# The Affinity-Labeled Residues in Antibody Active Sites. II. Nearest-Neighbor Analyses*

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ABSTRACT: By the method of affinity labeling, tyrosine residues in the active sites of a number of different antihapten IgG antibodies have been specifically modified. These residues occur on both heavy and light chains of the antibodies. In order to characterize these residues, small peptide fragments bearing the label have been prepared by Nagarse digestion, and have been isolated in a pure state in high yields, from heavy and light chains of rabbit and mouse anti-2,4-dinitrophenyl antibodies that were affinity labeled with [$^3$H]*m*-nitrobenzenediazonium fluoroborate. The isolated labeled peptides were shown to be dipeptides with *m*-nitrobenzeneazotyrosine as the carboxyl-terminal residue, and with a different characteristic amino acid as the predominant amino-terminal residue from each of three of the chain preparations. In the case of the light chains from the mouse antibodies, the predominant labeled dipeptide was aspartyl- (or asparginyl-) *m*-nitrobenzeneazotyrosine. By comparison with published sequences of mouse $\kappa$-type Bence–Jones proteins, this result establishes that the labeled tyrosine residue is not in the invariant half of the light chains. This proves that the variable half of the light chain participates in the antibody active site. The results are consistent with the suggestion that the labeled tyrosine residues on heavy and light chains are homologous; that is, that they occur at comparable positions in the amino acid sequences of both types of chains. This suggestion leads to the postulate that a light chain and the Fd piece of a heavy chain are related to one another by a dyad axis of pseudosymmetry in each half of an intact immunoglobin G molecule.

In our earlier studies of affinity labeling of the active sites of antibody (Ab)[1] molecules (Wofsy *et al.*, 1962; Singer and Doolittle, 1966; Good *et al.*, 1968), with rabbit IgG Ab specific to three different benzenoid haptens, and with anti-DNP Ab from four different mammalian species, it was found that tyrosine residues on both H and L chains were labeled by the specific diazonium reagents used. We concluded that these tyrosine residues were unique residues within H and L chains, but were common to all Ab sites studied. In order to test these conclusions, given the chemical heterogeneity of the affinity-labeled tryptic peptides of H and L chains (Doolittle and Singer, 1965), it was thought best to establish which amino acid residues were situated immediately adjacent to the labeled tyrosine residues. We therefore embarked several years ago on a program to prepare, isolate, and analyze small peptide fragments bearing the label from the H and L chains of affinity-labeled Ab.

In the experiments reported in this paper, anti-DNP Ab were raised in rabbits and in mice, and were affinity labeled with the tritiated diazonium reagent [$^3$H]*m*-nitrobenzenediazonium fluorobate (Good *et al.*, 1967; Traylor and Singer, 1967). Digestion of the labeled chains with the enzyme Nagarse released a large fraction of the label as dipeptides which were isolated in high yield and were analyzed. Most of the development and validation of the procedures used were carried out with the more plentiful rabbit Ab, and then applied to the more scarce mouse Ab. The great interest in the results with mouse Ab, however, is that some comparative amino acid sequence data are available for homogeneous mouse Bence–Jones proteins (Gray *et al.*, 1967). The results of our nearest-neighbor analyses with the labeled L chains of mouse anti-DNP Ab, combined with the Bence–Jones data, definitely establish that the affinity-labeled tyrosine residue is within the variable half of the L chains. The precise localization of the labeled tyrosine residue within the variable segment is considered, and three possible positions are indicated. The most likely possibility is Tyr-86. This provisional assignment has led us to propose a detailed model of the structure of IgG molecules in which the L chain and the Fd portion of the H chain in an Fab fragment are structurally related by a pseudodyad axis of symmetry. A preliminary account of these studies has been published (Singer and Thorpe, 1968).

## Materials and Methods

*Antibodies.* Rabbit anti-DNP Ab were prepared, pooled, isolated, affinity labeled with [$^3$H]*m*-nitrobenzenediazonium fluorobate, and separated into labeled H- and L-chain fractions, as in an earlier study (Good *et al.*, 1967). The "X" fraction, which appears to consist largely of aggregated H chains, was used only in exploratory studies, and not for any of the data reported in this paper.

* From the Department of Biology, University of California at San Diego, La Jolla, California 92037. *Received July 11, 1969.* This work was supported by Grants AI-06659 and GM-12027 from the National Institutes of Health.

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[1] Abbreviations used are: Ab, antibodies; H, heavy; L, light; PBS, phosphate-buffered saline, 0.01 M sodium phosphate–0.15 M NaCl, pH 7.4.

Mouse anti-DNP Ab were raised in Swiss–Webster mice[2] against the antigen DNP-keyhole limpet hemocyanin. The mice were given four intraperitoneal injections at weekly intervals of 0.05 mg of antigen in complete Freund's adjuvant. With the fourth injection, about $10^6$ Ehrlich ascites tumor cells were implanted intraperitoneally. One week later the ascites fluid was tapped and heparinized to prevent clotting. From 3 to 10 ml of supernatant was recovered from the cells in each fluid, and contained an average of 1–2 mg of anti-DNP Ab/ml, as measured by precipitation with DNP-bovine serum albumin. Since the Ab titers were quite uniform, the fluids were pooled before centrifugation. The supernatant was then brought to 40% saturation in $(NH_4)_2SO_4$, and the γ-globulin precipitate was washed twice in 40% saturated $(NH_4)_2SO_4$, and then stored until needed.

The mouse anti-DNP Ab were isolated by a modification of a method used by Eisen and Siskind (1964). To a solution of the γ-globulin fraction containing the Ab was added an equivalent amount of DNP-bovine serum albumin in PBS buffer. The mixture was held at 37° for 2 hr and cooled to 0°, and the precipitate formed was centrifuged and washed three times with cold 0.15 M NaCl. The precipitate was then dissolved at 37° in 0.1 M 2,4-dinitrophenol in the PBS buffer to a concentration of about 10 mg of Ab/ml. A small amount of residual precipitate was removed by centrifugation. The resultant solution was then passed through two jacketed columns successively. The first contained DEAE-cellulose equilibrated with 0.1 M 2,4-dinitrophenol in the PBS buffer, and was used to remove the DNP-bovine serum albumin. The second contained Dowex 1-X8 ion-exchange resin equilibrated with the PBS buffer, and removed the dinitrophenol including most of that bound to the Ab active sites (Farah *et al.*, 1960). Both columns were maintained at 37°, and were eluted with the PBS buffer at a constant rate of 0.9 ml/min. The anti-DNP Ab was resolved by this procedure into two fractions, A and B (Figure 1). Fraction B eluted in a widely spread band. Both fractions were separately affinity labeled (see below, Table I), but only the labeled fraction A was used in the other studies reported in this paper. The basis for this fractionation is not clear, since preliminary qualitative immunodiffusion and immunoelectrophoretic studies[3] showed the presence of at least the three immunoglobulin classes IgG1, IgG2a, and IgG2b in fraction A Ab.

Mouse anti-DNP Ab were affinity labeled with [$^3$H]*m*-nitrobenzenediazonium fluorobate, the labeled H and L chains were fractionated, and the degree of labeling was measured, according to the procedures previously used with rabbit Ab (Good *et al.*, 1967). Protected Ab refers to an Ab affinity labeled in the presence of *N*-DNP-ε-aminocaproate to protect the active sites.

*Enzymic Digestions of Labeled Chains.* The labeled chains were used as recovered from the G-100 Sephadex fractionation in 1 M propionic acid (Fleischman *et al.*, 1962), without any further chemical treatment. All of the enzymic digestions, with the exception of the peptic, were carried out as follows unless otherwise indicated. Lyophilized labeled chain (10 mg) was suspended in 1.0 ml of 0.2 M $NH_4HCO_3$ (pH ~8). (The medium for peptic digestions was 30% acetic acid.) Enzymes were added to obtain a weight ratio of chain/enzyme of 50, and the digests were incubated at 40° for 20 hr. Under these conditions, both H and L chains of rabbit Ab were mostly solubilized, with the exception of the tryptic digest of H chains. The Nagarse enzyme was obtained from the Enzyme Development Corp., New York, N. Y.

