fragments from normal $\gamma_2$ chain. The specific labeling of these fragments indicates preferential modification of their residues by MNBDF-$^{14}C$ compared with those of the C-terminal three-quarters of $\gamma_2$ chain, as illustrated in Figure 9.

*Some Primary Structural Characteristics of C-1-$a_2$.* Of the three main affinity-labeled fragments, two have counterparts from normal $\gamma_2$ chain which have known primary structures, C-1-$a_1$ (Birshtein and Cebra, 1971) and C-1-n (D. Benjamin, unpublished). To firmly position C-1-$a_2$, that fragment with the highest mole per cent of affinity label in all three preparations, this fragment from Ab1 was digested with trypsin and three peptides, T37, T41, and T42, were isolated (Figure 10). One of these, T37, had a composition identical with that of the N-terminal 20 residues of peptide T36-37, a methionine-containing tryptic peptide from $\gamma_2$ chain which overlaps the N-terminal sequence of fragment C-1-b with the C terminus of the adjoining CNBr fragment (Birshtein *et al.*, 1971b).

TABLE II: Amino Acid Compositions and Affinity Labeling of Constant Region CNBr Fragments of $\gamma_2$ Chain.[a]

| | C-1-b/C-1-c[b] | | | C-1-b[c] | | C-3 | | | | C-4 | | | | C-5[c] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | IgG(2) | Ab1 | Ab2 | IgG(2) | Ab3 | IgG(2) | Ab1 | Ab2 | Ab3 | IgG(2) | Ab1 | Ab2 | Ab3 | IgG(2) | Ab1 | Ab3 |
| Lys | 8 | 9.4 | 8.7 | 2 | 2.0 | 9 | 9.0 | 7.7 | 8.0 | 7 | 6.5 | 5.2 | 7.3 | 1 | 1.2 | 1.2 |
| His | 2 | 1.8 | 1.7 | 1 | 0.6 | 1 | 1.5 | 1.4 | 1.2 | 1 | 0.9 | 0.7 | 1.1 | 3 | 2.9 | 1.9 |
| Arg | 1 | 1.4 | 1.5 | | 0.7 | 4 | 4.7 | 3.4 | 4.4 | 2 | 2.1 | 1.8 | 2.6 | 1 | 1.2 | 1.1 |
| CMCys | 5 | 5.1 | 3.3 | 1 | 0.6 | 2 | 2.5 | 2.5 | 1.8 | 2 | 2.8 | 2.5 | | | | |
| Asp | 6 | 5.9 | 6.0 | 1 | 1.2 | 9 | 8.5 | 9.3 | 8.2 | 9 | 10.1 | 10.8 | 9.0 | 1 | 1.1 | 1.3 |
| Thr | 12 | 11.2 | 10.9 | 5 | 4.3 | 8 | 9.1 | 8.1 | 7.4 | 6 | 6.3 | 5.6 | 5.3 | 1 | 1.1 | 0.9 |
| Ser | 10 | 9.3 | 9.2 | 5 | 5.1 | 5 | 5.9 | 6.5 | 4.7 | 9 | 8.5 | 10.0 | 8.7 | 2 | 1.8 | 2.0 |
| Glu | 6 | 7.0 | 6.9 | 2 | 3.3 | 11 | 10.5 | 11.8 | 11.6 | 7 | 6.9 | 6.1 | 5.3 | 2 | 1.9 | 2.0 |
| Pro | 17 | 16.4 | 17.1 | 3 | 3.8 | 8 | 8.2 | 7.4 | 9.0 | 8 | 8.0 | 7.5 | 6.1 | 1 | 1.0 | 1.1 |
| Gly | 7 | 8.2 | 6.5 | 5 | 4.9 | 3 | 3.0 | 3.1 | 3.2 | 3 | 2.3 | 2.4 | 2.3 | 1 | 1.3 | 1.5 |
| Ala | 5 | 5.1 | 4.9 | 2 | 2.1 | 4 | 3.0 | 4.3 | 4.3 | 4 | 4.1 | 4.1 | 4.1 | 2 | 1.9 | 1.7 |
| Val | 11 | 10.8 | 10.5 | 5 | 4.1 | 11 | 10.6 | 9.8 | 10.6 | 9 | 9.0 | 8.4 | 10.6 | 1 | 0.9 | 1.0 |
| Ile | 2 | 2.0 | 2.3 | | | 4 | 3.7 | 3.5 | 3.9 | 3 | 3.2 | 3.1 | 3.8 | 1 | 1.0 | 1.2 |
| Leu | 8 | 7.6 | 7.8 | 6 | 4.8 | 5 | 4.3 | 4.6 | 4.4 | 5 | 5.3 | 5.1 | 5.7 | 1 | 1.1 | 1.2 |
| Tyr | 2 | 1.9 | 2.5 | 2 | 1.7 | 3 | 3.0 | 2.9 | 2.4 | 4 | 5.1 | 4.9 | 5.6 | | | |
| Phe | 4 | 4.0 | 5.1 | 2 | 1.7 | 3 | 3.4 | 3.9 | 2.8 | 3 | 3.3 | 3.6 | 4.2 | | | |
| Hsr | 2 | 2.0 | 1.5 | 1 | 1.2 | 1 | 0.9 | 0.7 | 0.9 | 1 | 1.1 | 0.9 | 0.7 | | | |
| Moles of label/ Mole of fragment $\times 10^3$ | | 0.6 | 6.0 | | 4.8 | | 1.0 | 5.0 | 8.2 | | 0.6 | 4.0 | 7.7 | | 0.5 | 0.7 |

