# Affinity Labeling of a Mouse Myeloma Protein Which Binds Nitrophenyl Ligands. Sequence and Position of a Labeled Tryptic Peptide*

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ABSTRACT: The nitrophenyl binding mouse myeloma protein 315 has been affinity labeled, and a 33 amino acid labeled peptide has been isolated and sequenced. By analogy with other light chains and particularly with Balb/c λ chains the labeled residue is Tyr-34. These results are consistent with preliminary data on conventional anti-dinitrophenyl antibodies. Functional and structural evidence strongly suggest that Tyr-34 is intimately related to the binding site.

The 33 amino acid peptide represents, therefore, the first substantial sequence of a region directly implicated in an immunoglobulin combining site. Ancillary data suggest that the 315 light chain, while λ like, may represent a new subtype of λ chains.

In previous papers we have described the reactivity of protein 315—a Balb/c IgA[1] myeloma protein known to bind nitrophenyl ligands—with affinity-labeling reagents (Metzger and Potter, 1968; Goetzl and Metzger, 1970a). It was demonstrated that the reaction with *m*-nitrobenzenediazonium fluoroborate (NBDF) led to enhanced azo derivatization of a single light-chain tyrosine. The reaction led to partial inactivation of all the combining sites and was markedly retarded by the presence of a nonreactive ligand. Kinetic studies confirmed that labeling was occurring by way of a reversible complex between NBDF and the protein 315 site. Finally, a 33 amino acid tryptic peptide isolated from the light chains contained all of the azotyrosine label.

The present paper presents the sequence of this peptide, and information regarding its position in the light chain. In addition evidence is presented that the 315 light chain is a λ-like chain with certain unusual features.

## Experimental Section

### *Methods*

*Isolation and Quantitation of the Labeled Tryptic Peptide.* The tryptic peptide was purified from a digest of labeled MOPC 315 light chains by previously described methods (Goetzl and Metzger, 1970a). The essential steps in this procedure are: gel filtration on Sephadex G-50 in 8 M urea, elution with a pyridine–acetic acid gradient from a Sephadex QAE (A-25) column equilibrated at pH 7.6, and selective precipitation of the labeled peptide from 10% pyridine–acetate at pH 3.0. The peptide was quantitated during the purification and prior to each further analytical or degradative procedure by the radioactivity of the label. The specific radioactivity of the light chains (counts per minute per mole of light chain) was measured and this was used to calculate the specific radioactivity of the peptide (counts per minute per mole of labeled peptide) assuming 1 mole of peptide/combining site and 1.4 sites/2 moles of light chains (Goetzl and Metzger, 1970a). Suspensions of the labeled peptide were generally also quantitated by analyzing released amino acids from 6 N HCl hydrolysates (105°, 24 hr) of known volumes of the suspension on a Beckman 120C AutoAnalyzer. The number of nanomoles of aspartic acid and of glycine were divided by 4, the number of residues of each amino acid in the peptide, and averaged to arrive at the number of nmoles of labeled tryptic peptide in the aliquot. This method generally gave values for the amount of peptide which were 10% higher than the corresponding ones estimated from radioactivity determinations. A portion of each preparation of peptide was hydrolyzed for compositional analysis prior to any enzymatic or chemical cleavage.

*Enzymatic Digestions of the Labeled Peptide.* Digestions were carried out on 200–300 nmoles of peptide suspended in 0.3 ml of the appropriate buffer. For thermolysin cleavage, the buffer was 0.2 M ammonium acetate–0.01 M in $CaCl_2$ (pH 8.2). Thermolysin 1% w/w was added initially and again at 8 and 16 hr. The digestion was conducted at 45° for 24 hr. Trypsin digestion was conducted at 45° in 0.05 M Tris-HCl buffer (pH 8.0) which was 0.01 M in $CaCl_2$. Trypsin 1% w/w was added at zero time, 8 hr, and 16 hr, and the incubation time was 24 hr. Subtilisin cleavage was performed in 0.1 M ammonium bicarbonate at a concentration of 2% subtilisin w/w for 4 hr at 25°. Chymotrypsin digestion was carried out in 0.2 M ammonium acetate which was 0.01 M in $CaCl_2$ (pH 8.2) using 2% enzyme w/w initially, and an additional 1% w/w at 12 and 24 hr. The digestion was performed at 45° for 36 hr. Subtilisin solubilized the labeled peptide rapidly, but thermolysin and chymotrypsin did not,

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[1] The standard nomenclature for immunoglobulins is used. Other abbreviations used are: NBDF, *m*-nitrobenzenediazonium fluoroborate; DNP, dinitrophenyl.

and an insoluble yellow residue remained even at the end of these digestions. The insolubility of the peptide required frequent swirling of the tube for resuspension in an attempt to maximize digestion. The suspension was much more stable at 45° than at 25°, and hence this temperature was used for the chymotrypsin and thermolysin procedures.

*Isolation of Peptides from Digestion Mixtures.* Digestion mixtures were lyophilized, dissolved in 0.1 ml of 5% $NH_4OH$, and spotted on Whatman No. 3MM chromatography paper. Descending chromatography using a butanol–acetic acid–water (4:1:5, v/v) system was followed by high-voltage electrophoresis in a pH 3.6 pyridine–acetic acid buffer in the standard fashion (Bennett, 1967). All maps, excluding those of the subtilisin digests, showed a yellow spot at the origin, which represented undigested tryptic peptide as shown by amino acid composition. Neither the untreated labeled tryptic peptide nor the insoluble radioactive residues from the thermolysin and chymotrypsin digests showed any mobility when chromatographed at a basic pH in a concentrated ammonium hydroxide–propanol–water (3:6:1, v/v) system (Moore and Baker, 1958). The paper was dried and sprayed lightly and repeatedly with a 0.025% ninhydrin solution in ethanol–2 N acetic acid (3:1, v/v). Peptide spots appearing after each successive spraying were excised and eluted with 5% $NH_4OH$.

*N-Bromosuccinimide Cleavage of Tryptic Peptide.* Tryptic peptide (250 nmoles) was dissolved in 0.05 ml of 8 M urea which had been titrated to pH 4.0 by the dropwise addition of 50% acetic acid. *N*-Bromosuccinimide (500 nmoles) was added in 0.01 ml of the same solvent. The reaction was allowed to proceed at 25° for 2.5 hr (Ramachandran and Witkop, 1967). Peptides in the digestion mixture were then separated from the *N*-bromosuccinimide and urea by passage over a 10-ml Sephadex G-10 column equilibrated and developed with 50% acetic acid. Dansyl-Edman analyses were carried out on this mixture. The products of digestion showed no mobility on paper during multiple attempts at either chromatography or electrophoresis under diverse conditions. They were resolved from the undigested peptide and recovered together in very low yield after gel filtration of the mixture on Sephadex G-25 in an 80-ml (1 × 100 cm) column developed with 50% acetic acid.