*Sizing Columns.* An estimate of the sizes of labeled peptide fragments was made by gel filtration in the solvent system phenol–acetic acid–water (1:1:1, v/v), using 60 × 1.0 cm columns of Bio-Gels P-2, P-4, and P-10 at room temperature. The columns of P-2 and P-4 were calibrated with the following compounds (molecular weights in parentheses): tyrosine (181), *m*-nitrobenzeneazotyrosine (330), glycylleucyltyrosine (351), (pyrrolidonecarboxylic acid)phenylalanylalanylarginine (488), and bradykinin (1288). The elution volumes for these compounds were quite reproducible and showed a monotonic decrease with increasing molecular weight of the compound. For the sizing experiments, 0.2-ml aliquots of the enzymic digests of the labeled chains were mixed with equal volumes of phenol and glacial acetic acid and the mixtures were applied to the column. Approximately 1.5-ml fractions were collected at 4–5-min intervals. Portions of the fractions were then removed, dried, and dissolved in 0.5 ml of Hyamine and 10 ml of scintillation fluid for radioactive counting.

*High-Voltage Paper Electrophoresis.* To Whatman No. 3MM paper was applied 40 μl of an enzymic digest of labeled chains. Electrophoresis on the Savant plate electrophoresis apparatus was carried out in 2% formic acid–8% acetic acid at pH 2 for 1 hr at 40 V/cm. Paper frames at 1-cm intervals were cut out, placed in scintillation vials, wetted with ~0.5 ml of toluene scintillation fluid, and then counted.

*Paper Chromatography.* Aliquots (40 μl) of an enzymic digest were spotted on Whatman No. 1 filter paper. The chromatograms were developed with 1-butanol–acetic acid–water (4:1:5). Paper frames at 2-cm intervals were cut out and counted as above.

*Purification of the Labeled Peptides.* The procedure that was finally adopted is described step by step, starting with the isolated labeled H and L chains. The two chain preparations were handled in the same way throughout.

1. NAGARASE DIGESTION. About 500 mg of the lyophilized labeled chains was dissolved or suspended in 0.2 M $NH_4HCO_3$ at a concentration of about 20 mg/ml. Nagarse enzyme was added to give a weight ratio of chain/enzyme of 20. The mixture was held at 37–40° for 16–20 hr. After this time, less than 1% of the radioactivity of the L chains and about 5–10% that of the H chains remained insoluble. The precipitates were removed by centrifugation and discarded.

2. BIO-GEL FILTRATION OF WHOLE NAGARSE DIGESTS. For preparative purposes, a Bio-Gel P-2 column of 95 × 2.5 cm, equilibrated with 0.05 M $NH_4HCO_3$, was employed to fractionate the Nagarse digests. The flow rate at room temperature was 40–50 ml/hr. This procedure was useful in separating the labeled dipeptide fraction from free *m*-nitrobenzeneazotyrosine, and because the labeled peptides were retarded in this solvent, from much of the unlabeled peptides and free amino acids. Up to 50 ml of Nagarse digest originating from 1200 mg of H chain could be applied to this column with good resolution of the labeled dipeptide fraction 4 (see Figures 3 and 4 below). The tubes making up this fraction were pooled

[2] For introducing us to the methods and for the generous donation of the Ehrlich tumor cells, we are greatly indebted to Drs. Leon Wofsy and Benjamin Burr.

[3] Dr. L. Herzenberg kindly performed the immunoelectrophoresis experiments for us.

as indicated in those figures, and the mixture was concentrated by rotary evaporation to a volume of 5–15 ml. The total recovery of radioactivity from the column was about 85% of that applied.

3. BINDING OF LABELED PEPTIDES TO ANTI-DNP AB. The solution of labeled peptides and a solution of rabbit anti-DNP Ab in PBS buffer were successively filtered through a Millipore filter into a suction flask. The amount of Ab used was the molar equivalent of the amount of label in the peptides, as determined by radioactivity measurements. (Ab sites were therefore in excess.) The Ab–peptide mixture was held at room temperature for 10 min, after which an equal volume of saturated $(NH_4)_2SO_4$ was added to it. This precipitated the labeled peptides bound to the Ab.[4] After 1 hr at 0°, the precipitate was centrifuged in the cold and the supernatant was discarded. The precipitate was washed four to five times in the cold with 50% saturated $(NH_4)_2SO_4$, and the washes were discarded.

4. DISSOCIATION OF LABELED PEPTIDES FROM ANTI-DNP AB. The washed Ab–peptide precipitate was dissolved in PBS buffer, and to the solution was added an amount of *N*-DNP-ε-aminocaproic acid (at a concentration of $10^{-2}$ M in 0.05 N NaOH) four times the molarity of the Ab. This compound binds so much more strongly to anti-DNP active sites than the mononitrobenzeneazo-labeled peptides that this small excess is adequate to completely displace the labeled peptides from the Ab sites. This mixture was held at room temperature for 10 min, after which an equal volume of saturated $(NH_4)_2SO_4$ was added. This precipitated the Ab bound to DNP-aminocaproate, and left the labeled peptides in the supernatant. After 1 hr at 0°, the precipitate was centrifuged and the supernatant was recovered.

5. EXTRACTION OF LABELED PEPTIDES INTO BUTANOL. The supernatant was then extracted three times with equal volumes of 1-butanol, into which the labeled peptides were preferentially distributed, along with the DNP-aminocaproate. This left behind in the aqueous phase most of the $(NH_4)_2SO_4$ and most of any remaining free amino acids and unlabeled peptides. The butanol extract was concentrated to ~5 ml by rotary evaporation, and was then carefully brought to dryness under a stream of $N_2$.

The butanol-extracted material at this stage accounted for 65–70% of the radioactivity in fraction 4 from the Bio-Gel filtration (step 2, above). From the ammonium sulfate washes of the antibody which was precipitated after the dissociation reaction (step 4), another 10% of the radioactivity could be extracted with butanol, but this was usually discarded.

6. FILTRATION THROUGH BIO-GEL P-2. The dried peptide material was dissolved in ~1 ml of dilute $NH_4OH$ and applied to a 60 × 1.0 cm column of Bio-Gel P-2 equilibrated and developed with 5% acetic acid. This step removed residual $(NH_4)_2SO_4$ from the labeled peptides and DNP-aminocaproate which were retarded together on the column. The tubes containing the radioactivity were pooled and brought to dryness in a stream of $N_2$. The recovery of radioactivity was generally about 80% of that applied to the column.

[4] If the Ab–peptide mixture was subjected to Sephadex gel filtration, the labeled peptides did not remain bound to the Ab. On the other hand, the labeled peptide remained bound upon precipitation of the Ab with $(NH_4)_2SO_4$.

7. CHROMATOGRAPHY ON PA-35 RESIN. The peptide material was then dissolved in 0.1 M pyridine acetate (pH 3.5) and applied to a 0.9 × 12 cm jacketed column of the sulfonated styrene copolymer (Custom Research Resin Type PA-35) supplied by the Beckman Co. for the Spinco amino acid analyzer. The column was kept at 50°, and was connected to a Beckman Accu-Flo pump which maintained a flow rate of 60 ml/hr. A pyridine acetate pH gradient was employed, using the Büchler gradient device; the mixing chamber contained 259 g of 0.1 M pyridine acetate (pH 3.5) and the reservoir 259 g of 2.0 M pyridine acetate (pH 5.0) as described by Benson *et al.* (1966). Fractions were collected at 3-min intervals and appropriate aliquots were removed for counting in either ethanol–toluene or Hyamine scintillation mixtures. About 90% of the radioactivity applied to the resin was recovered.

This final step in the peptide purification procedure accomplishes a separation of the labeled peptides from DNP-aminocaproate, and partially resolves the peptide mixture from rabbit chains into two fractions (see Figure 5 below).

*Edman Degradation Studies.* Preliminary experiments were carried out to assure the completeness of coupling and cleavage reactions with the labeled peptides. The procedure which was adopted is illustrated by the following example. A sample containing 0.0176 μmole of labeled peptide was coupled with an 84-fold excess of phenyl isothiocyanate in 100 μl of coupling buffer (Blombäck *et al.*, 1966) containing 0.4 M dimethylallylamine (pH 9.2). After incubation at 40° for 1 hr, the sample was kept under vacuum overnight to remove excess reagent and buffer. Cleavage of the phenylthiocarbamyl peptide was then effected in a mixture containing 50 μl of $H_2O$ and 100 μl of HCl-saturated glacial acetic acid at 40° for 2 hr. The sample was then applied without prior extraction to the PA-35 resin column and chromatographically fractionated in the pyridine acetate pH gradient described above. The radioactivity in the fractions was then measured to localize the components containing the label. Authentic samples of [$^3$H]*m*-nitrobenzeneazotyrosine and [$^3$H]*m*-nitroaniline were separately chromatographed on the same column. A second Edman degradation step was carried out in a similar manner on the mixture resulting from the first Edman step.