[a] The composition of fragments given under columns headed IgG(2) is based on the amino acid sequences of these fragments and is expressed in whole numbers (Birshtein *et al.*, 1971b; Turner and Cebra, 1971; D. Tracey and T. Trischmann, unpublished data). [b] The C-1-b/C-1-c was isolated as a pair of fragments joined by a disulfide bond from Ab2. The fragments C-1-b and C-1-c were obtained separately from Ab1 and their compositions and activities are summed. [c] Fragment C-1-c was not isolated from Ab3 and C-5 was not isolated from Ab2.

TABLE III: Amino Acid Compositions and Affinity Labeling of Variable Region CNBr Fragments of $\gamma_2$ Chain.[a]

| | C-1-n | | | | C-1-$a_1$ | | | | C-1-$a_2$ | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | IgG2 | Ab1 | Ab2 | Ab3 | IgG2 | Ab1 | Ab2 | Ab3 | IgG2 | Ab1 | Ab2 | Ab3 |
| Lys | 0.7 | 0.5 | 0.8 | 0.5 | 3.1 | 2.9 | 2.3 | 2.9 | 2.1 | 1.3 | 0.9 | 1.0 |
| His | | | | | | 0.2 | 0.1 | 0.1 | 0.6 | 0.3 | 0.4 | 0.4 |
| Arg | 1.0 | 1.0 | 1.4 | 1.0 | 3.0 | 2.6 | 2.3 | 3.4 | 3.0 | 2.6 | 3.1 | 2.8 |
| CMCys | 0.7 | 0.9 | 0.5 | 0.5 | | | | | 1.5 | 1.8 | 1.1 | 1.3 |
| Asp | 1.4 | 0.9 | 0.6 | 0.4 | 4.9 | 4.7 | 5.0 | 4.3 | 4.6 | 4.3 | 3.7 | 3.4 |
| Thr | 1.6 | 1.4 | 1.6 | 1.0 | 3.6 | 3.9 | 4.0 | 3.6 | 5.0 | 4.9 | 5.1 | 6.0 |
| Ser | 4.3 | 5.0 | 3.9 | 5.0 | 4.4 | 3.6 | 4.4 | 3.6 | 5.9 | 6.3 | 6.4 | 6.8 |
| Glu | 4.2 | 4.5 | 4.2 | 4.3 | 3.4 | 3.2 | 2.9 | 3.5 | 2.7 | 2.5 | 2.8 | 2.0 |
| Pro | 1.4 | 1.5 | 1.7 | 0.9 | 2.0 | 1.8 | 1.9 | 0.8 | 3.5 | 3.9 | 3.2 | 2.8 |
| Gly | 5.1 | 4.7 | 4.8 | 5.2 | 5.5 | 6.1 | 6.9 | 7.4 | 4.2 | 4.2 | 4.7 | 4.8 |
| Ala | 1.8 | 2.1 | 1.9 | 2.3 | 2.9 | 3.1 | 3.7 | 3.4 | 5.3 | 5.9 | 5.8 | 6.4 |
| Val | 3.2 | 3.1 | 2.9 | 3.7 | 2.9 | 2.5 | 3.6 | 2.3 | 4.7 | 5.2 | 5.2 | 5.2 |
| Ile | | | | | 3.0 | 3.1 | 2.9 | 3.3 | 1.6 | 1.3 | 0.9 | 0.7 |
| Leu | 3.9 | 3.8 | 4.1 | 3.7 | 3.6 | 3.5 | 3.7 | 3.7 | 3.5 | 3.4 | 3.7 | 3.7 |
| Tyr | 1.3 | 1.6 | 1.9 | 1.7 | 2.5 | 2.2 | 2.1 | 2.3 | 2.9 | 3.1 | 2.9 | 3.6 |
| Phe | 1.8 | 1.9 | 2.0 | 1.8 | 1.6 | 1.3 | 1.6 | 1.3 | 1.9 | 1.9 | 1.9 | 2.0 |
| Hsr | 1.0 | 0.9 | 1.1 | 0.9 | 1.3 | 1.2 | 1.0 | 1.1 | 0.9 | 0.9 | 1.1 | 0.8 |
| Moles of label/Mole of fragment $\times 10^3$ | | 9.7 | 60.0 | 18.3 | | 2.9 | 26.0 | 34.2 | | 25.7 | 69.0 | 37.5 |

