Affinity Labeling of a Mouse Myeloma Protein Which Binds Nitrophenyl Ligands. Kinetics of Labeling and Isolation of a Labeled Peptide*

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ABSTRACT: MOPC 315, a mouse myeloma IgA protein which binds nitrophenyl ligands, has been purified by affinity chromatography and affinity labeled with tritiated *m*-nitrobenzenediazonium fluoroborate.

The kinetics of labeling have been shown to follow the theoretically predicted curve at high reagent to protein concentration ratios. This is considered further evidence of the homogeneity of the binding sites. Labeling results in a 50-fold reduction in K_A, but the valence is unchanged from that of the native protein.

The labeled protein also exhibits the difference spectrum characteristic of native protein with dinitrophenylcaproate, but the extinction coefficient of the protein–ligand complex is reduced significantly. Labeling of only the light chain was confirmed. A tryptic peptide isolated from the labeled light chains contains all of the azotyrosine label. The composition of this peptide shows that it was derived from the variable region of the light chain.

The $MOPC_{315}$ protein is one of several human or mouse myeloma or monoclonal macroglobulin proteins to be described as capable of binding nitrophenyl ligands (Eisen *et al.*, 1967, 1968; Ashman and Metzger, 1969). This mouse IgA[1] immunoglobulin satisfies the other criteria for immunochemically defining an antibody in having high specificity and rigid stoichiometry for ligand binding, and localization of this binding to the Fab fragments. The characteristic difference spectra and fluorescence quenching seen with conventional antidinitrophenyl (anti-DNP) antibodies (Little and Eisen, 1967) have been obtained with this protein (Eisen *et al.*, 1968). Careful studies of purified $MOPC_{315}$ protein have shown homogeneous binding constants for all sites (Eisen *et al.*, 1968). Affinity labeling of this protein has been accomplished and found to be directed to tyrosine(s) on the light chains, resulting in partial site inactivation (Metzger and Potter, 1968). The present paper reports the kinetics of the affinity labeling, studies of the combining activity of the labeled protein, and isolation of a labeled tryptic peptide from the light chains. In addition, a method is described for the production and isolation of large quantities of this myeloma protein of suitable purity for structural studies.

Methods

Maintenance of Tumor. The plasmacytoma $MOPC_{315}$ was propagated in Balb/c mice by injecting 0.1 ml of a tumor homogenate in Locke's solution subcutaneously over the abdominal wall in the midline. This same tumor homogenate was injected intraperitoneally to produce ascites. Substantial ascites formed by 28 days on the average and the mice were routinely tapped for 1.5 ml on two or sometimes three occasions separated by 3 or 4 days. An average total yield of 3 ml/surviving mouse was obtained.[2] Ascites was frozen or alternatively stored for a few days at 4° prior to purification.

Purification of Protein 315. Several methods were tried using either the polymerized (native) or depolymerized (reduced alkylated) form of IgA protein and various immune adsorbents (see Results). The final procedure used was as follows. Ascites was dialyzed against 0.2 M Tris-HCl buffer (pH 8.6) and reduced with 0.01 M dithiothreitol for 1 hr at 25°. The solution was then diluted 1:1 with 0.2 M Tris-HCl buffer (pH 7.3) to achieve a final pH of 8.0. Sufficient iodoacetamide in distilled H_2O was then added to give a final molarity of 0.011 (Miller and Metzger, 1965). After 15 min at 25°, the solution was dialyzed *vs.* borate–NaCl buffer (pH 8.0). The reduced and alkylated ascites were diluted 1:1 with buffer and then applied to an ϵ-*N*-DNP-lysine–Sepharose adsorbent (below) equilibrated with the same buffer. Routinely 100 ml of diluted ascites was applied to a 150-ml (2.2 × 39 cm) column of the adsorbent. After washing with five to six bed volumes of buffer the effluent had less than 0.05 absorbancy unit at 280 mμ. The 315 protein was then specifically eluted by adding 0.1 M DNP-glycine (titrated to pH 8.6); 1 ml of the hapten solution/15 ml of the adsorbent column was used and the yellow eluate was collected, concentrated to 20 ml, and extensively dialyzed against 0.1 N NaCl–0.01 M sodium phosphate buffer (pH 5.7). The small amount of residual bound DNP-glycine was removed by passing the solution over a Dowex 1-X8 (20–50 mesh) column equilibrated with the same pH 5.7 sodium phosphate–NaCl buffer. A 10-ml column was adequate for processing 800 absorbancy units of protein.

Immunoadsorbents. Nitrophenyl–Sepharose adsorbents were

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[1] The standard nomenclature for immunoglobulins is used. Other abbreviation is: [^{3}H]MNBDF, tritiated *m*-nitrobenzenediazonium fluoroborate.

[2] An average of 50–60% of all injected mice survived to the stage of harvesting ascites.

prepared as follows (Cuatrecasas *et al.*, 1968; Cuatrecasas and Wilchek, 1968). Sepharose 4B (Pharmacia) was allowed to settle in distilled water to remove slowly sedimenting particles. Gravity-packed Sepharose (100 ml) was suspended in an equal volume of distilled water and the pH was adjusted to 11.0 with 4 N NaOH. A saturated solution of cyanogen bromide (125 ml) in distilled water (pH 11.0) was added to the Sepharose with stirring. The pH was maintained at 11.0 by the addition of 4 N NaOH over a 5–6-min period. The Sepharose was rapidly filtered dry on a sintered-glass funnel and washed with 300 ml of 0.1 N $NaHCO_3$. The activated Sepharose was quickly added to 150 ml of a solution containing either ϵ-*N*-PNP-lysine (80 mg dissolved in 0.1 N Na_2CO_3, pH 10.5) or ϵ-*N*-DNP-lysine (100 mg dissolved in 0.1 N $NaHCO_3$, pH 8.5), with stirring. It was then reacted 15–16 hr with magnetic stirring at 4°. After this it was filtered, washed with 100 ml of methanol and 500 ml of borate–saline buffer, and packed in columns.

Since the total number of absorbancy units added was known, the amount of derivatization could be estimated by subtracting the absorbancy units remaining in the pooled washes. Molar extinction coefficients of 1.75×10^4 at 362 mμ for ϵ-*N*-DNP-lysine and 2.04×10^4 at 418 mμ for ϵ-*N*-PNP-lysine were used for purposes of calculation (Ashman and Metzger, 1969). Per 100 ml of gravity-packed Sepharose, approximately 12–35 mg (4.5×10^{-2}–15×10^{-2} mmole) of PNP-lysine or 22–48 mg (7×10^{-2}–15×10^{-2} mmole) of DNP-lysine was conjugated using this procedure.

In our initial studies we found that upon application of the diluted ascites to the adsorbent, yellow color, having the characteristic spectrum of DNP-lysine, was evident in the effluent. Bovine serum albumin produced the same effect, and prevented subsequent elution of color by ascites added within 24 hr. The following washing procedure was therefore devised: 30 ml of 1% bovine serum albumin (Armour) in borate–sodium chloride buffer (pH 8.0) was added to the 150-ml Sepharose column and washed through with 300 ml of buffer; 40 ml of 25% acetic acid was then added and the column was washed with an additional 750 ml of buffer overnight. The purification of the 315 protein was conducted the following morning. After about six such purifications the albumin–wash procedure was found to be only necessary after every fourth or fifth purification run. Acetic acid washing was used after every run, however. On the average 100 absorbancy units (280 mμ) was removed by this procedure.