*Amino Acid Analyses.* These analyses were performed with a Spinco amino acid analyzer Model 120B, modified with the 6.6-cm light-path cuvets. An aliquot of a sample to be analyzed, containing a known amount of radioactivity, was hydrolyzed in 6 N HCl in an evacuated tube at 110° for 20 hr. For all analyses reported in this paper, a sample of the corresponding unhydrolyzed peptide containing the same amount of radioactivity was also analyzed, and the (generally small) amounts of free amino acids present were subtracted from those found in the hydrolyzed sample. Both long and short column runs were performed initially, but as it was found that the more highly purified labeled peptides contained no significant amounts of basic amino acids, only long column analyses were carried out in the final stages.

## Results

*Affinity Labeling of Mouse Anti-DNP Antibodies.* In our earlier studies with rabbit anti-DNP Ab (Good *et al.*, 1967), the specificity of affinity labeling with the reagent [$^3$H]*m*-

TABLE I: The Affinity Labeling of Anti-DNP Antibodies with [$^3$H]*m*-nitrobenzenediazonium Fluoroborate.[a]

| Source | Reaction Condn[b] | Whole Ab | H Chains | L Chains | 2 H + 2 L |
|---|---|---|---|---|---|
| Mouse, fraction A[c] | Unprotected | 0.676 | 0.164 | 0.126 | 0.58 |
| | Protected | 0.137 | 0.036 | 0.014 | 0.10 |
| Mouse, fraction B[c] | Unprotected | 0.579 | 0.177 | 0.097 | 0.55 |
| | Protected | 0.141 | 0.037 | 0.017 | 0.11 |
| Rabbit[d] | Unprotected | 0.66 | 0.23 | 0.113 | 0.69 |
| | Protected | 0.057 | 0.013 | 0.007 | 0.04 |

[a] Data expressed as moles of $^3$H label bound per mole of protein (molecular weight of whole Ab 160,000; of H chain 55,000; of L chain 25,000). [b] Protected samples were affinity labeled in the presence of a molar concentration of *N*-DNP-ε-aminocaproate 13 times that of Ab. [c] See Figure 1 for fraction designations. [d] From Table I, Good *et al.* (1967).

nitrobenzenediazonium fluoroborate was determined by measuring the amount of $^3$H that was covalently bound to the Ab and its H and L chains in two parallel experiments: (a) in the absence (unprotected) or (b) in the presence (protected) of a slight excess of the hapten *N*-DNP-ε-aminocaproate. The function of the latter hapten is solely to protect residues in the Ab active sites from reaction with the diazonium reagent. Exactly analogous experiments were carried out with the mouse anti-DNP Ab fractions A and B (Figure 1), with the results shown in Table I. (For comparison, previously published results with rabbit anti-DNP Ab are also given.) These results supplement the more preliminary data that were previously obtained with mouse anti-DNP Ab (Good *et al.*, 1968). They reveal a high specificity of labeling of the unprotected Ab and both the H and L chains. The average ratio of specific label on the H and L chains is about 1.5. The results are quite similar to those previously obtained with the rabbit Ab (Good *et al.*, 1967), except that the specificity of labeling of the latter was somewhat higher. With the rabbit chains, the $^3$H contents of the protected chains were uniformly less than 10% that of the unprotected; with the mouse chains, this figure varies from about 11% for the fraction A L chains to 22% for the fraction A H chains. These percentage values are measures of the degree of nonspecific modification of the chains, and will be referred to subsequently.

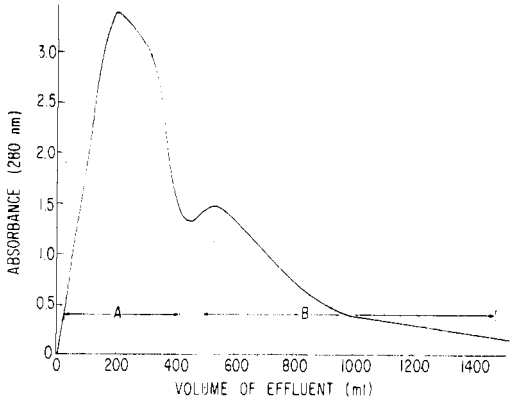


FIGURE 1: The fractionation of mouse anti-DNP Ab during isolation and purification on DEAE-cellulose. See text for details.

That the azo label is predominantly, if not exclusively, in the form of *m*-nitrobenzeneazotyrosine on H and L chains of both rabbit and mouse Ab is demonstrated in further experiments described below (also, see Discussion).

*Enzymic Fragmentation of Labeled Chains.* A number of proteolytic enzymes were investigated for their ability to degrade the labeled rabbit H and L chains mainly into labeled peptide fragments consisting of only two or three amino acids including the labeled residue. Aside from the mild reduction and alkylation required to cleave the H from the L chains, no further chemical modification of the chains was carried out prior to enzymic digestion. The digests

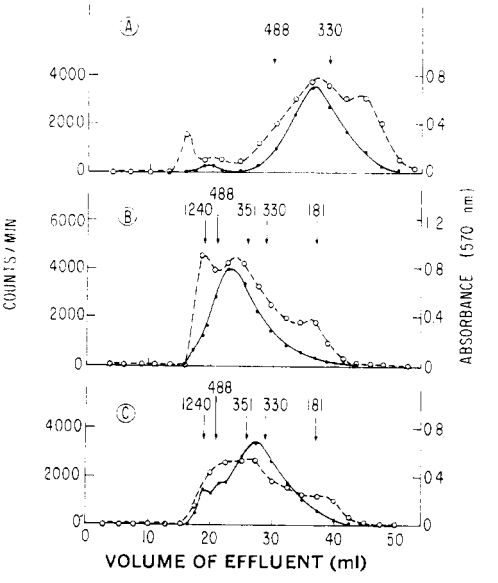


FIGURE 2: Sizing experiments with enzymic digests of affinity-labeled rabbit anti-DNP L chains, in the solvent phenol–acetic acid–water (1:1:1). The dashed curves and open circles correspond to ninhydrin analyses after alkaline hydrolysis of the peptide fractions (absorbance at 570 nm), and the full curves and closed circles measure the $^3$H affinity label in the fractions (counts per minute). The arrows above each figure give the peak positions of a number of compounds (see text) of molecular weights indicated above the arrows which were used to calibrate the sizing columns. (A) Nagarse digest, P-4 Bio Gel column. (B) Nagarse digest, P-2 Bio-Gel column. (C) Pronase digest, P-2 Bio-gel column.

TABLE II: Distribution of Radioactivity in Fractionated Nagarse Digests of Rabbit Chains.

| Fraction[a] | Radioactivity Recovd (%) | |
|---|---|---|
| | H Chains | L Chains |
| 1 | 10 | 3 |
| 2 | 16 | 7 |
| 3 | 16 | 11 |
| 4 | 44 | 53 |
| 5 | 7 | 11 |
| 6 | 7 | 15 |

[a] See Figure 4.

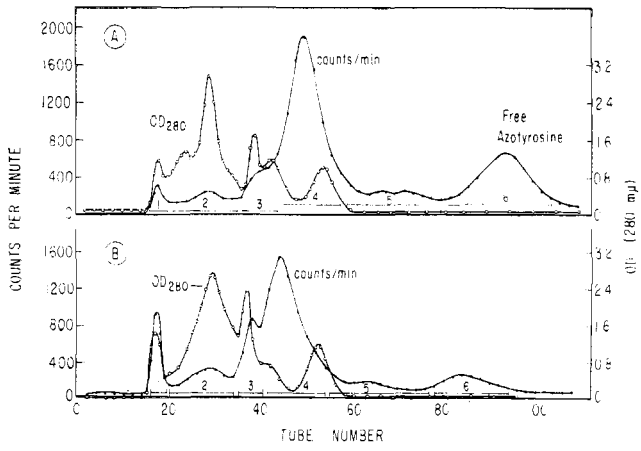


FIGURE 3: Nagarse digests of affinity-labeled rabbit anti-DNP chains fractionated on P-2 Bio-Gel columns in 0.5 M $NH_4HCO_3$. (A) From L chains and (B) from H chains. The tube contents were pooled as indicated (see Table II).

were screened by examination of the radioactivity of effluents from gel filtration on P-2, P-4, and P-10 Bio-Gel columns in phenol–acetic acid–water (1:1:1). This solvent system was used (Carnegie, 1965) to promote disaggregation and prevent undue retardation of hydrophobic peptides on the column. Elution profiles of radioactivity of Nagarse and pronase digests of labeled rabbit L chains are shown in Figure 2. The major fraction of the Nagarse-labeled material was of a size corresponding to a molecular weight of about 400. Since *m*-nitrobenzeneazotyrosine itself has a molecular weight of 330, this would correspond to the size of a dipeptide containing the azotyrosine residue. The average size of the labeled material from the pronase digest was smaller than this and the paper chromatographic and electrophoretic experiments confirmed that the enzyme released the label largely as free *m*-nitrobenzeneazotyrosine. The average size of labeled material released by chymotryptic digestion (not shown) was still larger than that of the Nagarse digest with an average size corresponding to tetrapeptide or larger. The labeled peptides released from H and L chains by trypsin and pepsin treatments were essentially excluded from Bio-Gel P-10, and were therefore certainly larger than ten residues average length. The Nagarse digests therefore appeared most suitable for our purposes, and were accordingly further investigated.