[a] The compositions of the fragments listed under IgG2 represent the average fragment compositions from five preparations from normal IgG2. The compositions of the fragments were 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 residues and one tryptophan residue).

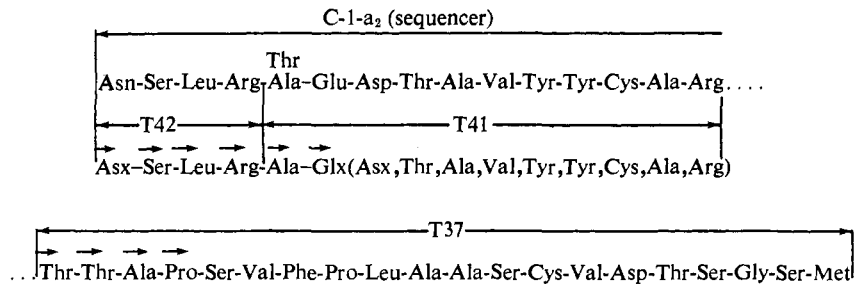


FIGURE 10: The partial amino acid sequence of C-1-$a_2$ from IgG(2) containing anti-Dnp antibody (Abl). The first 15 residues were determined using an automated sequencer and both gas chromatography and regeneration of amino acids to identify the residues (Birshtein and Cebra, 1971). The sequence of T37 is from Birshtein *et al.* (1971b) although the N-terminal four residues were confirmed using T37 from Abl. The → signifies a residue identified after one cycle of manual Edman degradation.

Determination of the first four amino acid residues to be Thr-Thr-Ala-Pro confirmed the placing of T37. Thus C-1-$a_2$ must immediately precede C-1-b in order toward the N terminus of $\gamma_2$ chain.

The other two tryptic peptides, T42 and T41, were identified as the N-terminal tryptic peptides of C-1-$a_2$ by using the automated amino acid sequencer to determine the first 15 residues of C-1-$a_2$, as shown in Figure 10. These data indicate that $\gamma_2$ chain must have a relatively constant primary structure throughout the 35 residue positions covered by the three tryptic peptides since purified antibody was not used in this analysis.