*Mild Acid Hydrolysis of Tryptic Peptide.* Tryptic peptide (200 nmoles) was dissolved in 0.4 ml of 0.06 N HCl which was 40% in acetic acid. The hydrolysis was carried out at 105° for 20 hr (Tsung and Fraenkel-Conrat, 1965). After lyophilization the hydrolysate was redissolved in 0.1 ml of 25% acetic acid and spotted on paper for a routine peptide map as described above. The origin and a yellow smear below it were cut out prior to ninhydrin spraying. All spots were eluted as above with 5% $NH_4OH$.

*Amino Acid Analysis.* All peptides isolated from paper were hydrolyzed in 0.15 ml of 6 N HCl in sealed tubes after repeated evacuation and flushing with water-purified nitrogen. After 24 hr at 105° the tubes were opened, the HCl lyophilized, and the contents dissolved in 0.4 ml of 0.01 N HCl for analysis of free amino acids on a Beckman 120C AutoAnalyzer. The 6 N HCl was made 5% in thioglycollic acid for all short-column analyses to maximize the yield of tryptophan (Matsubara and Lasaki, 1969). The corrected average yield of tryptophan was 71% using this method (Goetzl and Metzger, 1970a). Aminoethylcysteine was identified on a 15-cm basic column and quantitated using a known synthetic standard (Cole, 1967). The peptides obtained from any map were generally screened by analyzing their 6 N HCl hydrolysates electrophoretically. Samples and standards were spotted on a 46 × 20 cm sheet of Whatman paper and electrophoresced at pH 1.64 and 38° for 9000 V × hr as previously described (Kaplan and Metzger, 1969). The paper was dried and stained with a cadmium–ninhydrin solution (Dreyer and Bynum, 1967) by dipping. Only serine–valine and threonine–proline were unresolved under these conditions.

*Hydrazinolysis.* Peptide (10–15 nmoles) was lyophilized in a test tube in the presence of $P_2O_5$ and NaOH. The tube was constricted, 0.05 ml of anhydrous hydrazine was added, the tube was thoroughly flushed with nitrogen and sealed, and the mixture was incubated at 110° for 6 hr. The vials were opened and desiccated in the presence of concentrated $H_2SO_4$ and $P_2O_5$. The contents were immediately dissolved in 0.2 ml of 0.01 N HCl and the carboxy-terminal residue identified either on the amino acid analyzer or by high-voltage paper electrophoresis.

*Digestion of Peptides with Carboxypeptidase A or B.* Lyophilized peptide (10–15 nmoles) was dissolved in 0.1 ml of 0.1 M $NH_4HCO_3$ and digested with 2% w/w carboxypeptidase A or B. If a poor yield was obtained with carboxypeptidase A, the digestion was repeated on a fresh aliquot of peptide in 0.1 N NaCl buffered with 0.05 N $NaHCO_3$ (pH 7.5). Digestions were carried out at 25° for 24 hr with carboxypeptidase A and for 8–24 hr with carboxypeptidase B. Controls with no peptide were run simultaneously to quantitate amino acids released by enzymatic autodigestion. The carboxy-terminal residues were determined on the amino acid analyzer.

*Dansyl-Edman Degradation.* Sequential analysis of amino-terminal residues was performed as described by Gray (1967). Asparagine and glutamine were identified by dansylation of the free amino acid released after alkaline hydrolysis of the phenylthiohydantoin derivative (von Ehrenstein, 1966). Approximately a 10-nmole portion of the latter was hydrolyzed in 0.05 ml of 0.2 N NaOH in a sealed, nitrogen-flushed tube for 4 hr at 105°. The tube was opened and the pH of the solution was adjusted to 8 by exposure to the $CO_2$ atmosphere in a Dry Ice box for 3–5 min. After the addition of 0.04 ml of dansyl-Cl in acetone (3 mg/ml) the mixture was incubated for 60 min at 45°. The solution was extracted twice with 0.1 ml of water-saturated ethyl acetate, acidified with 0.2 N HCl to pH 4, and extracted with another 0.1 ml of ethyl acetate to obtain the dansyl-amino acid. This was identified by chromatography on polyamide sheets using appropriate standards on the reverse side of each sheet (Woods and Wang, 1967). Dansylation of the whole tryptic peptide, which was insoluble in 0.2 N $NaHCO_3$, was performed in 6 M urea (Gray, 1967). The dansylated peptide was passed over Sephadex G-10 (25% acetic acid) to remove urea prior to acid hydrolysis.

*Chymotryptic Digestion of Labeled MOPC 315 Light Chains.* Light chains (20 mg) were dissolved in 2.0 ml of 0.2 M $NH_4$ acetate buffer which was 0.01 M in $CaCl_2$, and digested at 37° for 24 hr with chymotrypsin added initially at 2% w/w and then 1% w/w at 8 hr and 16 hr. The mixture was lyophilized, redissolved in 0.2 ml of 0.1 M $NH_4HCO_3$, and applied to a 182-ml (1.76 × 75 cm) Sephadex G-25 column equilibrated with 0.1 M $NH_4HCO_3$.

TABLE I: Composition of Labeled Tryptic Peptide.

| | Moles[a] |
|---|---|
| Lys | 1.00 |
| His | 0.98 |
| Arg | 1.02 |
| Trp | 0.73 ± 0.07[b] |
| Asp | 3.82 ± 0.02 |
| Thr | 4.60 ± 0.10 |
| Ser | 3.27 ± 0.13 |
| Glu | 2.12 ± 0.02 |
| Pro | 1.12 ± 0.02 |
| Gly | 4.06 ± 0.05 |
| Ala | 2.09 ± 0.10 |
| Val | 1.00 ± 0.10 |
| Ile | 1.84 ± 0.14 |
| Phe | 1.91 ± 0.20 |
| Azo-Tyr | 1.00 |
| Total | 33 |

[a] Azotyrosine was determined by radioactivity and the other amino acids were quantitated relative to the number of moles of azotyrosine. [b] This value is an average of those from the spectral and hydrolytic determinations.

The two radioactive peaks (see Results) were each run on a paper map as detailed above. The single yellow spot on each map was cut out, eluted with 5% $NH_4OH$, and analyzed by 6 N HCl hydrolysis and the dansyl-Edman procedure as described above.