*Resolution and Characterization of the Labeled Material in Nagarse-Digested H and L Chains.* In an attempt to better resolve the labeled components in the Nagarse digests, and to do so on a preparative scale, the digests were gel filtered through a 95 × 2.5 cm column of P-2 Bio-Gel equilibrated with 0.05 M $NH_4HCO_3$. The elution profiles shown in Figure 3 were obtained with the rabbit Ab chains.

With 13 different Nagarse digests of affinity-labeled rabbit L chains carried out under the same conditions, very little deviation was observed from the results shown in Figure 3. Furthermore, variation in the time of Nagarse digestion from 1 to 22 hr did not affect the distribution of label.

The eluted material from the rabbit chain digests was pooled into six fractions as indicated in Figure 3. The total recovery of radioactivity was 86% for the H chains and 84% for the L chains, and the radioactivity was distributed among the six fractions as shown in Table II. The average size of the labeled peptides in these fractions was examined as described in the previous section (Figure 2). In fraction 6 from both H and L chains, the label was the same size as *m*-nitrobenzeneazotyrosine. (The considerable retardation of this component is presumably due to its hydrophobic interaction with the gel in 0.05 M $NH_4HCO_3$.) Fractions 3 and 4 contained labeled material of about the size of dipeptides, while the label in fractions 1 and 2 was for the most part excluded on the P-2 Bio-Gel sizing column. Fraction 4 alone accounted for about 50% of the label from both chains, and was therefore further purified and characterized as indicated in the next section.

Nagarse digests of labeled chains from the mouse fraction A anti-DNP Ab were treated similarly, with results shown in Figure 4. It is clear from Figures 3 and 4 that the radioactivity elution profiles are remarkably similar not only for the H and L chains of a given Ab, but also for the rabbit and mouse species.

*Purification of Labeled Peptide Fractions.* Fractions 4 in Figure 3 contained sufficiently large percentages of the label on the rabbit H and L chains to warrant the attempt to isolate and characterize their labeled peptides. (In the re-

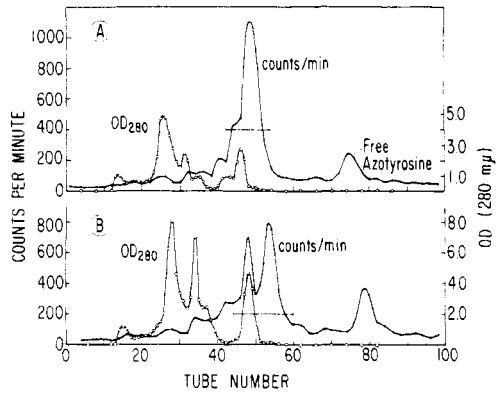


FIGURE 4: Nagarse digest of affinity-labeled mouse anti-DNP chains. (A) From L chains and (B) from H chains; fractionated on P-2 Bio-gel in 0.05 M $NH_4HCO_3$. The bar on each figure indicates the tube contents that were pooled, corresponding to the fractions 4 in Figure 3, for the isolation of the labeled dipeptides.

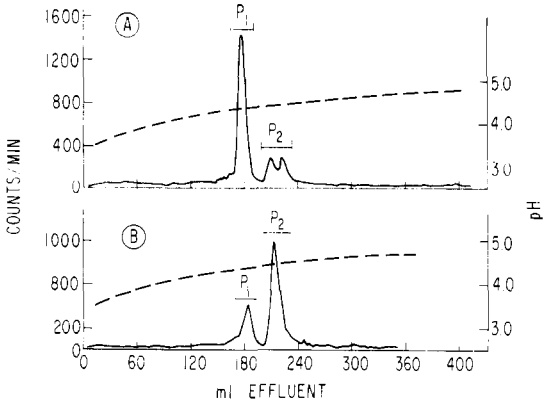


FIGURE 5: Purified labeled peptides from affinity-labeled rabbit anti-DNP chains fractionated on a column of PA-35 resin in a pyridine–acetate pH gradient (A) from H chains and (B) from L chains. The full curves give the elution profiles of the $^3$H affinity label; the dashed curves give the pH gradient. $P_1$ and $P_2$ designate the pooled fractions which were subjected to amino acid analyses (Table III, columns 6–8).

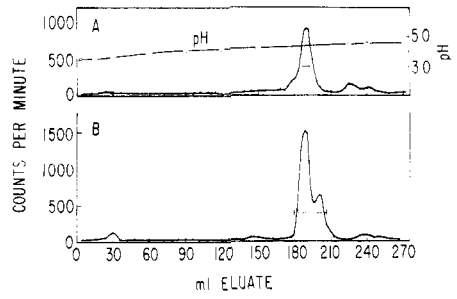


FIGURE 6: Purified labeled peptides from mouse anti-DNP chains, fractionated on the PA-35 resin as in Figure 5. (A) From H chains and (B) from L chains. The fractions indicated by the bars were pooled and subjected to amino acid analyses (Table III, columns 9 and 10).

mainder of this paper, the term "labeled peptides" refers to the labeled material from this fraction, unless otherwise stated.) These fractions, however, consisted mostly of unlabeled small peptides and amino acids. It was considered necessary to isolate the labeled peptides not only in a state of purity adequate for amino acid analyses, but also with a very high recovery. The use of unlabeled anti-DNP Ab to bind the affinity-labeled peptides in the mixture made possible what would otherwise have been a most difficult task. The details of the purification procedure that was devised are given in the section on Methods. The recovery of radioactivity attained at each step in the procedure was quite large. The final step in the procedure is a fractionation on the PA-35 resin in a pyridine acetate pH gradient. This step not only accomplished a separation of residual DNP-aminocaproate from the labeled peptides, but in the case of rabbit H and L chains, resolved the labeled peptides into two fractions, designated $P_1$ and $P_2$ (Figure 5). The ratio of label in $P_1$ and $P_2$ was about 70:30 for the H-chain peptides and 36:64 for the L.[5] These results indicated that chemical differences existed between the major portion of the labeled peptides from the rabbit H and L chains. This was confirmed by the analyses described below.

With the labeled peptides from Nagarse-digested mouse chains, purified by the same procedure used for the rabbit material, a fraction corresponding to $P_1$, but none corresponding to $P_2$, was observed with each chain (Figure 6). The $P_1$ fraction of the L-chain peptides was, however, partially resolved into two peaks.

*Characterization of Purified Labeled Peptides.* Up to this point, we have inferred but have not yet demonstrated directly that the azotyrosine label in the Nagarse fraction 4 was bound in peptide linkage to other amino acid residues. A simple demonstration of this was achieved by subjecting the labeled material to the action of the enzyme leucineaminopeptidase. At a weight ratio of peptide to enzyme of 40:1, the radioactivity of the purified labeled peptides of both the H and L chains of the rabbit Ab was converted almost completely into free *m*-nitrobenzeneazotyrosine, as determined by sizing experiments (as in Figure 2) and by chromatography on the PA-35 resin. These results, and the Edman degradation studies cited below, establish the peptide nature of the labeled material that was isolated.

The purified peptides were also examined spectrophotometrically to determine whether they contained any aromatic residues other than the azotyrosine, particularly tryptophan. In these experiments, the purification procedure was slightly different from that given in the section on Materials and Methods. After step 5, the peptides were passed through a Bio-Rad 1-X2 column and eluted with an ammonium acetate–acetic acid gradient from pH 6.0 to 1.5. The labeled peptides emerged as a single peak at pH 2.9 well resolved from the DNP-aminocaproate peak. After recovery of the labeled peptides, they were passed through a Bio-Gel P-2 column in 5% acetic acid to remove the ammonium acetate. The spectra were then obtained after making these solutions 0.15 N in NaOH. An authentic sample of [$^3$H]*m*-nitrobenzeneazotyrosine was also examined. The results in Figure 7 show that the labeled peptides from both H and L chains, and *m*-nitrobenzeneazotyrosine, have essentially superimposable spectra, and the absorbance in the range 270–290 nm indicates that no more than 0.05 mole of tryptophan could have been present per mole of azotyrosine label in these peptides. This is important because if tryptophan were present, it would have been destroyed by the acid hydrolysis prior to amino analysis of the labeled peptides. Similarly, negligible quantities of other aromatic amino acids were present, according to these spectrophotometric results. This was confirmed by the amino acid analytical data presented below.