Equilibrium Dialysis. Equilibrium dialysis was performed using Lucite dialysis chambers (Drummond Scientific Co.) of 0.1-ml capacity separated by 0.0008-in. Visking membranes. Hapten ([^{3}H]DNP-caproate) concentrations on both sides of the membrane were determined and the data were plotted by the method of Scatchard. The lines were drawn by the method of least squares. A mole of protein 315 was defined on the basis of an extinction coefficient (0.1%) at 280 mμ of 1.44 and a molecular weight of 1.2×10^5 daltons (Eisen *et al.*, 1968). The solvent was 0.01 M sodium phosphate–0.15 N NaCl buffer (pH 7.4) and dialyses were conducted at 4° for 18 hr.

Spectral Analyses. Absorbancies were routinely determined with a Zeiss PMQ_{II} spectrophotometer. Difference spectra were recorded with a Cary Model 14 recording spectrophotometer using pairs of quartz cells held in tandem in a special holder.

Immunoelectrophoresis. Immunoelectrophoresis was performed in the routine manner (Scheidegger, 1955).

Affinity-Labeling Studies. All labeling experiments were performed at 0° in an ice bath using purified protein in 0.5 M sodium acetate buffer (pH 5.0). The stock solution of labeling reagent was always tritiated [^{3}H]MNBDF at a concentration of 1.95×10^{-3} M in 0.001 M HCl. In kinetic studies, the labeling reagent at 0° was rapidly pipetted with a chilled lambda pipet into 0.5 ml of protein solution at 0°. The test tube was quickly shaken twice in the ice bath and the timing was started simultaneously. The reaction was stopped by the addition of a 500-fold excess of resorcinol added as a 0.7 M solution in 0.5 M sodium acetate buffer (pH 5.0). In these experiments, the protein was precipitated at 0° by the addition of absolute ethanol to achieve a final concentration of 70%. The labeled protein was then processed as previously described (Wofsy *et al.*, 1962; Metzger *et al.*, 1963). Counting and $OD_{280\,m\mu}$ measurements were performed after dissolving the precipitates in 0.5% sodium dodecyl sulfate in 0.02 M sodium phosphate buffer (pH 6.2) at 37°. Internal quench correction was carried out with [^{3}H]toluene (New England Nuclear). In all analytical experiments, a human IgG myeloma protein (IgG_{Waz}) was used as a control to estimate the degree of nonsite-directed labeling.

In preparative experiments, after resorcinol quenching, the labeled protein was dialyzed exhaustively against 0.01 M phosphate-buffered saline (pH 7.4).

Kinetic Analyses. The pertinent reactions for site-directed labeling of antibody (or enzyme) combining sites are as follows (Metzger *et al.*, 1963)

$$\mathrm{HpX} + \mathrm{Ab} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{C} \xrightarrow{k_3} \mathrm{L} \qquad (1)$$

where HpX is the unreacted labeling reagent, Ab the free active sites, C the specific reversible complex, L the irreversibly labeled product, and the k's are the rate constants for the reactions indicated. The initial rate of labeling, $\dot{L}_0$, according to eq 1 is given by

$$\dot{L}_0 = k_3[\mathrm{C}_0] \qquad (2)$$

where C_0 is the concentration of the reversible complex formed immediately upon mixing the antibody and HpX. The value of C_0 is of course determined by the relation

$$[\mathrm{C}_0] = K_\mathrm{A}([_1\mathrm{HpX}] - [\mathrm{C}_0])([_1\mathrm{Ab}] - [\mathrm{C}_0]) \qquad (3)$$

where K_A equals the association constant (k_1/k_2 in eq 1) and $[_1$HpX] and $[_1$Ab] are the initial concentrations of HpX and of Ab sites, respectively. At two different concentrations of HpX and Ab

$$\frac{[_1\mathrm{C}_0]}{[_2\mathrm{C}_0]} = \frac{([_1\mathrm{HpX}] - [_1\mathrm{C}_0])([_1\mathrm{Ab}] - [_1\mathrm{C}_0])}{([_2\mathrm{HpX}] - [_2\mathrm{C}_0])([_2\mathrm{Ab}] - [_2\mathrm{C}_0])} \qquad (4)$$

By eq 2, $[_1\mathrm{C}_0]/[_2\mathrm{C}_0] = {}_1\dot{L}_0/{}_2\dot{L}_0 = R$. We can write $_1$HpX and $_1$Ab as $W[_2$HpX] and $V[_2$Ab], respectively, so that $_2C_0$ can be determined by solving the quadratic

$$[_2\mathrm{C}_0^2](R^2 - R) + [_2\mathrm{C}_0][R(([_2\mathrm{Ab}] + [_2\mathrm{HpX}]) - (V[_2\mathrm{Ab}] + W[_2\mathrm{HpX}]))] - (R - WV)[_2\mathrm{HpX}_2\mathrm{Ab}] = 0 \qquad (5)$$

and K_A can be determined by eq 3.

For a homogeneous system reacting by eq 1 the integrated rate expression for the concentration of L is (Metzger *et al.*, 1963)

$$\frac{1}{K_A\sqrt{-q}} \ln\left(\frac{2x-\alpha}{2x-\beta}\right)\left(\frac{[2C_0]-\beta}{[2C_0]-\alpha}\right) - \ln\left(\frac{x^2 - Sx + P}{[C_0^2] - S[C_0] + P}\right) = k_3 t \quad (6)$$

where $L = x - K_A([_iAb] - x)([_iHpX] - x)$. Here $\alpha = S + \sqrt{-q}$, $\beta = S - \sqrt{-q}$, $q = 4P - S^2$, $S = [_iAb] + [_iHpX]$, $P = [_iAb][_iHpX]$, x is the concentration of occupied sites, t is the time in seconds after mixing the reactants at the initial concentrations $[_iAb]$ and $[_iHpX]$, and $[C_0]$ is defined as before. When $[_iAb] = [_iHpX]$ the above expression cannot be used and instead the appropriate expression is

$$\frac{1}{K_A}\left(\frac{1}{[_iAb] - x} - \frac{1}{[_iAb] - [C_0]}\right) - 2 \ln\left(\frac{[_iAb] - x}{[_iAb] - [C_0]}\right) = k_3 t \quad (6a)$$

where all the terms are as defined above. It is assumed in the above relations that reaction at only one amino acid chain in each combining site predominates.

Using eq 6 theoretical curves for the labeling reaction for various values of K_A and k_3 were generated using a computer program.

Separation of Polypeptide Chains. After affinity labeling 200 mg of protein, exhaustive dialysis was carried out against 0.1 M acetic acid. The protein solution was then made 6 M in urea by the addition of crystalline reagent and applied to a 412-ml (2.5 × 84 cm) G-200 Sephadex reverse-flow column equilibrated in 6 M urea–0.1 M acetic acid.

Reduction of Intrachain Disulfides, Aminoethylation. Light chains, which contained the major portion of the label, were dialyzed against 0.1 M acetic acid, lyophilized, and 50 mg was dissolved in 5 ml of 7 M guanidine hydrochloride buffered with 0.2 M Tris-HCl at pH 8.6. The intrachain disulfide bonds were reduced by adding 77.0 μl of a 0.065 M solution of dithiothreitol to the light-chain solution (final dithiothreitol concentration 0.001 M) and allowing the reduction to proceed under N_2 for 15 min. At this point, 75-μl aliquots of ethyleneimine were introduced three times at 20-min intervals (Raftery and Cole, 1963). The light chains were freed of excess reagent by passing them over a 100-ml G-10 Sephadex column equilibrated with 1.0 M acetic acid.