*N-Terminal and C-Terminal Analysis of the MOPC 315 Light Chain.* N-Terminal analysis of the light chain, which was blocked to Edman degradation, was performed on the subtilisin pentapeptide prepared as follows. To 20 mg of lyophilized light chains was added 2 ml of 0.1 M $NH_4HCO_3$ and 2% w/w of subtilisin. After 4 hr at 37° the mixture was lyophilized, redissolved in distilled water, and applied to a 15 × 1 cm Dowex 50 ($H^+$ form) column equilibrated with water. The optical density at 215 mμ of each 1-ml fraction was measured. The early peak tubes were pooled for analysis which included: 6 N HCl hydrolysis for amino acid composition, carboxypeptidase A digestion, and hydrazinolysis, all of which are described above. In addition, the amino-terminal pyrrolidonyl residue was cleaved off enzymatically using previously published methods (Doolittle and Armentrout, 1968; Kaplan and Metzger, 1969). The then accessible amino-terminal residues were sequentially determined using the routine dansyl-Edman procedure. A portion of the Dowex 50 column eluate was also applied to a paper map. The blocked peptide was located by using a starch–iodide peptide bond stain (Rydon and Smith, 1952), and the peptide recovered was analyzed by the same methods used for the crude eluate.

C-Terminal analysis of the light chain was performed in two ways. (a) Aminoethylated light chains were prepared by partial reduction of euglobulin from MOPC 315 ascites in 0.2 M Tris-HCl buffer (pH 8.6) with dithiothreitol and subsequent aminoethylation (Goetzl and Metzger, 1970a). These light chains were purified by standard methods and the C-terminal residue was identified by hydrazinolysis or carboxypeptidase A digestion. The penultimate residue (aminoethylcysteine) was identified by carboxypeptidase B digestion or hydrazinolysis of a 24-hr carboxypeptidase A digest. Carboxypeptidase A digestion was carried out on 1 mg of light chains as described above. Carboxypeptidase B digestion was done as previously detailed but conducted for 24 hr in 0.05 M Tris (pH 9.5) with addition of 1% enzyme w/w initially and at 8 hr, since yields were poor under standard conditions. Hydrazinolysis was modified for light chains to use 0.2 ml of hydrazine and 1 mg of protein. The hydrazine-treated protein was lyophilized, dissolved in distilled water, and passed over a 0.5-ml column of Amberlite IRC 50 resin ($H^+$ form) equilibrated with water. Acid and neutral amino acids were eluted directly, and basic amino acids were removed with 0.1 N $NH_4OH$ (de La Llosa *et al.*, 1964). A portion of the carboxypeptidase B digest was reexposed to 2% w/w carboxypeptidase A for 24 hr. Half of this solution was put on the amino acid analyzer long column directly, and half was applied after 1 N HCl hydrolysis for 8 hr at 105° to convert asparagine to aspartic acid. Prior to 1 N HCl hydrolysis, enzymes and partially digested light chains were precipitated by adding absolute ethanol to the solution to a final concentration of 70%. Asparagine was quantitated by using the difference in the areas under the serine–asparagine peak (which are not resolved in our system) before and after HCl hydrolysis, and dividing by the asparagine–ninhydrin color coefficient. For this series of analyses, both enzyme control tubes and a known human λ light chain (λ Riggs) were run in parallel with MOPC 315 light chains. (b) MOPC 315 light chains from partially reduced and iodoacetamide alkylated 315 protein (Goetzl and Metzger, 1970a) were digested with carboxypeptidase A for 8, 24, and 48 hr, using 1% w/w enzyme added initially and at 8 and 24 hr. The *S*-carboxamidomethylcysteine released was quantitated on the amino acid analyzer using a synthetic standard.

*Materials*

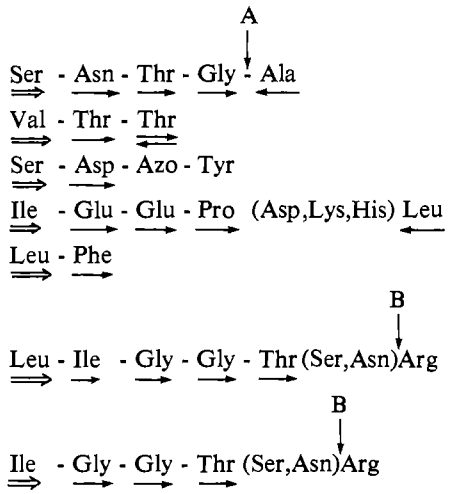
Mice bearing the MOPC 315 plasmacytoma were the generous gift of Dr. Michael Potter (National Cancer Institute, National Institutes of Health. The Bence–Jones protein, λ Riggs, was given by Dr. D. Ein, and the λ–γ$M_{Koh}$ by Dr. J. Johnson. Synthesis and handling of the labeling reagent [$^3$H]NBDF have been previously described (Goetzl and Metzger, 1970a).

Trypsin treated with L-(tosylamido-2-phenyl)ethyl chloromethyl ketone and chymotrypsin were purchased from Worthington Biochemical Corp. and stored as 1% solutions in 0.001 N HCl at −20°. Carboxypeptidase A treated with DFP (Worthington Biochemical Corp.) was obtained as a suspension, and stored as a 1% solution in 2 M $NH_4HCO_3$ at −80° in sealed tubes. Carboxypeptidase B treated with DFP (Worthington) was stored at −20° as a stock solution (6.1 mg/ml) and diluted prior to each use. Subtilisin was purchased from Nutritional Biochemical Corp. and stored at −80° as a 1% solution in 0.001 N HCl. Thermolysin was purchased from California Biochemical Corp. and recrystallized from a concentrated solution by pH change prior to use (Endo, 1962). It was stored over silica gel at −20° and a fresh solution made up for each digestion. Pyrrolidonyl-peptidase (Doolittle and Armentrout, 1968) was a gift from Dr. Leroy Hood.

TABLE II: Thermolysin Peptides: Compositions and Summary of Sequence Procedures.

| | I | II | III | IV | V | VI | VII |
|---|---|---|---|---|---|---|---|
| Lys | | | | 1.00 | | | |
| His | | | | 0.86 | | | |
| Arg | | | | | | 0.92 | 0.97 |
| Azo-Tyr[a] | | | 1.00 | | | | |
| Asp | 1.00 | | 0.95 | 1.14 | | 1.00 | 1.00 |
| Thr | 0.82 | 1.89 | | | | 0.73 | 0.89 |
| Ser | 0.72 | | 0.69 | | | 0.69 | 0.84 |
| Glu | | | | 1.78 | | | |
| Pro | | | | 0.97 | | | |
| Gly | 0.96 | | | | | 1.88 | 1.92 |
| Ala | 1.00 | | | | | | |
| Val | | 1.00 | | | | | |
| Ile | | | | 0.97 | | 0.87 | 0.89 |
| Leu | | | | 0.89 | 1.00 | 0.68 | |
| Phe | | | | | 0.82 | | |
| Yield (%) | 22 | 25 | 54 | 18 | 11 | 14 | 30 |

Sequence Procedures




[a] Azotyrosine was quantitated by radioactivity. ⇒ = dansyl analysis; → = dansyl-Edman procedure; ← = hydrazinolysis; A = carboxypeptidase A digestion; B = carboxypeptidase B digestion.