A comparison was also made of the concentration of label in the purified labeled peptides as determined independently by spectral analysis in 0.1 N NaOH for azotyrosine and by radioactivity measurements. For the spectral analysis, the extinction coefficient of *N*-chloracetyl-*m*-nitrobenzeneazotyrosine at 490 nm in 0.1 N NaOH, $1.00 \times 10^4$, was used (Traylor and Singer, 1967). The radioactivity measurements were converted into molar concentrations using the specific radioactivity of the parent diazonium compound (Traylor

[5] The resolution of the labeled dipeptides into two major fractions on the PA-35 column is probably a reflection of the relative hydrophobicity of the $NH_2$-terminal amino acid residues in the dipeptides. Neither fraction contained a pure dipeptide (see Tables III and IV below).

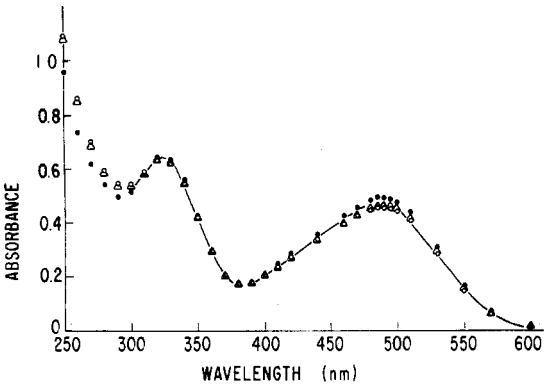


FIGURE 7: Absorption spectra of the purified labeled peptides from affinity-labeled rabbit anti-DNP chains: (○) from L chains; (Δ) from H chains, compared to that (●) for the model compound *m*-nitrobenzeneazotyrosine, all in 0.15 M NaOH.

and Singer, 1967). The results were as follows: for the L-chain peptides, the spectral and radioactivity assays of concentration of label gave 0.082 and 0.075 μmole per ml, respectively, while for the H chain peptides, the corresponding figures were 0.076 and 0.069 μmole per ml. The agreement is excellent, providing another demonstration that azotyrosine is the exclusive azo product in the labeled material.

To obtain information about the sequential arrangement of the azotyrosine residue within the labeled peptides, Edman degradation studies were carried out. The purified labeled peptides, prior to chromatography on the PA-35 resin (*i.e.*, prior to step 7 of the purification procedure), were coupled with phenyl isothiocyanate and were cleaved to the phenylthiohydantoin stage, as indicated in the Methods section. However, in contrast to the usual Edman procedure which involves an extraction of the phenylthiohydantoin at this stage, the entire sample after the cleavage step was applied directly to the PA-35 resin, and the radioactivity of the effluent was monitored. As is shown in Figure 8 for the labeled peptides from rabbit H chains, after one step of the Edman procedure the radioactivity is almost entirely (at least 90%) in the form of free *m*-nitrobenzeneazotyrosine. If the labeled mixture after one Edman degradation was subjected to a second degradation, almost all of the radioactivity now appeared at the effluent volume corresponding to free *m*-nitroaniline.[6] In separate experiments, it was shown that a one-step Edman degradation carried out with authentic free [$^3$H]*m*-nitrobenzeneazotyrosine converted it almost completely into [$^3$H]*m*-nitroaniline.

Entirely similar results were obtained with purified labeled peptides from rabbit L chains and from mouse L and H chains.

The results of the Edman analyses are precisely those to be expected if the purified labeled peptides from the rabbit and mouse H and L chains in each case were exclusively dipeptide with the [$^3$H]*m*-nitrobenzeneazotyrosine residue carboxyl terminal. If any substantial fraction of the labeled peptides contained the *m*-nitrobenzeneazotyrosine residue at the amino terminus, the corresponding amount of free *m*-nitrobenzeneazotyrosine would not have been produced

[6] *m*-Nitroaniline is produced by cleavage of the azotyrosine linkage.

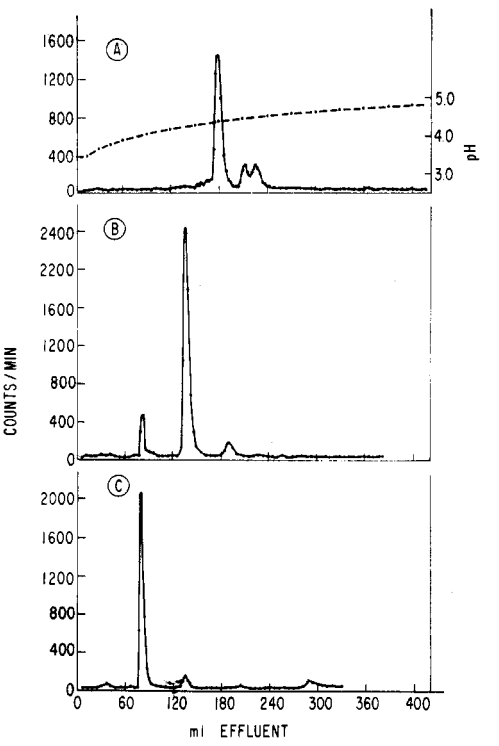


FIGURE 8: Analysis on the PA-35 resin column of Figures 5 and 6 of the products of Edman degradations of purified peptides from the H chains of affinity-labeled rabbit anti-DNP Ab (see text for details). The radioactivity profiles are shown for: (A) the original labeled peptides; (B) after one step of the degradation; and (C) after two steps. The major peak of radioactivity in (B) was eluted at the position corresponding to an authentic sample of [$^3$H]*m*-nitrobenzeneazotyrosine, and in (C) to [$^3$H]*m*-nitroaniline.

after one step of the Edman procedure. Likewise, if any appreciable fraction of the labeled peptides were tripeptides or larger species, one step of the Edman procedure would have released the radioactivity of such peptides as a fraction readily distinguishable from free *m*-nitrobenzeneazotyrosine on the PA-35 resin and in sizing experiments.

Sufficient quantities of labeled peptides from only the rabbit H chain were available to carry out a direct analysis of the phenylthiohydantoin released after one step of the Edman procedure. The $P_1$ fraction eluted from the PA-35 resin (Figure 6) was used. This same fraction upon amino acid analysis (see next section) showed 0.51 mole of threonine present per mole of label. After the coupling reaction and the cleavage as described in the Methods section, the material was applied to the solvent III chromatographic system (Sjöquist, 1960). The phenylthiohydantoin of threonine was the only major spot located; it was eluted from the paper, and its amount was compared spectrally with known standard amounts of the phenylthiohydantoin of threonine applied to the paper and eluted in parallel. By this method, 0.6 mole of $NH_2$-terminal threonine/mole of label was found in the $P_1$ peptide, in good agreement with the total amino acid analysis described below.

*Amino Acid Analyses of Purified Labeled Peptides.* Total amino acid analyses of the labeled peptides from rabbit H and L chains were carried out at a number of stages during the process of refining the purification procedure described

TABLE III: Amino Acid Analyses of Purified Labeled Peptides.[a]

| | Rabbit Chain Samples | | | | | | | Mouse Chain Sample | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | | 2 | | 3 | | | | |
| Amino Acid | H | L | H | L | H(P1) | L(P2) | H(P2) | H | L |
| Lys | 0.002 | 0.001 | 0.0004 | | | | | | |
| His | 0.0005 | 0 | <0.0004 | 0 | | | | | |
| Arg | 0.010 | 0.004 | 0 | 0 | | | | | |
| Asp | 0.003 | 0.003 | 0.002 | 0 | 0.005 | 0.0002 | 0.010 | 0.008 | *0.052* |
| Thr | *0.016* | 0.003 | *0.016* | 0.003 | *0.050* | 0.001 | 0.011 | 0.011 | 0.015 |
| Ser | 0.009 | 0.008 | 0.003 | 0.004 | 0.013 | 0 | 0.015 | 0.019 | 0.011 |
| Glu | 0.004 | 0.003 | 0.002 | 0.002 | 0.002 | 0.005 | 0.012 | 0.005 | 0.006 |
| Pro | 0.010 | 0.003 | 0 | 0 | | | 0.006 | <0.001 | 0.003 |
| Gly | 0.011 | 0.006 | 0.002 | 0.003 | 0.013 | 0.003 | 0.017 | 0.016 | 0.005 |
| Ala | 0.003 | 0.001 | 0.0003 | 0 | 0.005 | 0.002 | 0.011 | 0.005 | 0.002 |
| ½-Cys | 0 | 0 | 0 | 0 | | | | | |
| Val | 0.004 | *0.018* | 0.002 | *0.014* | 0.003 | *0.041* | 0.033 | 0.003 | 0.002 |
| Met | 0 | 0 | 0 | 0 | | | 0.002 | | |
| Ile | 0.004 | 0.001 | 0.001 | 0.001 | 0.003 | 0.001 | 0.022 | 0.003 | 0.001 |
| Leu | 0.002 | 0.002 | 0.0005 | 0.001 | 0.001 | 0.001 | 0.022 | 0.002 | 0.002 |
| Tyr | 0.001 | 0.001 | <0.0005 | Trace | 0.001 | 0.0005 | 0.005 | 0.003 | 0.002 |
| Phe | 0.012 | 0.005 | <0.0005 | Trace | 0.001 | 0.0005 | 0.003 | 0.003 | |
| Total applied | 0.050 | 0.050 | 0.052 | 0.057 | 0.098 | 0.078 | 0.120 | 0.054 | 0.090 |
| Total recovered | 0.091 | 0.060 | 0.030 | 0.028 | 0.097 | 0.055 | 0.169 | 0.078 | 0.101 |