Tryptic Digestion. The light chains were lyophilized and 50 mg of aminoethylated light chains was dissolved in 1.0 ml of 8 M urea buffered at pH 8.0 with 0.05 M Tris-HCl. This solution was diluted with 9.0 ml of 0.05 M Tris-HCl buffer (pH 8.0) which was 0.01 M in $CaCl_2$. Trypsin (0.5 mg) was added to the now opalescent fluid followed by incubation at 37° for varying lengths of time. In some experiments, a second 0.5 mg of trypsin was added at 7 hr and the digestion was allowed to proceed at 37° for 14–20 hr. In preparative runs the chains were digested with one dose of trypsin for 45 min. Digestions were stopped by adding 10 ml of 10 M urea, and applying the sample to a 600-ml (3.8 × 52.5 cm) G-50 Sephadex column equilibrated in 8 M urea. The major peak of radioactivity was further purified on a 16-ml QAE (1.0 × 20 cm) Sephadex column equilibrated with 0.2 M pyridine acetate (pH 7.6) buffer.

The column was cleared of urea by washing with two bed volumes of pyridine acetate buffer. It was then developed with a concave gradient by adding 0.1 M acetic acid to the starting buffer reservoir which had a volume of 800 ml. Ninhydrin determinations were performed on aliquots from fractions over the entire gradient pattern to pH 5.0; 1 ml of a standard ninhydrin reagent (Moore and Stein, 1954) was added to 1 ml of peptide solution or 0.2 ml of solution after alkaline hydrolysis (13 N NaOH, 20 min at 120°). After reacting 15 min in a boiling-water bath, the absorbance was measured at 570 mμ. If necessary, appropriate dilution was performed with 50% ethanol prior to spectrophotometric determinations. The major peak of radioactivity was further purified after lyophilization by dissolution in 10% pyridine and dropwise titration with glacial acetic acid to pH 3.0 which resulted in the precipitation of essentially all of the radioactivity and color after 24–72 hr at 0°.

Quantitation of Labeled Peptide. The specific activity of the light chains (counts per minute per mole of light chain) was determined by two methods. After reduction and aminoethylation, light chains were lyophilized from 1 N acetic acid and dried for 72 hr over silica gel at 37°. A 5-mg portion was dissolved in 8 M urea and aliquots were counted in triplicate. Assuming a light-chain molecular weight of 22,500, a specific activity was calculated from this data. Some of the same dried sample was dissolved in 0.1 N sodium hydroxide, and the concentration of azotyrosine was quantitated from the absorbancy at 490 mμ based on the known extinction coefficient (Good *et al.*, 1967). Aliquots of this solution were also counted in triplicate with internal quench correction ([^{3}H]toluene). Assuming 1 mole of azotyrosine label/active site, and 1.2 sites/2 moles of light chain a specific activity (counts per minute per mole of light chain) was calculated. At equivalent quench levels, the spectral and dry weight specific activities always agreed within ±3%. The peptide could be quantitated from the above specific activity.

Characterization of Labeled Peptide. High-voltage electrophoresis and descending paper chromatography were carried out by standard methods (Bennett, 1967).

Similarly 6 N HCl hydrolysis of the peptide was performed in the routine manner and released amino acids quantitated on a Beckman 120C Autoanalyzer.

Tryptophan was determined using either a spectral method (Edelhoch, 1967) or by amino acid analysis of a 6 N HCl hydrolysate which had been made 5% in thioglycollic acid (Matsubara and Lasaki, 1969). In the former method, peptide was prepared by passage through a G-10 Sephadex column equilibrated with 6 M guanidine buffered with 0.02 M sodium phosphate (pH 6.5). The spectrum of the model compound *N*-chloroacetyl-*m*-nitrophenylazotyrosine, synthesized as previously described (Traylor and Singer, 1967), was taken in the same buffered 6 M guanidine and subtracted from the spectrum of an equimolar concentration of peptide I. For the acid hydrolyses, egg-white lysozyme was used as a standard. The average yield of tryptophan from triplicate hydrolyses was 71% of the expected number (Matsubara and Lasaki, 1969; Canfield, 1963).

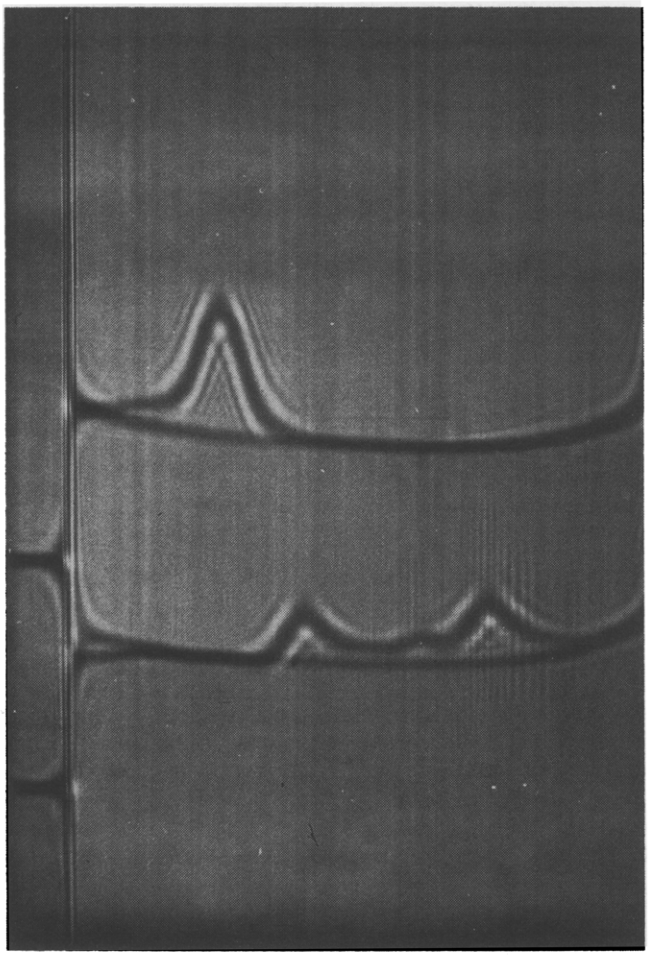

FIGURE 1: Ultracentrifuge study of reduced-alkylated monomer (top cell) and PNP-lysine–Sepharose-purified polymer (bottom cell). The photograph was taken after 32 min at 56,000 rpm in a Spinco Model E centrifuge. The buffer was 0.2 M Tris (pH 8.6), and the temperature was 28.6°. Sedimentation from left to right.

Materials

Mice bearing the $MOPC_{315}$ plasmacytoma were the generous gift of Dr. Michael Potter (National Cancer Institute, National Institutes of Health).

A rabbit anti-whole ascites antiserum was produced by injecting a 10-mg total of protein 315 in Freund's adjuvant (Difco) into the footpads of two white rabbits. These animals were boosted at 3 weeks after initial immunization with another 10 mg of ascites protein given intravenously. They were bled 1 week later and the sera were pooled.