Butanol (Allied Chemical Co.) and pyridine (Eastman Organic Chemicals) were reagent grade and redistilled prior to use. Urea was deionized as an 8 M solution by passage over a Rexyn I 300 (Fisher) mixed-bed resin column. Hydrazine was purchased from Matheson, Coleman and Bell and was redistilled *in vacuo* over $CaCl_2$ and stored in 0.5-ml aliquots in sealed vials at −80°. Dithiothreitol was purchased from California Biochemical Corp. and was sublimated prior to use. Iodoacetamide (California Biochemical Corp.) was recrystallized twice from boiling water. Ethylenimine was purchased from Matheson, Coleman and Bell and stored at 4° stabilized over KOH pellets.

Butyl acetate and ethyl acetate for the dansyl-Edman procedure were reagent grade and redistilled before use. These reagents and the redistilled pyridine were periodically checked for peroxide content by methods previously detailed (Kaplan and Metzger, 1969). Phenyl isothiocyanate from Eastman was redistilled *in vacuo* and stored at −20° in sealed vials. Dansyl-Cl and dansyl-amino acids were purchased from Pierce Chemical Co. Polyamide chromatography sheets were obtained from Cheng Chin Trading Co., Ltd. (Gallard-Schlesinger Chemical Manufacturing Co.).

Dowex cation-exchange resin AG-50W-X2 (100–200 mesh) from Bio-Rad Laboratories and Amberlite IRC 50 cation-exchange resin from Mallinckrodt were used in the $H^+$ form and successively washed prior to use with 2 N NaOH–water–2 N HCl, and finally water until the effluent was neutral.

## Results

*Composition of Labeled Peptide.* The amino acid composition of the labeled tryptic peptide has been previously published and is given in Table I. Thirty-one of the thirty-three amino acids have been arranged in sequence by the proce-

TABLE III: Subtilisin Peptides: Compositions and Summary of Sequence Procedures.

| | I | II | III | IV | V | VI | VII |
|---|---|---|---|---|---|---|---|
| Lys | | | | 1.06 | | | |
| His | | | | 0.98 | | | |
| Arg | | | | | | | 1.00 |
| Azo-Tyr[a] | | | 1.00 | | | | |
| Asp | 1.00 | | 0.84 | 1.00 | | | 0.93 |
| Thr | 1.06 | 0.81 | | | 0.97 | 1.00 | |
| Ser | 0.94 | | | | | | 0.78 |
| Glu | | | | 1.00 | | | |
| Pro | | | | 1.03 | | | |
| Gly | 0.94 | | | | 1.00 | 1.81 | |
| Ala | 0.95 | | | | | | |
| Val | | 1.00 | | | | | |
| Ile | | | | | | 0.85 | |
| Leu | | | | 0.98 | 0.87 | | |
| Phe | | | | | 0.84 | | |
| Yield (%) | 18 | 15 | 42 | 18 | 27 | 25 | 19 |

Sequence Procedures

I Ser ⟹ - Asn → - Thr → - Gly → - Ala →

II Val ⟹ - Thr →

III Asp ⟹ - Azo-Tyr

IV Glu ⟹ - Pro → - Asp → - Lys → - His ↓A - Leu ←

V Phe ⟹ - Thr → - Gly → - Leu ←

VI Ile ⟹ - Gly → - Gly → - Thr ⇄

VII Ser ⟹ - Asn ↓B - Arg →

[a] Refer to Table II for the meanings of the symbols and abbreviations.

dures outlined below. The remaining two amino acids were positioned on the basis of the compositional data as well as by homology with related sequences.

*Carboxy-Terminal Residue.* Carboxypeptidase B digestion yielded only arginine. Forty-four and ninety per cent of the theoretical yields were obtained after 6 and 24 hr of digestion, respectively. Carboxypeptidase A failed to release any amino acids above enzyme control values.

*Amino-Terminal Sequence.* By conducting the dansylation in 6 M urea sequential dansyl-Edman analysis yielded three clear-cut residues: Ser-Asn-Thr (Table V). This sequence was subsequently confirmed with the thermolysin and subtilisin peptides. A previously reported Ser-Gly sequence (Goetzl and Metzger, 1970b) obtained by dansylation in bicarbonate buffer appears to have resulted from a combination of low yields and consequently disproportionately high contaminants.

*Thermolysin Peptides.* Those peptides which were recovered in greater than 10% yield from two independent thermolysin digestions of the labeled peptide are shown in Table II. The sequence of peptide I is consistent with the *N*-terminal sequence of the whole tryptic peptide (above). The azotyrosine residue in peptide III was quantitated by radioactivity measurements alone. Free tryptophan but no tryptophan-containing peptides were recovered.

*Subtilisin Peptides.* Each of the subtilisin peptides shown in Table III was obtained in relatively high yield from at least two preparative fingerprints. Subtilisin alone, of all the enzymes used, completely solubilized the tryptic peptide. None of the subtilisin digestion mixtures showed color or radioactivity at the origin. Histidine in peptide IV was positioned by inference from the peptide composition and the sequence. Despite long carboxypeptidase A or B digestions of this peptide, after removal of leucine with carboxypeptidase A, only 5% or less of the theoretical amount of histidine was released.

*Chymotryptic Peptides.* Table IV contains the data on all peptides recovered in at least 10% yield from two preparative fingerprints of the chymotryptic digest of tryptic peptide. Free tryptophan in 30–40% yield was obtained but no tryptophan-containing peptides. Chymotryptic digestion of whole labeled light chains yielded two major labeled peptides which could be separated on a Sephadex G-25 column (Figure 1) and further purified on paper (Table IV). Peptide $C_2$ proved to be identical with thermolysin peptide III and chymotryptic peptide II. Peptide $C_1$ was a tetrapeptide having an additional residue of threonine N terminal to the serine in peptide $C_2$.

*Tryptic Cleavage of Labeled Peptide.* Despite vigorous digestion conditions, the smaller labeled peptide which

TABLE IV: Chymotrypsin Peptides: Compositions and Summary of Sequence Procedures.

| | Tryptic Peptide | | | | | | Light Chains | |
|---|---|---|---|---|---|---|---|---|
| | I | II | III | IV | V | VI | $C_1$ | $C_2$ |
| Arg | | | | 0.93 | | 0.88 | | |
| Azo-Tyr[a] | | 1.00 | | | | | 1.00 | 1.00 |
| Asp | | 1.00 | | 1.00 | | 1.00 | 1.02 | 1.00 |
| Thr | 1.09 | | 0.72 | 1.03 | 1.03 | | 0.77 | |
| Ser | | 0.75 | | 1.00 | | 0.68 | 0.70 | 0.72 |
| Gly | 1.00 | | 1.00 | 1.86 | 2.00 | | | |
| Ala | 0.77 | | | | | | | |
| Val | 0.80 | | | | | | | |
| Ile | | | | 0.86 | 0.88 | | | |
| Leu | | | 0.84 | | | | | |
| Yield (%) | 18 | 20 | 58 | 32 | 23 | 27 | 29 | 25 |