[a] Data given in micromoles.

in the section on Materials and Methods. One criterion used to determine whether the peptides were adequately pure was based on the recovery of free amino acids per mole of labeled peptide that was hydrolyzed and applied to the analyzer column. In separate experiments, it was shown that free *m*-nitrobenzeneazotyrosine, when subjected to the hydrolysis conditions used, yielded no ninhydrin-positive material on amino acid analysis. Since the results described in the previous section demonstrated that the labeled material was essentially all dipeptide containing the azotyrosine residue, a recovery of a total of close to 1 mole of amino acid residues/mole of label was expected if the labeled peptides were pure.

Amino acid analytical data are given in Table III, columns 2 and 3, for one set of labeled peptides recovered from the Ab purification procedure (step 5, section on Methods and Materials) with no further chromatographic purification. In these preparations, the total moles of amino acids recovered exceeded the moles of label applied; however, the excess was not great. These data are shown for several reasons. In the first place, they demonstrate the remarkable efficiency of the Ab purification step. We estimate that the labeled peptides were purified in this step by a factor of at least 100 with no loss of the label other than mechanical. Secondly, even at this stage of purification, it is clear that threonine was the most prominent amino acid occurring in the H chain labeled peptides, and valine in the L chain ones. Thirdly, these analyses were atypical in that significant amounts of the basic amino acids lysine and arginine were present, as well as proline and phenylalanine. In other analyses carried out at this stage, and as the labeled peptides were further purified, these amino acids were absent. The results in Table III, columns 4 and 5, for example, were obtained with purified labeled peptides from H and L chains which had undergone, in addition to the Ab purification step, chromatography on Bio-Rad AG 1-X2. Again, threonine and valine predominate for the H and L peptides, respectively. Lysine, arginine, proline, and phenylalanine are absent to within the experimental uncertainties. (The reason for the low recovery of amino acids in the example shown is not known.)

Subsequent to these analyses, it was found that a last step of chromatography of the purified peptides on the PA-35 resin improved the procedure, and also resolved the labeled peptides from rabbit chains into the two fractions $P_1$ and $P_2$ (Figure 5). Accordingly, the $P_1$ (major) and $P_2$ (minor) fractions from the H-chain peptides, and the $P_2$ (major) fraction from the L-chain peptides, were obtained in sufficient quantities for reasonably accurate amino acid analyses. The $P_1$ fraction from the L-chain peptides was isolated in amounts adequate only for semiquantitative analysis. Only long-column runs were carried out, since the previous data had shown the absence of basic amino acids. The analytical results are shown in Table III, columns 6–8. These data show that: (a) threonine was the major amino acid present in the $P_1$ fraction of the H chain peptides, accounting for about 50% of the label applied; (b) valine was the major amino acid present in the $P_2$ fraction of the L peptides, accounting for over 50% of the label applied; (c) the amount of the major amino acid exceeds by at least a factor of four for the H-chain and ten for the L-chain peptides the next most prominent amino acid: and (d) the $P_2$ fraction of the H-chain peptides

was more hetergeneous than the $P_1$ fraction: valine accounted for about 25% of the label applied, and the hydrophobic amino acids valine, leucine, and isoleucine together for about 65%.

After these experiments had been carried out, and the techniques involved had been refined with the rabbit chain peptides, a single amino acid analytical experiment was performed with the corresponding material from the mouse fraction A Ab. The fractions demarcated in Figure 4 of Nagarse digests of labeled mouse H and L chains were taken through the entire Ab purification procedure given in the section on Materials and Methods. The fractions shown in Figure 6, obtained from chromatography on the PA-35 column, were analyzed for amino acid content with the results shown in Table III, columns 9 and 10. These data indicate that: (a) the labeled peptides from mouse L chains contained predominantly aspartic acid or asparagine, accounting for over 50% of the label applied; the next most prominent amino acid, threonine, was present in only one-quarter that amount; and (b) in the mouse H-chain peptides a number of amino acids, in the decreasing order serine, glycine, threonine, aspartic acid or asparagine, and glutamic acid or glutamine, were present in significant amounts. This latter result may be due to the presence of multiple classes or subclasses of H chains in the preparation, as is discussed below.

## Discussion

At an earlier stage in this series of investigations, when quantitative studies of the affinity labeling of three different rabbit antihapten antibodies had been carried out, followed by a partial characterization of the labeled tryptic peptides from one of these antibodies, Singer and Doolittle (1966) drew a number of conclusions and inferences about antibody and immunoglobulin structure from the data. First of all, it was concluded that the specific labeling reaction occurred with residues in the active sites of the antibodies. Secondly, from the fact that both H and L chains of all three antibodies were affinity labeled, it was concluded that both chains contribute amino acid residues to each active site of an antibody molecule. Furthermore, the similar manner in which H and L chains were affinity labeled at tyrosine residues, and the gross similarities in the labeled tryptic peptides from both chains, led us to infer that H and L chains might be chemically homologous in their active site regions. This in turn suggested that H and L chains might have had a common evolutionary ancestry, and some fragmentary amino acid sequence results reported by others were adduced to support this conclusion. This evolutionary relationship was firmly and independently established by the detailed sequence studies of Hill and coworkers (1966).

Thirdly, from the remarkable constancy of the ratio of affinity label on the H and L chains from different antibodies, it was inferred that a single and characteristic tyrosine residue on different L chains, and a single and characteristic tyrosine residue on different H chains of a given class, were being affinity labeled in the different antibodies. On the other hand, the gross heterogeneity of the labeled tryptic peptides from either the H or L chains (Doolittle and Singer, 1965), in conjunction with the structure studies of Hilschmann and Craig (1965), suggested that these characteristic tyrosine residues were within those segments of their respective chains which varied in sequence from one molecule to another in the population. The experiments reported in this paper strongly support and extend all of these conclusions and inferences.

*Representative Nature of the Labeled Dipeptides.* In order to meet the objectives of these experiments, we required first that most of the label of both chains be converted into small peptide fragments, and second, that the labeled fragments be recovered in high yield. In this way, any labeled peptides that were ultimately characterized would be clearly representative of the whole chain population.

The first criterion was satisfied by Nagarse digestion. About 50% of the label from H and L chains of both rabbit and mouse Ab was reproducibly obtained as the dipeptide fraction (Figures 3 and 4) which was subsequently purified and characterized. Relatively little label appeared as free *m*-nitrobenzeneazotyrosine. This is important because the release of free *m*-nitrobenzeneazotyrosine might indicate the presence of chains in which the labeled tyrosine residue had a different amino-terminal neighboring residue than the ones found in the dipeptide fraction. As it is, the small amount of free *m*-nitrobenzeneazotyrosine may well have arisen by the slow digestion of the main dipeptide fraction. Nagarse fractions 1 and 2 (Figures 3 and 4), which eluted more rapidly than the main dipeptide fraction in 0.05 M $NH_4HCO_3$, may have contained some aggregated dipeptide, since parts of these fractions were retarded on P-2 Bio-Gel in the dissociating solvent phenol–acetic acid–water. Therefore, these considerations suggest that the Nagarse-labeled dipeptide fractions that were purified and analyzed represented a substantial fraction of the label of the original labeled chains.

The second criterion, that the labeled peptides were to be recovered in high yield, was efficiently achieved by the use of anti-DNP Ab to sequester the *m*-nitrobenzeazo-labeled peptides. The losses incurred at each step in the purification procedure were small and were not likely to produce any fractionation of the labeled peptides.