The labeling reagent [^{3}H]MNBDF was synthesized and its specific activity was quantitated by previously described methods (Traylor and Singer, 1967). It was stored as a 1.95×10^{-3} M solution in 0.001 M HCl at −20°. The specific activity was 17.5 mCi/mmole. ϵ-*N*-DNP-L-lysine, *N*-DNP-ϵNH_2-caproate, [^{3}H]N-DNP-ϵNH_2-caproate, and DNP-glycine were synthesized by previously described methods (Porter, 1950; Ashman and Metzger, 1969).

ϵ-*N*-PNP-L-lysine was synthesized by adding 1.08 g of 1-fluoro-4 nitrobenzene in 30 ml of absolute ethanol to 3.54 g of α-*N*-carbobenzoxy-L-lysine (Cyclo Chemical Corp.) dissolved in 0.5 M Na_2CO_3 (pH 11.0). After stirring for 72 hr at 60° in the dark the solution was flash evaporated to 30 ml, titrated to pH 9.0 with 50% HCl, and extracted twice with 50 ml of ether to remove unreacted 1-fluoro-4-nitrobenzene. The pH was lowered to 1.5 by further addition of 50% HCl and the product was extracted with 50 ml of dichloroethane. This was flash evaporated to an oil, dissolved in 60 ml of glacial acetic acid, and reacted with 20 g of HBr for 1 hr at 25° to remove the carbobenzoxy group (Greenstein and Winitz, 1961). The mixture was flash evaporated repeatedly after serial additions of 30 ml of H_2O to remove HBr completely.

Upon raising the pH to 7.0 with 2 N NaOH the yellow product precipitated rapidly and was recrystallized twice from boiling water. The yellow powder crystals melted at 181° with a color change at 165–167°. At pH 8.5 it had a molar absorption of 20,400 at 418 mμ.

S-Aminoethylcysteine was synthesized from L-cysteine-HCl (5.7 M in 0.01 N NaOH) and a 1.5 molar excess of ethyleneimine under N_2. The product was crystallized as described previously (Cavallini *et al.*, 1955) and had a melting point of 192°.

Dithiothreitol was a Calbiochem product and was sublimated prior to use. [^{14}C]Iodoacetamide was purchased from New England Nuclear and recrystallized with [^{12}C]iodoacetamide from water. It had a final specific activity of 1 mCi/mmole. Ethyleneimine was purchased from Matheson, Coleman & Bell. Lysozyme was a Schwarz BioResearch, Inc., product. Trypsin which had been treated with L-(tosylamido-2-phenyl)ethyl chloromethyl ketone was purchased from Worthington Biochemicals Corp.

Results

Purification of Protein 315. Unreduced ascites contain a large fraction of the IgA as a heterogeneous polymer. When euglobulin from these unreduced ascites was applied to a DNP-lysine–Sepharose column, DNP-glycine removed only 5–8%, and PNP-caproate only 6–10% of the total bound protein. DNP-caproate removed 55–70% of the bound protein; however, neither exhaustive dialysis nor dialysis and ion-exchange columns could completely separate the myeloma protein from the bound DNP-caproate. PNP-lysine–Sepharose columns bound only the polymeric IgA protein, with a resulting loss of the monomer fraction which amounted to 10–35% of the total IgA. This latter point was demonstrated by ultracentrifugal studies of the PNP–Sepharose-purified protein, using reduced-alkylated monomer IgA as a reference standard (Figure 1). The heterogeneous polymer which bound to PNP–Sepharose was clearly free of any monomer IgA. This native monomer could be purified by passing the effluent and wash buffer from the PNP columns over a DNP–Sepharose column and eluting it with DNP-glycine. Purified native monomer was identical with reduced-alkylated monomeric IgA when compared by immunoelectrophoresis. There was a tendency for the native monomer to aggregate and precipitate at ionic strengths lower than 0.3, regardless of pH. A single sharp band, identical with reduced-alkylated monomer, was obtained when the native monomer was electrophoresed at higher ionic strengths.

For highest yields and easiest hapten–protein separations, partially reduced-alkylated IgA was put over DNP-lysine–Sepharose columns, and eluted with DNP-glycine. The

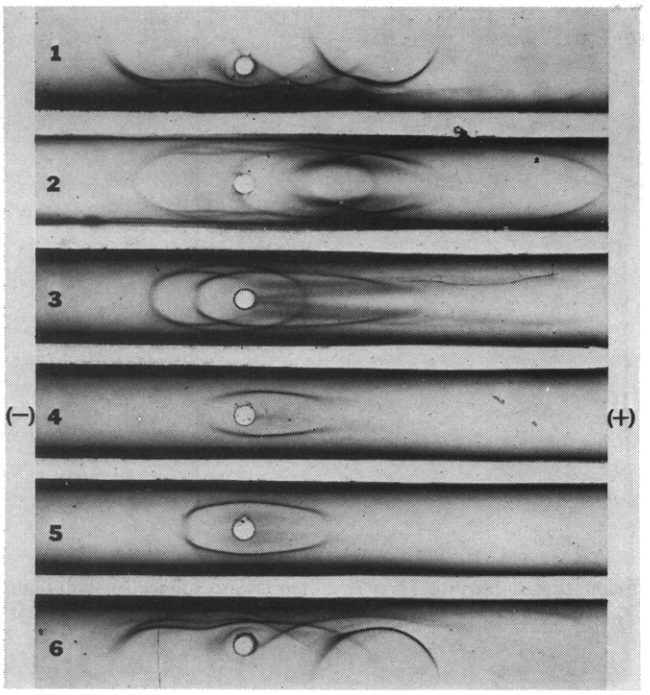

FIGURE 2: Immunoelectrophoresis of 315 protein preparations. The anode is at the right. Wells contain (from top to bottom): (1) crude ascites (40 mg/ml), (2) 58% saturated ammonium sulfate fraction (20 mg/ml), (3) euglobulin preparation from 2 (20 mg/ml), (4) native polymer from PNP-lysine–Sepharose column (5 mg/ml), (5) reduced-alkylated monomer (6 mg/ml), and (6) ascites (40 mg/ml). All troughs contained rabbit anti-whole ascites serum.

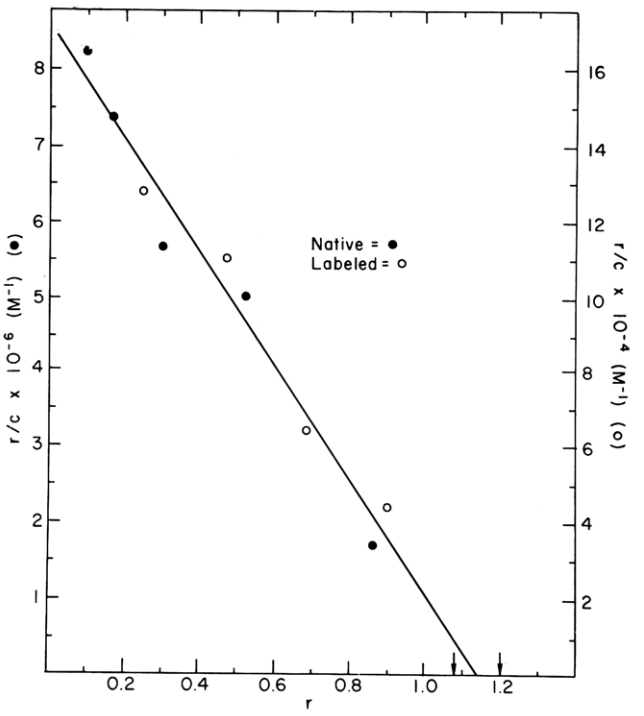

FIGURE 3: Equilibrium dialysis of native and labeled protein 315. The hapten was [^{3}H]DNP-caproate, and the solvent was 0.01 M sodium phosphate–0.15 M NaCl (pH 7.4). r = moles of bound hapten/mole of protein. The left arrow on the abscissa indicates the exact valence of the labeled protein and the right arrow shows the native protein valence.

purity of this IgA can be seen by immunoelectrophoresis developed with anti-whole ascites serum (Figure 2). This slide also demonstrates the impurities remaining after the ammonium sulfate and euglobulin purification steps. These steps were not used in subsequent preparative runs, and reduced-alkylated crude ascites was applied directly to the DNP-lysine–Sepharose columns. The complete protocol is given in Methods. Average yields were 3.0–4.0 absorbancy units (at 280 mμ)/ml of undiluted ascites.