Sequence Procedures

I: Gly - Ala - Val - Thr

II and $C_2$: Ser - Asp - Azo-Tyr

III: Thr - Gly - Leu

IV: Ile - Gly - Gly(Thr,Ser,Asn) [B] Arg

V: Ile - Gly - Gly - Thr

VI: Ser - Asn [B] - Arg

$C_1$: Thr - Ser - Asp - Azo-Tyr

[a] Refer to Table II for the meanings of the symbols and abbreviations.

would result from a break at Lys-43 was never isolated in pure form. The yield of the lysyl peptide was estimated from the radioactivity recovered when the tryptic digest was analyzed on a Sephadex G-25 column equilibrated with 50% acetic acid. The radioactivity which was found in a peak at 50% bed volume amounted to 30% of the radioactivity in the earlier peak corresponding to undigested label peptide. This smaller peptide eluted at a position identical with that of the smaller labeled peptide (II in Goetzl and Metzger, 1970a) resulting from long tryptic digests of 315 L chain. This peptide did not move with electrophoresis or chromatography on paper, and was recovered from ion-exchange resin gradients in less than 1% yield. It therefore cannot be conclusively identified as the peptide spanning residues 24–43.

We previously reported a chymotryptic peptide sequence of Leu-Ala-Phe on the basis of composition and a dansyl-Edman sequence Leu-Ala (Goetzl and Metzger, 1970b). Without going into detail, we are convinced that this peptide arose as a contaminant and is not present in the tryptic peptide. We were never able to isolate such a peptide again but were able to identify a Leu-Phe dipeptide from thermolysin digest peptide maps on two occasions and in good yields (Table II).

*N-Bromosuccinimide Cleavage of Tryptic Peptide.* The products of *N*-bromosuccinimide cleavage were not easily separable from the uncleaved peptide nor from each other (see Methods). Dansylation of the mixture revealed serine and isoleucine. The dansyl-Edman procedure yielded on three subsequent cycles aspartic and glutamic, threonine and glutamic, and proline, respectively. These results are consistent with the *N*-terminal sequences for thermolysin peptides I and IV (Table II). The finding of this single break

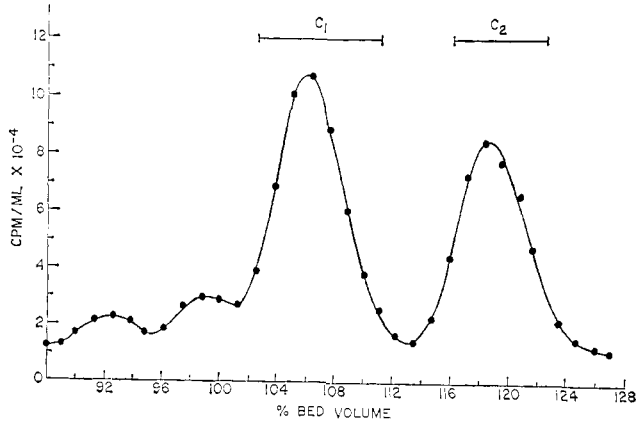
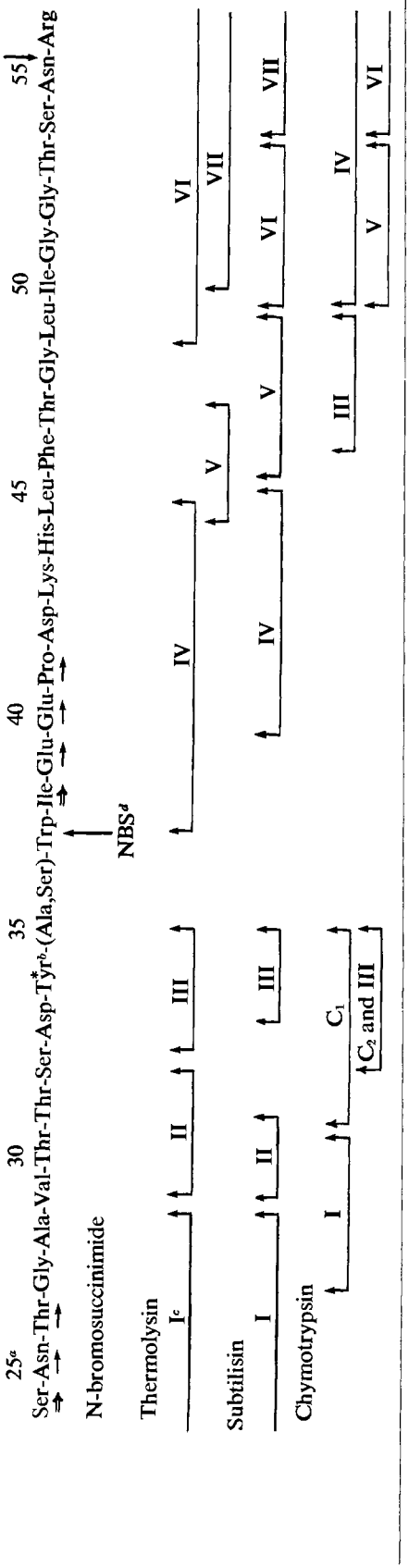



FIGURE 1: Sephadex G-25 fractionation of a chymotryptic digest of labeled 315 λ chains. The digestion was carried out in 0.2 M ammonium acetate buffer–0.01 M in $CaCl_2$ at 37°. The column was equilibrated with 0.1 M ammonium bicarbonate. Fractions were counted in Bray's solution without quench correction. Peak $C_1$ represents the labeled tetrapeptide and $C_2$, the tripeptide.

TABLE V: Composite Sequence of Labeled Tryptic Peptide.




[a] The numbering system used is that adopted by M. Weigert. [b] Tyr* = azotyrosine. [c] The code for the peptide cleavage products is from Tables II–IV. [d] *N*-Bromosuccinimide cleavage. Refer to Table II for the meanings of the other symbols and abbreviations.

TABLE VI: N-Terminal Analysis of MOPC 315 λ Chains with Sequence Homologies.

| | | |
|---|---|---|
| | P ↓ | A ↓ |
| 315 | Glp - Ala - Val - Thr | -Val |
| | 1.07[a] 0.93 | 1.77 0.87 |
| RPC 20, MOPC 104 S178, xs 104 | Glp - Ala - Val - Val | - Thr |
| | Glp - Ala - Val - Val | (Thr, ....) |

[a] Composition of the pentapeptide using an average of the glutamic and alanine amino acid recoveries as 1.00. P = pyrrolidonylpeptidase digestion. Refer back to Table II for the meanings of the other symbols and abbreviations. The RPC 20 and MOPC 104 sequences are from Appella and Perham (1968), and the S 178 and xs 104 sequences are from M. Weigert (personal communication, 1970).

in the tryptic peptide under conditions selective for peptide bonds involving the carboxyl group of tryptophan positions the tryptophan immediately N terminal to isoleucine (Table V). By the use of isoleucine standards we were able to estimate the extent of cleavage by *N*-bromosuccinimide as approximately 20%.