## Discussion

The schematic shown in Figure 9 does indicate the relative modification of a set of CNBr fragments which can account for the entirety of $\gamma_2$ chain (Benjamin *et al.*, 1972). However, one might question how well Figure 9 portrays the distribution of affinity label in the total population of anti-Dnp antibody. Indeed Figures 3, 5, and 8 clearly indicate that fractions of $\gamma_2$ chain or antibody not used directly in the scheme presented to isolate C-1-n, C-1-$a_1$, and C-1-$a_2$ also contained appreciable affinity label. Much of this bound affinity label could also be recovered in the form of these same three fragments comprising the N-terminal ~140 residues of $\gamma_2$ chain and had about the same specific labeling as shown for the fragments isolated directly and portrayed in Figure 9. For instance, the affinity label in pool CA3 (Figure 3) could be separated from C-3 and C-1-c–C-1-b (Figure 5) and then part of it could be released from the "C-1-a fraction" by reductive cleavage of disulfide bonds and be recovered in the C-1-n fragment (D. Benjamin, unpublished). Both the remaining labeled material and that revealed in Figure 8, pool II, could occur on fragments from the N-terminal 140 residues formed from $\gamma_2$ chains lacking a methionine residue at either positions 34 or 83. So far, evidence for $\gamma_2$ chains having alternative amino acids to methionines at these positions is lacking. Pools CA1 and CA2 (Figure 3) each gave rise to labeled C-1-n and C-1-$a_2$ upon reduction of all disulfide bonds. Thus, the distribution of modified residues shown schematically for Ab1, Ab2, and Ab3 in Figure 9 is likely to be a fair representation for the entire population of antibodies in each of these preparations.

A future report will more thoroughly document the residue positions in the $\gamma_2$ chain of antibody which are specifically modified by affinity label. However, Figures 2C and D show spectra of modified C-1-n and C-1-$a_2$ which are consistent with both bearing azotyrosine. Figure 11 presents a summary of the primary structure of the N-terminal 140 residues of normal $\gamma_2$ chain, as far as it is known (Birshtein and Cebra, 1971; D. Benjamin, unpublished). The fragment C-1-n contains one tyrosine at position N-33 and, when it derives from anti-Dnp antibody, another full tyrosine residue at N-32 (see Table III). Thus it is likely that the affinity-labeled residue is at N-32 and/or N-33. These positions are within the short variable segment from N-27 to N-35 at the C terminus of C-1-n and including the first residue position of C-1-$a_1$ (D. Benjamin, unpublished). Although the spectrum of modified C-1-$a_1$ has not been obtained, it appears that at least some of its label is present on the tyrosine at N-60. The affinity-labeled chymotryptic peptide, including residues N-51 to -60, has been isolated from purified antibody, and carboxypeptidase-A was found to remove the label along with C-terminal tyrosine. The tyrosine at N-60 falls just at the end of a short segment of C-1-$a_1$ having a highly variable primary structure in normal IgG (Birshtein and Cebra, 1971). Finally, although the spectrum of modified C-1-$a_2$ shows azotyrosine (Figure 2D), no label could be isolated along with tryptic peptide T41, although it contains tyrosine residues at N-94 and N-95 (see Figures 10 and 11). Furthermore, sequential Edman degradation of C-1-$a_2$ for 15 steps failed to remove the residue with affinity label. Thus it seems that the modified residue(s) may be in the segment from N-99 to -119, which has a highly variable primary structure in normal C-1-$a_2$. These placements of modified residues, together with the complementary identification of residue positions in normal $\gamma_2$ chain which have alternative amino acids, suggest the value of both types of markers, "variability" and affinity label, in locating sections of the molecule involved in antigen binding specificity.

The pattern of affinity labeling shown in Figure 9, in which C-1-n, C-1-$a_1$, and C-1-$a_2$ each bear at least one modified residue, may reflect a mixture of primary structures in the population of purified antibodies and/or alternative residue positions at which the MNBDF, first bound in the site, has a significant probability of reacting. Since Good *et al.* (1967) showed that the diazonium salt MNBDF was generally effective for specifically modifying antigen binding sites of anti-Dnp antibodies raised in mice, guinea pigs, and rabbits, this reagent has been used in detailed structural studies of both antibodies and Dnp-binding myeloma proteins. Franék (1971) has summarized the available data indicating that MNBDF can be used to label up to 40% of the antigen binding sites before nonspecific modification becomes excessive. His own preparations of pig anti-dinitrophenyl antibodies were substituted with MNBDF to 31 mole %—or at a maximum of 16% of the binding sites (Franék, 1971). About one-third