Properties of Protein 315. An extinction coefficient was determined on the purified material using the dry weight method. The extinction coefficient at 280 mμ for a 0.1% solution was 1.44 in borate-buffered saline (pH 8.0) and 1.21 in 0.5% sodium dodecyl sulfate buffered at pH 6.2. A bovine serum albumin control measured in 0.25 N acetic acid was within 1.5% of the literature value (Foster and Sterman, 1956).

Equilibrium dialysis was performed using [^{3}H]DNP-caproate. Typical data are given in Figure 3. The K_A (7.5×10^6) cannot be directly compared with that obtained in a previous study since the latter employed DNP-L-lysine, but the valence is in excellent agreement with the value of 1.2 obtained in that work (Eisen *et al.*, 1968).

Sedimentation studies were performed in a Spinco Model E ultracentrifuge. The sedimentation pattern of the reduced-alkylated column purified monomer 315 (Figure 1) shows one major peak at 6.6 S. There is some trailing of lighter material, which may represent dissociated light chains.

Affinity Labeling of Protein 315. It was previously reported that protein 315 reacted rapidly with the reagent MNBDF (Metzger and Potter, 1968). The reaction appeared to yield exclusively the azotyrosine derivative, led to partial inactivation of the combining sites, and could be markedly inhibited by the presence of the nonreactive competing ligand DNP-caproate.

We now report more detailed studies on the labeling reaction and the properties of the affinity-labeled protein.

Kinetic Studies. In order to study whether the labeling reaction followed the theoretically expected rate (see eq 6 in Methods) it was necessary to determine the association constant of protein 315 for the reagent MNBDF. This was obtained by comparing the initial rates of labeling at two different reagent–protein concentrations. At a protein concentration of 4.04×10^{-5} M (7.0 OD_{280}/ml) and 2.02×10^{-5} M (3.5 OD_{280}/ml) and MNBDF concentrations of 5.68×10^{-5} and 2.84×10^{-5} M, respectively, the ratio of the initial rates of labeling was 3.7 ± 0.2 during the first 5 min. By eq 3 and 5 (Methods) a K_A of 1.8×10^3 M^{-1} was calculated.

Figure 4 shows the kinetics of labeling at a protein concentration of 3.02×10^{-5} M and MNBDF concentration of 4.67×10^{-5} M. The rate constant, k_3, was determined to be 1.2×10^{-2} sec^{-1}. The theoretical curve for the K_A and k_3 determined is shown as well as theoretical curves for various multiples of the K_A and k_3. The experimental data do not fit any of the theoretical curves adequately. For a variety of reasons (see Discussion) we postulated that a better fit might be obtained at higher reagent: protein concentrations. Figure 5 gives the result of such an experiment in which the

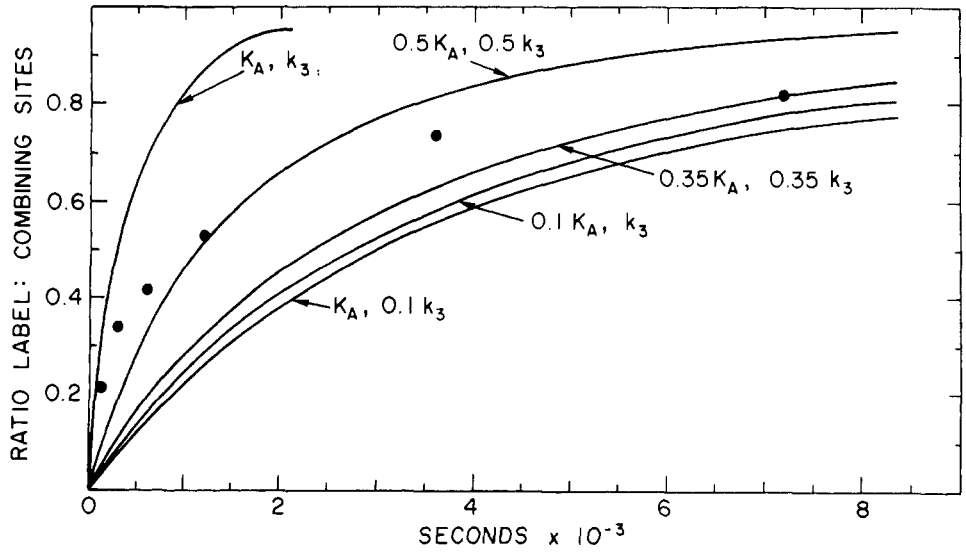

FIGURE 4: The kinetics of labeling protein 315 at a low reagent to protein concentration ratio. Theoretical curves are solid lines with data points as closed circles (●). The concentrations were protein 315 = 3.02×10^{-5} M, and MNBDF = 4.67×10^{-5} M. The buffer was 0.5 M sodium acetate (pH 5.0).

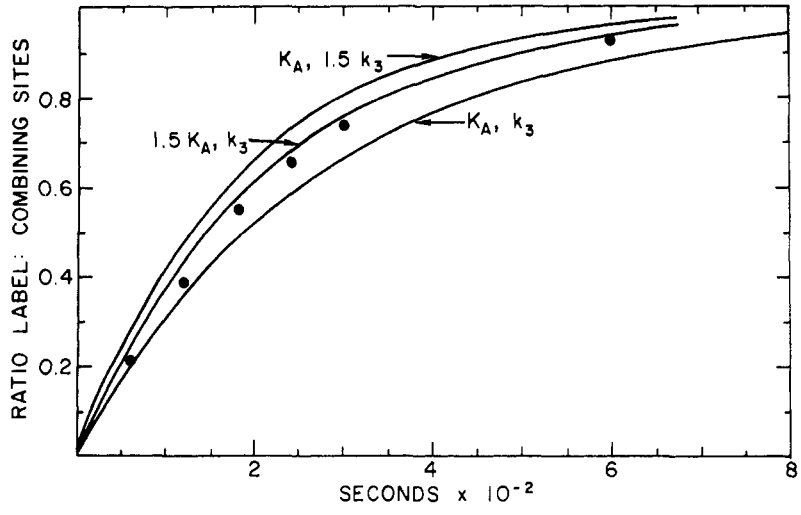

FIGURE 5: The kinetics of labeling protein 315 at a high reagent to protein concentration ratio. The actual data points are shown as closed circles (●) between three theoretical curves. The concentrations were: protein 315 = 1.55×10^{-5} M, MNBDF = 2.54×10^{-4} M. The buffer was 0.5 M sodium acetate (pH 5.0).

protein concentration was 1.55×10^{-5} M and the reagent concentration 2.54×10^{-4} M. It is clear that the experimental data now fit eq 6 (Methods) considerably better.