*Mild Acid Hydrolysis of Tryptic Peptide.* Mild acid hydrolysis did not completely solubilize the peptide and approximately 60% of the total radioactivity was eluted with 50% acetic acid from the origin of the peptide map performed on the hydrolysate. Free serine and free arginine were obtained in 25–40% yields from two separate preparations—which is consistent with the N and C penultimate asparagines already positioned. Unfortunately, no other peptides were recovered in significant yields. The yellow smear near the origin contained 10% of the radioactivity and had the same amino acid composition as the labeled tryptic peptide.

*Sequence of Labeled Tryptic Peptide.* Table V lists the peptides recovered by the enzymatic degradations as well as by chemical cleavage. There is sufficient information to unambiguously position 31 of the 33 residues found by composition (Table I). The only place where the unsequenced alanine and serine fit is between the azotyrosine and tryptophan. These positions are consistent, also, with the sequence data of M. Weigert (see Discussion).

As will be discussed below, the sequence of the labeled peptide made us suspicious that the light chain might not be a κ chain as was previously suggested from fingerprint data (Eisen *et al.*, 1968). We therefore investigated the N- and C-terminal regions of the light chain.

*Amino-Terminal Peptide of Whole Light Chain.* Neither performic acid oxidized nor completely reduced and aminoethylated light chains yielded a free amino group when reacted by the Edman procedure. A blocked pentapeptide was recovered in 70% yield from the Dowex 50 eluate of a subtilisin digest of the light chains. This was shown to be a single peptide by analysis of the Dowex 50 eluate on paper which revealed one starch–iodide spot that had not been

TABLE VII: Sequence Homologies—MOPC 315, BALB/C λ, Human λ.[a]

| | 25 | | | | 30 | | | | 35 | | | | 40 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MOPC 315 | Ser- | Asn- | Thr- | Gly- | Ala- | Val- | Thr- | Thr- | Ser- | Asp- | Tyr(Ala,Ser) | | Trp- | Ile- | Glu- | Glu- |
| Balb/c 178 | Ser- | Asn- | Thr- | Gly- | Ala- | Val- | Thr- | Thr- | Ser- | Asx- | Tyr- | Ala- | Asx- | Trp- | Val(Glx,Glx, | |
| Others | | Ser | | | | | | | | | | | | | | |
| Human HA | Gly- | Gly- | Ser- | Ser- | Asn- | Gly- | Thr- | Gly- | Asn- | Asn- | Tyr- | Val- | Tyr- | Trp- | Tyr- | Gln- |
| Others | | Thr | Lys | Thr | Asp | Val | Leu | Arg | Asp | Tyr | Asp | Ala | Ala | | His | His |
| | | Asp | Asn | — | — | — | Ile | Glu | Gly | Thr | Phe | | Cys | | Phe | |
| | | | | | | | Gly | Asn | Lys | Lys | | | Ser | | | |
| | 41 | | | | 45 | | | | 50 | | | | | 55 | | |
| MOPC 315 | Pro- | Asp- | Lys- | His- | Leu- | Phe- | Thr- | Gly- | Leu- | Ile- | Gly- | Gly- | Thr- | Ser- | Asn- | Arg |
| Balb/c 178 | Pro, | Asx, | Lys, | His, | Leu, | Phe) | Thr- | Gly- | Leu- | Ile- | Gly- | Asn- | Thr- | Asn- | Asx- | Arg |
| Others | | | | | | | | | Leu | | Gly | | | | | |
| Human HA | Leu- | Pro- | Gly- | Thr- | Ala- | Pro- | Lys- | Leu- | Leu- | Ile- | Tyr- | Arg- | Asp- | Asp- | Lys- | Arg |
| Others | Arg | | Arg | Ser | Leu | | Val | | Phe | Gln | Arg | Glu | Asn | | | |
| | Lys | | Gln | | Val | | | | | Gly | His | Ser | Gln | | | |
| | His | | | | | | | | | Glu | Val | Asn | Glu | | | |
| | | | | | | | | | | Ser | | | | | | |

[a] The numbering system used is that adopted by M. Weigert. The S 178 and xs 104 sequences are from M. Weigert (personal communication, 1970). The human λ-chain sequences are taken from a review of the literature (Wu and Kabat, 1970).

seen when the map was sprayed initially with ninhydrin. The composition of this peptide as well as the procedure used to sequence it are given in Table VI. The exposure of an amino-terminal alanine after treatment of the peptide with pyrrolidonylcarboxylase is strong evidence that the N-terminal residue is pyrrolidonecarboxylic acid.

*Carboxy-Terminal Residues of Whole Light Chain.* Hydrazinolysis of the 315 light chains gave a 66% yield of leucine. A control human λ chain (λ Kohly) gave 52% serine. Similarly, carboxypeptidase A digestion of aminoethylated or iodoacetamide alkylated chains gave 80–90% yields of leucine after 24 hr. The penultimate cysteine was identified by three approaches: (1) hydrazinolysis of a 24-hr carboxypeptidase A digest of aminoethylated chains, (2) carboxypeptidase A digestion of iodoacetamide alkylated light chains, and (3) carboxypeptidase B digestion of a carboxypeptidase A digest of aminoethylated light chains. In each case yields were comparable to yields obtained with known human λ chains. Even prolonged carboxypeptidase A digestion of reduced iodoacetamide alkylated light chains from which the penultimate cysteines had been cleaved in 30–40% yield failed to give additional amino acids in significant amounts (Table II).

## Discussion

*Sequence Analysis.* The major difficulty encountered in this study was the insolubility of the purified labeled peptide in aqueous solvents. This property, while facilitating purification, led to poor yields from digestions with all enzymes except subtilisin. Higher temperatures and longer digestion times gave better cleavage but apparently led to more extensive breakdown of the released peptides. This may account for our failure to isolate tryptophan-containing peptides although free tryptophan was regularly recovered.

*Position of the Peptide.* The amino acid composition of this peptide, its large size, the presence of lysine, arginine, and tryptophan, and the absence of aminoethylcysteine first suggested to us that the peptide spanned approximately residues 24 to 56 (Goetzl and Metzger, 1970a). The poor homology with limited-sequence data from human κ and λ as well as mouse κ chains made exact positioning impossible. The completion of our own study and the availability of sequence data from several mouse λ chains now allow us to specify the position of this peptide more precisely. The pertinent data are given in Table VII. The extraordinary homology with residues 24–56 from Balb/c λ chains is obvious. The 315 light chain N- and C-terminal analyses are consistent with those of λ (or λ like (see below)) chains and available antisera to light chains from protein 315 and several Balb/c λ chains fail to discriminate between 315 and these other λ chains (E. J. Goetzl and K. R. McIntire, unpublished observations).