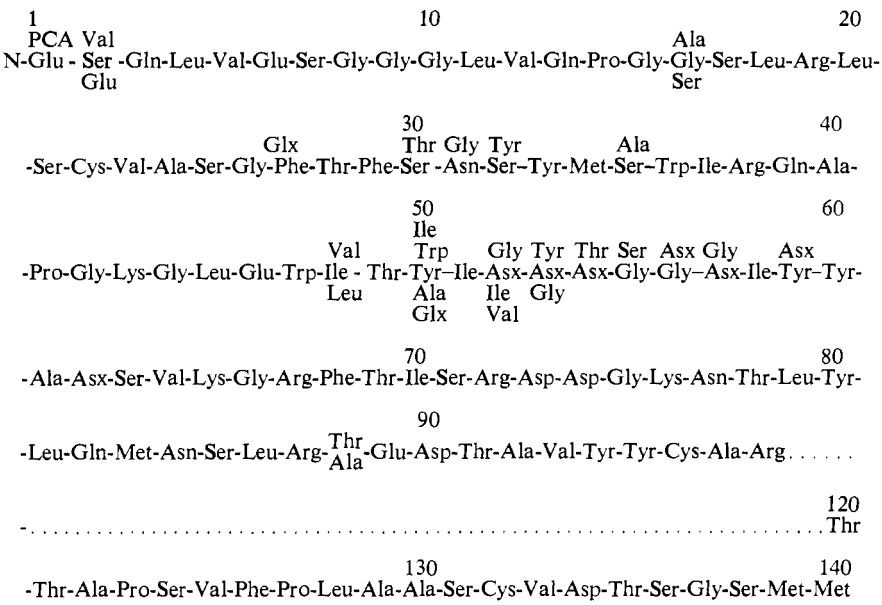


FIGURE 11: The primary structure of the N-terminal 140 residues of $\gamma_2$ chain from normal guinea pig IgG2, excepting residues N-99 to -119. The data not included in this paper are from Birshtein and Cebra (1971) and D. Benjamin (unpublished data). The dotted line signifies that the segment from N-99 to -119 has a variable primary structure.

of the label was associated with the λ chains of the pig antibody, and about 90% of this was distributed between the tyrosines at positions N-33 and N-93 in a ratio of 2:1, respectively. In contrast to the distribution of azotyrosine within pig antibodies, Goetzl and Metzger (1970) found that MNBDF modified only a single tyrosine on the λ chain of the mouse myeloma protein MOPC 315, which binds dinitrophenyl ligands. The azotyrosine residue, isolated in a 33-residue tryptic peptide, was assigned position N-34 by comparison of its primary structure to other homologous sequences found in mouse light chains. However, the labeling of an immunoglobulin at several residue positions is not necessarily diagnostic of the heterogeneity of the antibody preparation. Givol *et al.* (1971), using an homologous series of bromoacetyl derivatives of Dnp ligands to modify the same MOPC-315 myeloma portein, found that some of the reagents, such as bromoacetyl-Dnp-ornithine, reacted with residues on both the heavy and light chains. The modified residue of the light chain was designated as the tyrosine at N-34 (Givol *et al.*, 1971), the same residue labeled by MNBDF (Goetzl and Metzger, 1970).

The only data available concerning the placement of affinity label in the primary structure of heavy chain comes from an analysis of rabbit antibodies raised to the Anp group and reacted with Anp-lysine after its photoactivation to the nitrene derivative (Fleet *et al.*, 1969). The ratio of label in heavy to light chain was about 3.5:1 and 90% of the modified residues of heavy chain were in its N-terminal half (Press *et al.*, 1971). Although the label appeared to be associated with various peptides, about 40% of that on the heavy chain could be accounted for associated with the modified residues at N-97 and N-98. Thus, though it is not unreasonable to suppose some variability in primary structure among antibodies reactive with a given hapten, it seems equally probable that those residue positions which are specifically modified depend also on the chemical structure of the affinity label. The residue positions found labeled in our guinea pig anti-dinitrophenyl antibodies, N-32–33 and N-60 and within the section N-99 to -119, and those found to be modified in pig anti-dinitrophenyl antibodies (Franék, 1971), mouse myeloma protein MOPC-315 (Goetzl and Metzger, 1970; Givol *et al.*, 1971), and rabbit anti-Anp antibodies (Press *et al.*, 1971) all fall within or just without those segments of heavy and light chain known to have a variable primary structure in normal Igs or a "hypervariable" sequence in subgroups of myeloma proteins. It is likely that these segments, marked by variability in primary structure and affinity label, occur rather close to each other in the antibody molecule. For instance, Givol *et al.* (1971), using a bifunctional affinity label, were able to specifically cross-link H and L chain of MOPC-315, probably through reaction of the reagent with N-54 and N-34, respectively. In the guinea pig heavy chain, C-1-n and C-1-$a_2$ are joined by a disulfide bond (Figure 6) formed by the half-cystines at N-22 and N-96 (Figure 11). Each of these half-cystines precedes a segment of normal $\gamma_2$ chain with a variable primary structure, which must be close to each other in the native antibody.