Properties of Affinity-Labeled Protein 315. A preparation of affinity-labeled protein 315 was studied by equilibrium dialysis with [^{3}H]DNP-caproate. Surprisingly it was found that the combining sites had not been fully inactivated but instead showed a 50-fold reduction in combining affinity. The results of this study are plotted in Figure 3 along with the data on the unlabeled preparation. We considered the possibility that affinity labeling of one site on the myeloma protein had simply facilitated detection of a second preexisting site on the same molecules. We therefore restudied the native preparation at sufficiently high concentrations of hapten so that sites having a 50-fold lower intrinsic association constant than that previously determined could be readily detected. Under these conditions however the valence remained 1.14 ± 0.03.

Combining activity of the fully labeled protein 315 could also be detected by difference spectroscopy. Figure 6 shows the difference spectra for DNP-caproate bound either to the native or to the affinity-labeled protein 315. The spectra for both preparations show the distinctive maxima at 381 and 468 mμ and the minimum at 405 mμ previously described (Eisen *et al.*, 1968).

Extinction coefficients for the difference peaks at 468 mμ for the native and labeled proteins were calculated. The concentration of complex was in each case calculated on the basis of the association constant found by equilibrium dialysis. For the native protein, at a concentration of 1.64×10^{-5} M and a DNP-caproate concentration of 1.39×10^{-5} M, 80% site saturation was expected. For the labeled protein 315, a site saturation of 46% was expected using a protein concentration of 3.49×10^{-5} M and a DNP-caproate concentration of 2.22×10^{-5} M. For the native protein the extinction coefficient per mole of bound hapten was 990 ± 43 and for the labeled protein 447 ± 3.

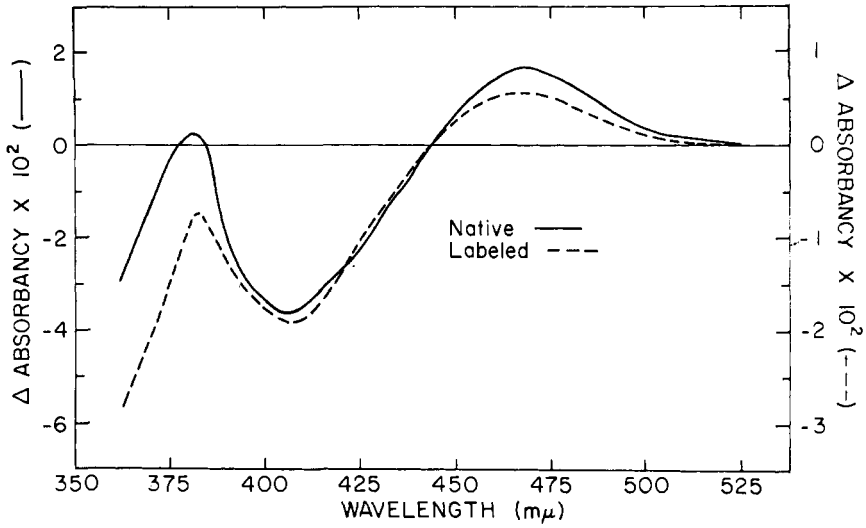

FIGURE 6: Difference spectra of labeled and native 315 protein. The spectra are corrected to the same base-line reading at 525 mμ. In each case 100 μl of DNP-caproate (2×10^{-4} M) was added to 1 ml of protein solution. Concentrations initially were 1.90 mg/ml of native and 3.26 mg/ml of labeled protein. The solvent was 0.01 M sodium phosphate–0.15 M NaCl (pH 7.4).

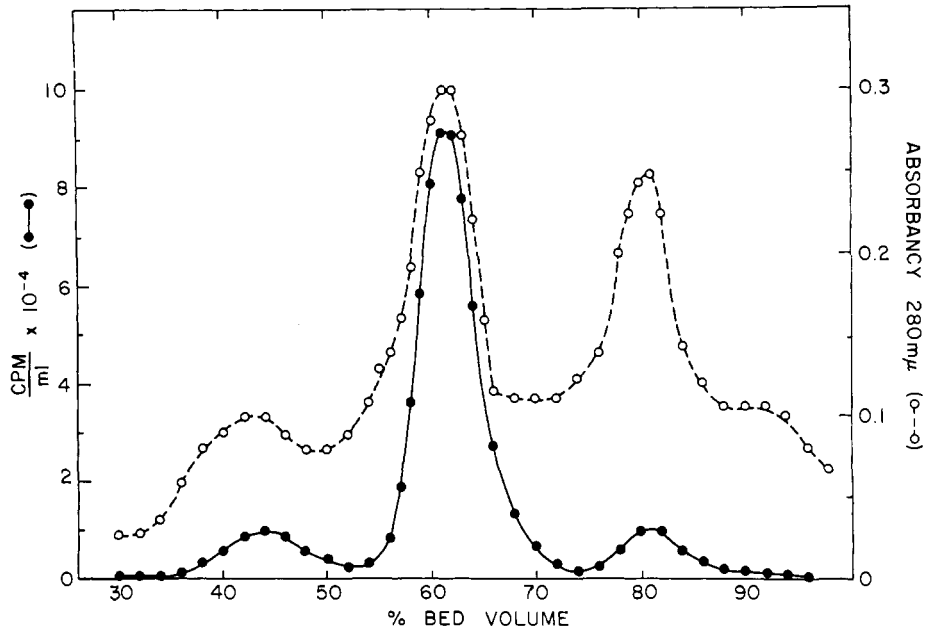

FIGURE 7: Sephadex G-50 fractionation of a 45-min tryptic digest of 315 light chains. The column was equilibrated and developed with 8 M urea. Counting was in Bray's solution without quench correction. The first peak represents undigested light chains, and the second and third peaks are peptide I and II, respectively.

Localization and Isolation of the Labeled Tyrosine Peptide. For all the preparative work to be described, protein 315 labeled at a 1.6:1 reagent to site ratio was employed. It was previously reported that the labeled tyrosine(s) appeared to be located predominantly on the light chains of protein 315 (Metzger and Potter, 1968). Repeated chain separations performed in the current study confirmed this observation. Over 90% of the total tritium counts were routinely found under the light-chain peak.

The labeled residue was isolated in the form of a tryptic peptide as follows. Conditions were sought under which complete reduction of the two intrachain disulfides could be reproducibly achieved in the absence of significant alteration of the azotyrosine label. Using alkylation with [^{14}C]iodoacetamide of released sulfhydryls as an assay for the cleavage of the disulfide bonds, it was found that 0.001 M dithiothreitol for 15 min at pH 8.6 in 7 M guanidine-HCl–0.2 M Tris-HCl was adequate to achieve complete reduction. Analysis indicated 3.9 ± 0.4 released sulfhydryls/light chain under these conditions. Alkylation either before reduction or after aminoethylation yielded less than 1% of the counts found after reduction and prior to aminoethylation testifying to the

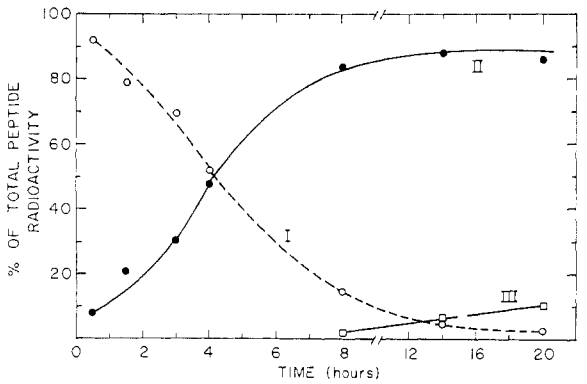

FIGURE 8: Time course of tryptic digestion of labeled light chains from protein 315. The per cent total radioactivity was corrected for the radioactivity in the undigested light-chain peak and expressed as per cent peptide radioactivity. The digestion mixture was buffered in 0.05 M Tris-HCl–0.01 M $CaCl_2$ (pH 8.0). Digestion at 37°.

adequacy of the analysis and the completeness of aminoethylation. It is known that there are two intrachain disulfide bridges in mouse light chains (Dayhoff, 1969).