The precise position number of the labeled tyrosine is dependent on homologous sequence data of Weigert and colleagues and will ultimately have to be verified by the detailed sequence of the 315 light chain. With these reservations in mind the composite data indicate that the labeled residue is homologous to Tyr-34 of Balb/c λ chains.

*Nature of MOPC 315 Light Chains.* While the sequence data and serological results cited above indicate that the 315 light chains are λ like, the C-terminal leucine is clearly unusual (M. Weigert, personal communication; Appella *et al.*, 1967). It is possible that this leucine resulted from a somatic mutation in the λ genome of the cell which gave rise to tumor 315. The 315 tryptic peptide fingerprints, however, are not characteristic for λ chains (M. Potter, personal communication). These results suggest that there may be multiple differences between the common region of 315 and those of other λ chains. The 315 chain may also contain a new subgroup of mouse λ-variable regions. There is an apparent inversion of amino-terminal residues 4 and 5

(Table VI), and the variation between 315 and all other Balb/c λ chains in the region 24–56 (Table VII) is greater than has been so far noted for these chains. For the 6 Balb/c sequences available in this region (M. Weigert, personal communication), the greatest difference between any two is two substitutions. The 315 chain differs by at least three and as many as five residues from any one of these sequences. Thus, it would appear that the 315 chain might represent a combination of a new common region subtype and a previously undocumented variable region subgroup. The role of strain variation[2] remains to be determined.

*Comparisons with Conventional Anti-DNP Antibodies.* Many similarities between the combining sites of protein 315 and those of conventionally induced anti-DNP antibodies have been cited already (Eisen *et al.*, 1968; Metzger and Potter, 1968; Goetzl and Metzger, 1970a).[3] The isolation and sequencing of the site-labeled peptide and the exact positioning of the labeled tyrosine in protein 315 now permit further comparisons.

Labeling of mouse light chains from anti-DNP antibodies with NBDF led to the isolation of an aspartylazotyrosine dipeptide (Singer and Thorpe, 1968; Thorpe and Singer, 1969). While arguments were advanced for the labeled residue being Tyr-86 there were insufficient data to determine this directly. Recently, two peptides have been isolated from a chymotryptic digest of labeled mouse light chains (S. J. Singer, personal communication) by the antibody-sequestering method (Thorpe and Singer, 1969). They have the *compositions* (Thr, Ser, Asx, and Azo-Tyr) and (Ser, Asx, and Azo-Tyr). It is not known what fraction of the total label is represented by these peptides but they are thought to be among the major labeled fragments. The similarity of these results to ours on protein 315 (see peptides $C_1$ and $C_2$) is provocative though of course not definitive. Even more striking are the results of Franěk and Novotny (1969) on the location of the azotyrosine in NBDF-labeled λ light chains from pig anti-DNP antibodies. Over two-thirds of the label on the light chains was recovered in a peptide whose composition was identical with that of the N-terminal 47 amino acid peptide from λ chains of nonspecific pig IgG. Since there was no tyrosine N terminal to Cys-22, these workers concluded that the labeled tyrosine must lie between positions 23 and 47. The same region also contained one tryptophan.[4] The similarity to the MOPC 315 results is clear.

While the 315 protein is a λ-type γA-immunoglobulin the heterogeneous population of anti-DNP antibodies raised by conventional immunization of mice are overwhelmingly κ-type γG-immunoglobulins. Furthermore, affinity labeling of such conventionally raised anti-DNP leads to labeling of heavy chains twice as frequently as light chains (Singer and Thorpe, 1968). This latter difference has been considered in detail in our previous paper (Goetzl and Metzger, 1970a). Thus, while our present results are encouraging and provide additional support for the usefulness of functional myeloma proteins as models for conventional antibodies, some caution must be exercised before these results are too widely generalized.

*Relationship of Labeled Peptide to Combining Site of Protein 315.* We have previously cited the enhanced rate of reaction of NBDF with protein 315, its reaction with a unique tyrosine on the light chain, the ability to block the reaction with a nonreactive hapten, the partial inactivation of the sites resulting from the NBDF reaction, and the first-order kinetics of the labeling reaction as evidence that the labeling is occurring within the 315 combining sites as these can be operationally defined. In addition the stereochemical similarity of the reagent to known ligands and the proximity of the reactive diazonium moiety to areas of the ligand which can be expected to interact with the combining site are favorable factors. Experience with affinity labeling of enzymes of known crystallographic structure, *e.g.*, chymotrypsin (Lawson and Schramm, 1965; Schoellmann and Shaw, 1963; Erlanger and Cohen, 1963; Matthews *et al.*, 1967), ribonuclease (Crestfield *et al.*, 1963; Marfey *et al.*, 1965; Kartha *et al.*, 1967), and, more recently, staphylococcal nuclease (Cuatrecasas *et al.*, 1969; Cuatrecasas, 1970) has produced evidence that such functional criteria are meaningful. That is, modifications which inactivate the binding site, which can be blocked by substrate, which are specific for a particular amino acid side chain, and which proceed at an unusually rapid rate generally have been found to involve a contact residue or one immediately adjacent to it (Stryer, 1968). The extent of inactivation is not necessarily a measure of the proximity of the modified residue to the site. For example, labeling of Tyr-85 in staphylococcal nuclease produced only partial inactivation, yet this tyrosine appears to be a contact amino acid (P. Cuatrecasas, personal communication). Reagents which closely resemble the native substrates are more likely to label contact amino acids. Thus, the residues labeled in chrymotrypsin with substrate-like reagents are all relevant to the combining site, whereas some of those labeled in staphylococcal nuclease appear only distantly related (P. Cuatrecasas, personal communication). In the latter instance some of the reagents used were rather large and/or resembled known substrates rather poorly.

These functional considerations are complemented by certain structural inferences. Sequence data on mouse and human light chains indicate that there are two and perhaps three "hypervariable" sections in the variable regions of these chains: residues 25–35, 52–55, and 89–96 (reviewed in Wu and Kabat, 1970). The first of these in particular not only

[2] MOPC 315 tumor arose in a mouse which had representation from both Balb/c and C57BL genomes. The $F_1$ mice from a Balb/c × C57BL mating were backcrossed with Balb/c mice for seven generations with selection for C57BL heavy-chain allotypic markers prior to each backcross. The 315 tumor arose in a mouse from this seventh generation.

[3] It has been shown that MOPC 315 resembles rabbit anti-DNP antibodies with qualitatively and quantitatively similar fluorescence quenching and difference spectrum, and high specificity for nitrophenyl ligands (Eisen *et al.*, 1968). The only difference documented so far is the inability of MOPC 315 to bind the dye 2-(2,4-dinitrophenylazo)-1-naphthol-3,6-disulfonate at alkaline pH values, although there is significant binding in the acid pH range. This dye is strongly bound by rabbit anti-DNP and pooled mouse anti-DNP antibodies (Metzger *et al.*, 1963; H. Metzger and M. Potter, unpublished observations) both in the alkaline and acid pH range.