*The Properties of the Labeled Dipeptides.* The Edman degradation studies show that for rabbit and mouse H and L chains, essentially all of the purified labeled peptide was dipeptide in nature, with the azotyrosine residues carboxyl terminal (Figure 8). On the other hand, the amino acid analyses (Table III, columns 6–10) indicate that each purified labeled peptide contained significant amounts of several amino acids, their sum being more or less equal to the amount of label. It follows that each fraction was a mixture of labeled dipeptides, containing as their amino-terminal residues those amino acids that were detected by analysis of the hydrolysate. The major and significant minor labeled dipeptides found with each chain preparation are collected in Table IV. For the rabbit H and L chains, and for the mouse L chains, a different single dipeptide predominated. For the mouse H chains, several dipeptides were present in appreciable amounts. The presence of several labeled dipeptides from each chain may have arisen from any of several sources, among which are the following: (1) some nonspecific modification of tyrosine residues not in the active sites certainly occurred during the affinity-labeling reaction. From the data of Table I, somewhat less than 10% of the label on the rabbit chains, and from 10 to 20% of the label on the mouse chains, was nonspecific. (2) Several classes and subclasses of L and H

TABLE IV: Major Labeled Dipeptides from Affinity-Labeled Chains.[a]

| Rabbit[b] | | Mouse | |
|---|---|---|---|
| H | L | H | L |
| -Thr-Tyr- (50) | -Val-Tyr- (75) | -Ser-Tyr- (24) | -Asp-Tyr- (52) |
| -Ser-Tyr- (13) | | -Gly-Tyr- (20) | -Thr-Tyr- (15) |
| -Gly-Tyr- (13) | | -Thr-Tyr- (14) | -Ser-Tyr- (11) |
| | | -Asp-Tyr- (10) | |

[a] Numbers in parentheses are calculated from the per cent of amino acid recovered in the purified samples analyzed in Table III. [b] In the P1 fraction (see Table III).

chains, each with its own characteristic tyrosine residue to contribute to an Ab-active site, might have been present in the Ab population. This was certainly the case with the mouse H chains; immunodiffusion and immunoelectrophoresis experiments indicated that at least the γG1, γG2a, and γG2b classes were present in the fraction A (Figure 1) of mouse anti-DNP Ab used in these studies. (3) A single class of chains might have been present in other cases and a unique tyrosine residue might indeed have been labeled, but the amino acid residues amino terminal to the labeled tyrosine might have differed among different chains of that same class. This last factor might well be involved since, as will be shown below, the labeled tyrosines are within the variable segments of the chains.

From these considerations it is evident that the observed heterogeneity of the labeled dipeptides from any particular chain preparation is not difficult to rationalize. On the contrary, these considerations make it all the more remarkable and significant that any one dipeptide predominated in these mixtures.

*The Position of the Labeled Tyrosine Residue in Mouse L Chains.* In the case of the mouse L chains, the predominant labeled dipeptide was aspartyl- (or asparaginyl-) azotyrosine. L chains from mouse IgG globulins are known to be primarily of the κ class (Potter and Lieberman, 1967). Our results may therefore be compared with the nearly complete amino acid sequences that have been reported (Gray *et al.*, 1967) for two mouse κ-type Bence–Jones proteins. In the constant, or carboxyl-terminal, halves of these two chains, four tyrosine residues are found. (Throughout the remainder of this paper the numbering system of Gray *et al.* (1967) will be utilized.) These occur in the following dipeptidyl sequences: (1) -phenylalanyl-tyrosyl- at positions 139–140 from the amino-terminal end of the chain; (2) -threonyl-tyrosyl-, at 172–173; (3) -glutamyl- (or glutaminyl-) tyrosyl-, at 185–186; and (4) -seryl-tyrosyl-, at 191–192. Taken together, these data demonstrate that the affinity-labeled tyrosine cannot be within the constant half of the mouse L chains, and must therefore be in the variable half. Since the labeled tyrosine is in the active site (Singer and Doolittle, 1966), this proves that the active site is, at least partly, in the variable segment.

Since the time that Hilschmann and Craig (1965) announced their discovery that Bence–Jones proteins were characterized by variable amino-terminal and constant carboxyl-terminal halves, it has been inferred that the variable segments of the chains form at least part of the antibody active site. The results herein reported constitute the first proof that this inference is correct. It should perhaps be noted that this need not have been the case; it is conceivable that the active site region might have been located in the constant segment of the L chains, but that its conformation and specificity were influenced by the variable segment.

Precise positioning of the labeled tyrosine is difficult to do with certainty at this time. Although the amino acid sequences of two mouse κ-type Bence–Jones proteins provide all the information necessary to completely define the constant segments of the mouse L chains, they only inadequately define the variable segments. One can be certain, for example, that not all the possible dipeptidyl sequences involving tyrosine residues are represented in the two mouse variable segments so far analyzed. It is appropriate, however, given the close correspondence of the variable segments of mouse and human L chains (Gray *et al.*, 1967), to examine also the more extensive amino acid sequence data obtained with human κ-type Bence–Jones proteins. The positions of all reported tyrosine residues, so far as we are aware, within the variable regions of κ chains are given in Table V, which includes in addition some representative sequence data useful for the discussion.

The tyrosine residues which occur at positions 36, 49, 87, 91, and 96 would appear to be eliminated as the labeled residue because each of their amino-terminal neighbors is invariant and is not aspartic acid or asparagine. The tyrosine residues which occur infrequently at positions 32 and 71, and the invariant tyrosine at position 86, however, are all possibilities, since aspartic acid or asparagine can be the amino-terminal residue in each case. We have earlier suggested (Singer and Thorpe, 1968) the assignment of the labeled residue to tyrosine-86. A primary argument for this assignment is that the affinity labeling of antibodies from a variety of animal species (Good *et al.*, 1968) and of a range of different specificities (Singer and Doolittle, 1966) occurs at tyrosine residues on both H and L chains in the active sites; this suggests that the tyrosine residues in question are invariant residues within the variable segments of the H and L chains (Singer and Doolittle, 1966). If tyrosine-32, tyrosine-71, or any other tyrosine that is only infrequently found within Bence–Jones protein sequences were to be the labeled residue on L chains, a very strong selection for chains containing that tyrosine would be required to produce a variety of specific antibody molecules. This does not seem very likely. Another argument in favor of tyrosine-86 is as follows. Among human and mouse Bence–Jones proteins, position 85 is variable; aspartic acid, valine, and threonine are the residues most commonly found at that position. These are precisely the residues (Table IV) which are the predominant amino-terminal neighbors of the affinity-labeled tyrosine in mouse L, rabbit L, and *rabbit H* anti-DNP chains, respectively.

These facts led us to propose (Singer and Thorpe, 1968) that the H chains as well as the L chains of different species possess a tyrosine residue at a position homologous to tyrosine-86 in mouse and human L chains, and that these are

TABLE V: The Tyrosine Residues and Neighboring Residues in Variable Segments of Bence–Jones Proteins.[a]

| | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | | 48 | 49 | | 68 | 69 | 70 | 71 | 72 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Mouse 41 | Gly | Ser | Leu | Ser | Asx | Trp | Leu | Glx | | Ile | Tyr | | Gly | Ser | *Asp* | *Tyr* | Ser |
| Mouse 70 | Ile | Ser | Phe | Met | Asn | Trp | Phe | Glx | | Ile | Tyr | | Gly | Thr | Asp | Phe | Ser |
| Human Ag | Gly | Ser | Phe | Leu | Asn | Trp | Tyr | Gln | | Ile | Tyr | | Gly | Thr | Asp | Phe | Thr |
| Human Roy[b] | Ser | Ile | Phe | Leu | Asn | Trp | Tyr | Gln | | Ile | Tyr | | Gly | Thr | Asp | Phe | Thr |
| Human 3 | Gly | *Asx* | *Tyr* | Leu | Asp | Trp | Tyr | Leu | | Ile | Tyr | | Gly | Thr | Asp | Phe | Thr |
| Human Cum[b] | Gly | Thr | Tyr | Leu | Asn | Trp | Tyr | Leu | | Ile | Tyr | | Gly | Thr | Asp | Phe | Thr |

| | 83 | 84 | 85 | 86 | 87 | 88 | 89 | 90 | 91 | 92 | 93 | 94 | 95 | 96 | 97 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Mouse 41 | Phe | Val | *Asp* | *Tyr* | | Cys | Leu | Gln | Tyr | Ala | Ser | Ser | Pro | Trp | Thr |
| Mouse 70 | Thr | Ala | Met | Tyr | Phe | Cys | Glx | Glx | Ser | Lys | Glu | Val | Pro | Trp | Thr |
| Human Ag | Ile | Ala | Thr | Tyr | Tyr | Cys | Gln | Gln | Tyr | Asp | Thr | Leu | Pro | Arg | Thr |
| Human Roy | Ile | Ala | Thr | Tyr | Tyr | Cys | Gln | Gln | Phe | Asp | Asn | Leu | Pro | Leu | Thr |
| Human 3 | Val | Gly | Val | Tyr | Tyr | Cys | Met | Gln | Ala | Leu | Thr | | Pro | Leu | Thr |
| Human Cum | Val | Gly | Val | Tyr | Tyr | Cys | Gln | Met | Arg | Leu | Glu | Ile | Pro | Tyr | Thr |