Reduced-aminoethylated labeled light chains were digested with trypsin for varying time periods prior to applying them to a G-50 Sephadex column equilibrated with 8 M urea. The solvent was 0.05 M Tris-HCl (pH 8.0) which was 0.01 M in $CaCl_2$, and 1% by weight trypsin was employed. Figure 7 illustrates the elution pattern obtained after 45–50-min digestion. About 10% of the radioactivity due to the labeled tyrosine is eluted at the position of undigested light chain, 83% as peptide I, and 7% as peptide II (and 0% as peptide III) (Figure 8). Figure 8 illustrates the kinetics of the digestion over 20 hr. It can be seen that from 30 min to 3 hr peptide I predominates and that thereafter peptide II becomes the major component. Peptide III does not become a quantitatively significant fraction even after 20 hr.

It is clear from Figure 8 that peptide I must be larger than peptide II and that by virtue of the high ratio of radioactivity to absorption at 280 mμ it is substantially cleaner than peptide II. It, therefore, was clearly a more suitable peptide to try to isolate. Further purification was achieved by subjecting peptide I to ion-exchange chromatography on QAE-Sephadex, using a pyridine acetate gradient (Figure 9). An average of 3–4% of the total counts were washed through the column from the time of application of the sample in 8 M urea to the time the gradient was begun. The ninhydrin pattern suggests that the major labeled peptide peak is reasonably free of contaminating unlabeled peptides. The main radioactivity peak eluted at pH 6.7–6.5. Minor peaks constituting about 10–15% of the total radioactivity recoverable were frequently seen at pH 7.0, 5.9, or 5.6. These were considered to be artifacts of the gradient or possibly due to variable amide content of the peptide, since after acid hydrolysis of these peaks the compositions were identical with that of the main peak. Average total radioactivity yields were 60–65% of the starting material with precipitation in the column as the major reason for losses. This latter point was supported by the frequently seen precipitation of radioactive peptide in the buffer collected directly from the column. The final purification step, precipitation from acetic acid (pH 3.0), gave a 95% yield of radioactivity. The average yields at each step of the purification process and the cumulative net yields are shown in Table I. These values are the average of four separately quantitated purifications runs. It should be noted that the only procedure which led to a significant loss was precipitation on the QAE-Sephadex column.

The final labeled peptide product was free of smaller contaminating peptides by both electrophoretic and chromato-

TABLE I: Average Yields of Label from Affinity-Labeled Protein 315.

	Absolute[a]	Rel[b]
Protein	100	100
Label	92[c]	92
Label under light-chain peak	90	98
Label after reduction and aminoethylation	83	92
Label recovered under peptide I peak	74	90
Label recovered under main QAE-Sephadex peak	48	65
Label recovered as pure peptide	46	95

[a] Moles per mole of starting material. [b] Moles per mole at previous step. [c] Nonspecific labeling has been accounted for on the basis of the IgG control.

TABLE II: Amino Acid Composition of Tryptic Peptide from Labeled Light Chain of 315.[a]

Amino Acid	Moles/Mole of Labeled Tyrosine
Lys	1.00
His	0.98
Arg	1.02
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
Ala	2.09 ± 0.10
Gly	4.06 ± 0.05
Val	1.00 ± 0.10
Ile	1.84 ± 0.14
Leu	1.91 ± 0.20
Phe	0.95 ± 0.05
Trp	0.73 ± 0.07[b]
Total	33–34[c]

[a] The values given are the averages of analyses on three peptide preparations. [b] This value is the average of the spectral and amino acid hydrolysis determinations (see Results). [c] The serine value may represent four or five residues per tryptic peptide.

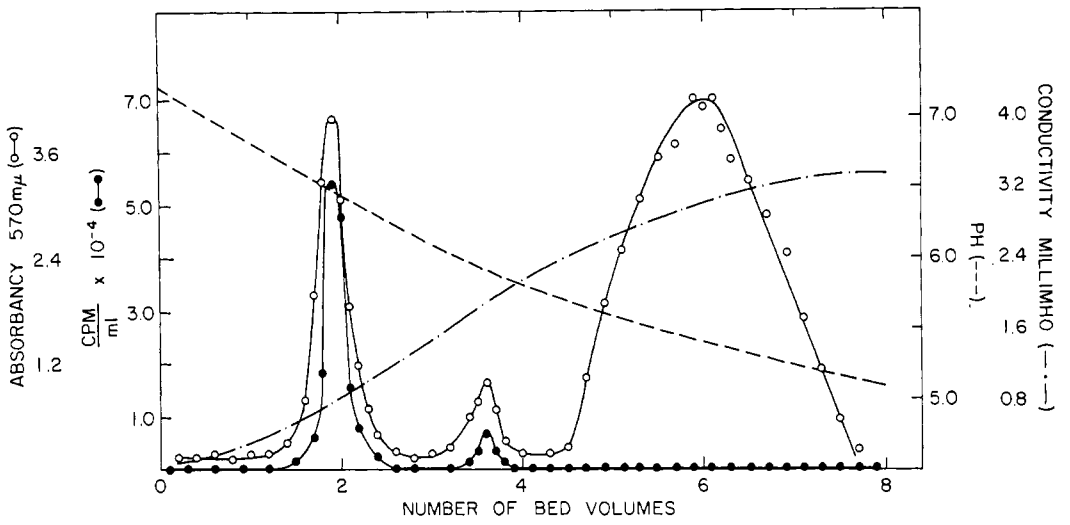

FIGURE 9: QAE-Sephadex gradient purification of peptide I. The pattern shown represents bed volumes from the initiation of the gradient, after washing the column for two to three bed volumes with starting buffer, 0.2 M pyridine acetate (pH 7.6). The counting was performed in Bray's solution without quench corrections.

graphic analysis. When 0.5-cm paper strips from electrophoretic and chromatographic runs were counted, all radioactivity was found at the origin indicating no movement of peptide I during either procedure. Peptide-bond staining of paper chromatographic strips showed three discrete nonradioactive spots in the major radioactivity peak from the QAE-Sephadex gradient run. These spots were present in the acetic acid supernatant but not in the redissolved precipitate.

Amino Acid Composition. Table II shows the amino acid composition of peptide I based on analyses of peptide prepared in three separate experiments. The azotyrosine label was counted as one residue. The yield of tryptophan determined spectrally was 0.8 ± 0.2 nmole/nmole of peptide (Figure 10) and by acid hydrolysis with 6 N HCl with 5% thioglycollic acid was 0.66 nmole/nmole of peptide corrected for the yield obtained with a standard (lysozyme). Peptide I contained no *S*-aminoethylcysteine when analyzed on a 15-cm basic column which separates synthetic *S*-aminoethylcysteine from lysine (Cole, 1967).