[4] A tryptophan residue has been implicated in the binding sites of both conventional anti-DNP antibodies (Little and Eisen, 1968) and myeloma anti-DNP immunoglobulins (Eisen *et al.*, 1968), including 315, by studies of fluorescence quenching and difference spectra. It is tempting to consider that Trp-37, because of its linear proximity to Tyr-34, plays a critical role in these spectral phenomena.

contains numerous amino acid substitutions but also deletions (or insertions) and is also the most frequent site for attachment of carbohydrate moieties which may be present in light chains on occasion (Melchers, 1969; Sox and Hood, 1970). The proximity of these sections to the N-terminal intrachain disulfide bond has suggested to several authors that these sections may be topologically adjacent, and that they may in turn be related to comparable areas on the heavy chains by an axis of pseudosymmetry (Singer and Thorpe, 1968). It is striking that in three instances where affinity-labeled residues have been positioned with more or less assurance, the label is situated in these suspected regions: in our own study Tyr-34 is labeled; in the study of Franěk and Novotny (1969) a tyrosine between 23 and 47 is implicated; and in a study by Porter and associates on rabbit antibodies to 4-azido-2-nitrophenyl determinants at least some of the label appears to be close to Cys-97 on the heavy chains (R. R. Porter, personal communication).[5]

These considerations taken together make it likely that light-chain Tyr-34 intimately participates in the nitrophenyl binding site of protein 315. The present results appear to represent, therefore, the first substantial sequence of a region directly implicated in an immunoglobulin antigen combining site.

## Acknowledgment

This study was greatly facilitated by the dedicated work of Mr. George Poy who performed most of the amino acid analyses and prepared much of the purified protein 315.

## References

Appella, E., McIntire, K. R., and Perham, R. N. (1967), *J. Mol. Biol. 27*, 391.

Appella, E., and Perham, R. N. (1968), *J. Mol. Biol. 33*, 963.

Bennett, J. C. (1967), *Methods Enzymol. 11*, 330.

Cole, D. R. (1967), *Methods Enzymol. 11*, 316.

Crestfield, A. M., Stein, W. H., and Moore, S. (1963), *J. Biol. Chem. 238*, 2413.

Cuatrecasas, P. (1970), *J. Biol. Chem. 245*, 574.

Cuatrecasas, P., Wilchek, M., and Anfinsen, C. B. (1969), *J. Biol. Chem. 244*, 4316.

de La Llosa, P., Tertrin, C., and Jutisz, M. (1964), *Experientia 20*, 204.

Doolittle, R. F., and Armentrout, R. W. (1968), *Biochemistry 7*, 516.

Dreyer, W. J., and Bynum, E. (1967), *Methods Enzymol. 11*, 37.

Eisen, H. N., Simms, E. S., and Potter, M. (1968), *Biochemistry 7*, 4126.

Endo, S. (1962), *J. Fermentation Technol. 40*, 346.

Erlanger, B. F., and Cohen, W. (1963), *J. Amer. Chem. Soc. 85*, 348.

Franěk, F., and Novotny, J. (1969), *Eur. J. Biochem. 11*, 165.

Goetzl, E. J., and Metzger, H. (1970a), *Biochemistry 9*, 1267.

Goetzl, E. J., and Metzger, H. (1970b), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29*, 438.

Gray, W. R. (1967), *Methods Enzymol. 11*, 139.

Green, N. M. (1969), *Advan. Immunol. 11*, 1.

Kaplan, A. P., and Metzger, H. (1969), *Biochemistry 8*, 3944.

Kartha, G., Bello, J., and Harker, D. (1967), *Nature 213*, 862.

Lawson, W. B,, and Schramm, H. (1965), *Biochemistry 4*, 377.

Little, J. R., and Eisen, H. N. (1968), *Biochemistry 7*, 711.

Marfey, P. S., Uziel, M., and Little, J. (1965), *J. Biol. Chem. 240*, 3270.

Matsubara, H., and Lasaki, R. M. (1969), *Biochem. Biophys. Res. Commun. 35*, 175.

Matthews, B. W., Sigler, P. B., Henderson, R., and Blow, D. M. (1967), *Nature 214*, 652.

Melchers, F. (1969), *Biochemistry 8*, 938.

Metzger, H., and Potter, M. (1968), *Science 162*, 1398.

Metzger, H., Wofsy, L., and Singer, S. J. (1963), *Arch. Biochem. Biophys. 103*, 206.

Moore, T. B., and Baker, C. G. (1958), *J. Chromatog. 1*, 513.

Ramachandran, L. K., and Witkop, B. (1967), *Methods Enzymol. 11*, 283.

Rydon, H. N., and Smith, R. W. G. (1952), *Nature 169*, 922.

Schoellmann, G., and Shaw, E. (1963), *Biochemistry 2*, 252.

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

Sox, H. C., and Hood, L. (1970), *Biochemistry 9* (in press).

Stryer, L. (1968), *Annu. Rev. Biochem. 37*, 25.

Thorpe, N. O., and Singer, S. J. (1969), *Biochemistry 8*, 4523.

Tsung, C. M., and Fraenkel-Conrat, H. (1965), *Biochemistry 4*, 793.

von Ehrenstein, G. (1966), *Cold Spring Harbor Symp. Quant. Biol. 31*, 705.

Woods, K. R., and Wang, K. T. (1967), *Biochim. Biophys. Acta 133*, 369.

Wu, T. T., and Kabat, E. A. (1970), *J. Exp. Med. 132*, 211.

[5] It is interesting to speculate about whether the location of the labeled residue may depend on some general considerations regarding the stereochemistry of the labeling reagent and its orientation in the site.

If the electron microscopic findings on the complexes formed by anti-DNP antibodies and short bis-DNP ligands are interpreted simply, it would suggest that the nitrophenyl group was oriented parallel to the axis of pseudosymmetry mentioned above. The *p*-nitro substituent would point toward the center of the antibody molecule (see Figure 3 in Green (1969)) while the *m*-nitro substituent could be directed toward the light or heavy chain alternatively. In general, then, the immunodominant determinants would tend to point toward the hinge region of the antibody.

If we make the further simplifying assumption that the amino-terminal ends of the heavy and light chains are topologically the most peripheral portions of the molecule it suggests the following. If the reactive group (X) of an affinity-labeling reagent is distal to the immunodominant portion of the reagent (as it is in NBDF) it will tend to react with an amino acid toward the amino-terminal end of the light or heavy chain; if X is ortho to, or actually a part of, the immunodominant determinant (as it is in the 4-azido-2-nitrophenyl reagent) a region further from the amino terminus will be modified. This speculation is consistent with the presently available data and is testable.