If affinity-labeled positions so far found mark sections involved in determining antigen binding specificity, and these are indeed also marked by their variability in primary structure in normal molecules (see Figure 11), then one should expect to find that these sections have a simpler primary structure in specific antibodies and that their sequences will be correlated with antigen reactivity. We have found that the primary structure of C-1-n and C-1-$a_1$ from guinea pig anti-Dnp antibodies has a greatly restricted variability and that their distinctive sequences occur repeatedly in different preparations of purified antibody (Cebra *et al.*, 1971). Examination of a series of antibodies of different specificities, after their reaction with specific affinity label, is now under way.

## Acknowledgment

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# Effects of Hydrogen Bonding and Solvents upon the Tryptophanyl $^1L_a$ Absorption Band. Studies Using 2,3-Dimethylindole†

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ABSTRACT: To gain information about the spectral properties of tryptophanyl side chains buried within proteins, the absorption spectra of 2,3-dimethylindole were examined in a variety of solvent systems. Comparing the spectra of 2,3-dimethylindole and 3-methylindole indicates that methylation at the 2 position causes the 0–0 $^1L_a$ band to red shift away from the 0–0 $^1L_b$ band even in the nonpolar solvent methylcyclohexane. Thus 2,3-dimethylindole can be used to measure the red shift of the $^1L_a$ band due to hydrogen bonding the indolyl >NH group in a nonpolar solvent. At low concentrations of hydrogen acceptor molecules, the $^1L_a$ red shifts are: 3–5 nm for 1-butanol or ethyl acetate, 6–8 nm for *N*,*N*-dimethylacetamide, and 7–9 nm for 1,2-dimethylimidazole. Hydrogen bonding either indole or 2,3-dimethylindole causes only a 0.5–1.5-nm red shift of the 0–0 $^1L_b$ band. With 1-methylindole, which cannot form a hydrogen bond, no significant spectral shifts occur at low acceptor concentrations. Altering the bulk solvent (water and perfluorocarbons), however, shifts the absorption bands of both >$NCH_3$ derivatives (1-methylindole, 1,2-dimethylindole) and >NH derivatives (indole, 3-methylindole, 2,3-dimethylindole). Interestingly, water shifts the $^1L_a$ band to longer wavelengths and the $^1L_b$ band to shorter wavelengths, whereas perfluorohexane shifts both bands to shorter wavelengths relative to their positions in methylcyclohexane. The results from studying model compounds suggest that in proteins the $^1L_a$ band may be red shifted by about 3–10 nm due to hydrogen bonding the indolyl >NH to other protein moieties. The largest red shifts are expected for the following hydrogen acceptors: carboxylate ions, —N̈= of histidyl side chain, and the carbonyl oxygens of the peptide backbone and of side-chain amides. Even when an indolyl ring is not hydrogen bonded, the wavelength of the $^1L_a$ band may be shifted due to the local polarizability and due to nearby polar groups of either the protein or the solvent.

The near-ultraviolet absorption spectrum of tryptophan consists of two overlapping electronic transitions (the $^1L_a$ and $^1L_b$ bands). Both of these $\pi$–$\pi^*$ bands are polarized in the plane of the indolyl ring with their transition directions oriented nearly perpendicular to each other (Bernardin, 1970; Konev, 1967). The $^1L_b$ transition has well-resolved vibronic

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