[a] Taken from the compilation of Dreyer *et al.* (1967). [b] Hilschmann (1967).

the tyrosine residues which become affinity labeled in the Ab active sites. Among the many structural predictions which follow from this hypothesis and from our labeled peptide data are that normal rabbit L chains should have the predominant sequence -valyl-tyrosyl- homologous to positions 85 and 86 of mouse L chains, and that normal rabbit H chains should have the predominant sequence -threonyl-tyrosyl- in the corresponding positions. The latter prediction has now been confirmed by the sequence analysis of normal rabbit H chains carried out by I. A. O'Donnell and R. R. Porter (personal communication). In their numbering system, residues 94–97 from the amino termini of the H chains have the predominant sequence Thr-Tyr-Phe-Cys-, which is certainly homologous to the sequences from 85 to 88 of mouse and human Bence–Jones proteins (Table V). Not only is a tyrosine residue present in the rabbit H chains in a position homologous to tyrosine-86 in mouse L chains, but its amino-terminal neighbor is predominantly threonine, as predicted.

Other evidence, involving the nature of the labeled tryptic peptides from L chains of mouse anti-DNP Ab, is also consistent with the assignment of the affinity-labeled tyrosine on mouse L chains to position 86 (J. W. Fenton and S. J. Singer, in preparation).

If it is true that homologous tyrosine residues on H and L chains are both present in Ab active sites, it followed that the Fab fragment of an IgG molecule most probably possesses certain symmetry properties (Singer and Thorpe, 1968). A schematic structural model showing this symmetry is given in Figure 9. In this model, each H chain is divided into four nonidentical, approximately 100 amino acid long, segments and each L chain into two. This segmentation is based on evolutionary considerations, and was first proposed by Singer and Doolittle (1966); it has been firmly established by the sequence data of many investigators, especially of Hill *et al.* (1966) and Edelman *et al.* (1969). It is proposed that the L chain and the Fd portion of the H chain, which together comprise the Fab unit, are structurally related by a pseudo-

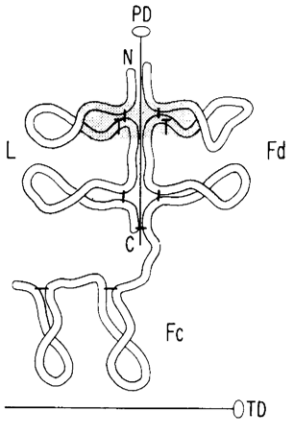


FIGURE 9: A schematic model for half of an IgG molecule (Singer and Thorpe, 1968). PD is a proposed dyad axis of pseudosymmetry which relates the L chain and Fd fragment of the H chain. TD is the true dyad axis presumably relating the two identical halves of the IgG molecule. N and C refer to the amino and carboxyl termini, respectively, of the L chain. The six similarly, but not identically, folded segments, four on the H chain and two on the L, represent the approximately 100 amino acid long sequences which are evolutionarily related (see text). The solid bars indicated the four intrachain disulfide bridges and the one H–L interchain bridge in the Fab fragment. The T symbols represent the proposed locations of the tyrosine residues on H and L chain which become affinity labeled, and the stippled region is a schematic view of the active site and its pseudosymmetry.

dyad axis of rotation, such as relates the $\alpha$ and $\beta$ subunits of a hemoglobin molecule (Muirhead *et al.*, 1967). The tyrosine residues on the H and L chains that become affinity labeled and that are in the active site are related by this pseudosymmetry axis. It is further proposed that the affinity-labeling reagent, when reversibly bound to a site, lies along the axis of pseudosymmetry such that there is a relatively constant probability for it to react with either the H- or the L-chain tyrosine in a site.

The existence of this pseudosymmetry relationship between H and L chains in the active site region (embodying the variable segments of the H and L chains) of different immunoglobulin molecules suggests that the variability of H and L chains produced in a single cell is not achieved independently, but is somehow coordinated. This inference arises from other kinds of studies as well, particularly those of Dorrington *et al.* (1967) which show that specific optical rotatory effects accompany the recombination of H and L chains derived from the same molecule, but not for chains from different molecules. These structural conclusions have important implications for the biosynthesis of antibodies, as we suggested some time ago (Singer and Doolittle, 1966). They form the basis of a theory by Cohn (1968) of the origin of immunoglobulin variability in which the selection of properly matched H and L chains in a single cell is a critical element. In this theory, the genes coding for the two chains undergo repeated somatic mutations until they become stabilized by H–L chain pairing of a suitably large affinity.

Acknowledgment

We gratefully acknowledge the expert technical assistance of Mrs. Birgitta Kiefer. For many fruitful discussions, and much helpful advice, we express our appreciation to Professor Russell F. Doolittle.

References

Benson, J. V., Jr., Jones, R. T., Cormick, J., and Patterson, J. A. (1966), *Anal. Biochem. 16*, 91.

Blombäck, B., Blombäck, M., Edman, P., and Hessel, B. (1966), *Biochim. Biophys. Acta, 115*, 371.

Carnegie, P. R. (1965), *Biochem. J. 95*, 9p.

Cohn, M. (1968), *in* Nucleic Acids in Immunology, Plescia, O. J., and Braun, W., Ed., New York, N. Y., Springer-Verlag, p 671.

Doolittle, R. F., and Singer, S. J. (1965), *Proc. Natl. Acad. Sci. U. S. 54*, 1773.

Dorrington, K. J., Zarlengo, M. H., and Tanford, C. (1967), *Proc. Natl. Acad. Sci. U. S. 58*, 996.

Dreyer, W. J., Gray, W. R., and Hood, L. E. (1967), *Cold Spring Harbor Symp. Quant. Biol. 32*, 353.

Edelman, G. M., Cunningham, B. A., Gall, W. E., Gottlieb, P. D., Rutishauser, U., and Waxdal, M. J. (1969), *Proc. Natl. Acad. Sci. U. S. 63*, 78.

Eisen, H. N., and Siskind, G. W. (1964), *Biochemistry 3*, 996.

Farah, F. S., Kern, M., and Eisen, H. N. (1960), *J. Exptl. Med. 112*, 1195.

Fleischman, J. B., Pain, R. H., and Porter, R. R. (1962), *Arch. Biochem. Biophys., Suppl. 1*, 174.

Good, A. H., Ovary, Z., and Singer, S. J. (1968), *Biochemistry 7*, 1304.

Good, A. H., Traylor, P. S., and Singer, S. J. (1967), *Biochemistry 6*, 873.

Gray, W. R., Dreyer, W. J., and Hood, L. (1967), *Science 155*, 465.

Hill, R. L., Delaney, R., Lebovitz, H. E., and Fellows, R. E., Jr. (1966), *Proc. Roy. Soc. (London) B166*, 159.

Hilschmann, N. (1967), *Z. Physiol. Chem. 348*, 1077.

Hilschmann, N., and Craig, L. C. (1965), *Proc. Natl. Acad. Sci. U. S. 53*, 1403.

Muirhead, H., Cox, J. M., Mazzarella, L., and Perutz, M. F. (1967), *J. Mol. Biol. 28*, 117.

Potter, M., and Lieberman, R. (1967), *Advan. Immunol. 7*, 92.

Singer, S. J., and Doolittle, R. F. (1966), *Science 153*, 13.

Singer, S. J., Slobin, L. I., Thorpe, N. O., and Fenton, J. W., II (1967), *Cold Spring Harbor Symp. Quant. Biol. 32*, 99.

Singer, S. J., and Thorpe, N. O. (1968), *Proc. Natl. Acad. Sci. U. S. 60*, 1371.

Sjöquist, J. (1960), *Biochim. Biophys. Acta 41*, 20.

Traylor, P. S., and Singer, S. J. (1967), *Biochemistry 6*, 881.

Wofsy, L., Kimura, J., Bing, D. H., and Parker, O. C. (1967), *Biochemistry 6*, 1981.

Wofsy, L., Metzger, H., and Singer, S. J. (1962), *Biochemistry 1*, 1031.