Discussion

Purification of Protein 315. The method of affinity chromatography using Sepharose–ligand adsorbents was chosen for the purification of protein 315 because of the simplicity by which such adsorbents can be made and their generally favorable properties with respect to capacity, nonspecific sticking of proteins, and stability (Porath *et al.*, 1967; Cuatrecasas *et al.*, 1968). The problems presented by our particular project were twofold: (1) Since the native protein had polymeric heterogeneity, adsorbents suitable for binding polymer (*e.g.*, PNP-lysyl–Sepharose) were not adequate for binding the monovalent monomer, and (2) a hapten (DNP-caproate) which bound to the combining sites of 315 with sufficient strength to cause elution of both polymer and monomer from the DNP-lysyl adsorbent was difficult to remove from the purified protein by dialysis or ion-exchange chromatography. Prior reduction and alkylation of the 315 which converted all of it into the monomer form, adsorption on a DNP-lysyl–Sepharose column, and elution with DNP-glycine proved to be effective. Immunodiffusion studies indicated that all of the 315 protein was bound and that no significant amounts of other proteins were eluted. Although we have no precise determinations of the overall yield, about 65% of column-

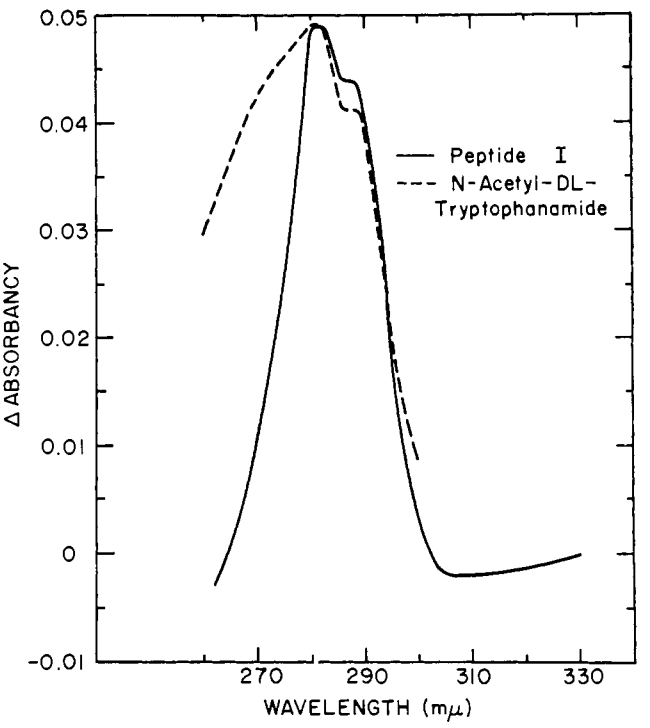

FIGURE 10: Peptide I absorption spectrum. The buffer for both spectra was 6 M guanidine–0.02 M sodium phosphate (pH 6.5). The spectrum for an equimolar concentration of *N*-chloroacetyl-*m*-nitrophenylazotyrosine was subtracted from the spectrum of peptide I.

purified material was recovered when it was rerun through the procedure.

Properties of Protein 315. Some of the functional and structural properties of protein 315 have been described (Eisen *et al.*, 1968). We have confirmed the extinction coefficient previously determined for this protein, the homogeneity of the binding sites, and the fact that the subunit appears to be univalent, even when studied in the presence of a very large excess of free hapten. The valence of 1.1–1.2 is based on a molecular weight of 1.2×10^5 daltons (Eisen *et al.*, 1968; Abel and Grey, 1968). Recent results suggest that this may be too low and that the true molecular weight is closer to 1.5×10^5 daltons (H. Eisen, 1969, personal communication). This would then make the valence 1.38–1.50. The discrepancy with the data on all IgG proteins (Porter, 1959; Nisonoff *et al.*, 1960) and some IgM proteins (Ashman and Metzger, 1969) which show one combining site for each heavy–light-chain pair remains unexplained. Further comments with respect to this point will be reserved for the discussion below on the labeled peptide.

Affinity Labeling of Protein 315. Preliminary findings on the labeling of protein 315 with the affinity-labeling reagent *m*-nitrobenzenediazonium fluoroborate have been described (Metzger and Potter, 1968). These showed that the reaction had an initial rate close to 100-fold faster with protein 315 than with a non-DNP binding IgG myeloma, was specific in that it could be blocked by the unreactive hapten, DNP-caproate, and was unique in that only tyrosines on the light chains were modified. At least partial inactivation of the combining sites was observed. All of these findings are consistent with the reaction occurring in the combining sites of protein 315 as these can be operationally defined. Three new findings with respect to the labeling reaction are worthy of discussion.

(1) We found that under appropriate conditions (*i.e.*, high reagent to site ratio) the labeling reaction followed the theoretical kinetics expected for a set of homogeneous binding sites being labeled by the mechanism originally envisioned. This in itself is strong evidence that the labeling is occurring at the combining sites of protein 315 (again as these can be *operationally* defined). It is of interest that both the association constant for the reagent to the sites on protein 315 and the rate constant, k_3 (see eq 6 in Methods), are so close to those determined for rabbit anti-DNP antibodies (Metzger *et al.*, 1963). It was previously shown (Metzger and Potter, 1968) that the labeling of protein 315 with the three reagents *p*-nitrobenzenediazonium fluoroborate, *m*-nitrobenzenediazonium fluoroborate, and carboxybenzenediazonium fluoroborate was similar to that observed with rabbit antibodies with respect to the relative efficiency of labeling. Since the labeling reaction should be exquisitely sensitive to the configuration of the combining site, the similarity in the labeling of the rabbit antibodies and protein 315 suggest common features in their combining sites. It is striking in this regard that tyrosine is generally found to be labeled since the reagent can also react readily with histidine and lysine. Only rarely has affinity labeling of histidine been observed (Wofsy and Parker, 1967).

On the other hand, there is a marked difference in the results obtained with protein 315 and those obtained with rabbit anti-DNP (IgG) antibodies. In the latter case, labeling always occurred on both heavy and light chains, the amount on the former usually predominating in a 2:1 ratio (Metzger *et al.*, 1964; Good *et al.*, 1967). Similar findings have been obtained with IgG antibodies having different specificities (Wofsy and Parker, 1967; Wofsy *et al.*, 1967) and anti-DNP antibodies from different species (Good *et al.*, 1968). Only in a few instances has labeling been seen exclusively on one chain and in those instances it was always the heavy chain (Wofsy *et al.*, 1969). Labeling of non-IgG conventional antibodies has not yet been described.

It is premature to ascribe this difference to any one of the following possibilities: (a) labeling of homogeneous immunoglobulin combining sites will tend to show labeling on one or another of the chains preferentially, (b) light chains make a somewhat different contribution to the combining sites in IgA molecules than they do in IgG molecules, (c) the combining sites on 315 are not really directed to DNP groups but to some determinant considerably different than those for which we have data from conventional antibodies, (d) the combining sites of myeloma proteins are different than those found in conventionally raised antibodies, and (e) protein 315 is unique.

The many similarities of the combining sites on 315 to those of conventional anti-DNP antibodies as evidenced by binding studies and reactions with labeling reagents (above) make us favor alternatives a and/or b.

(2) Affinity-labeled protein 315 was found to be only partially inactivated. At maximal labeling all the sites were still capable of binding DNP-caproate although the binding constant was reduced 50-fold (Figure 3). Significantly the characteristic shift in the spectrum of the bound ligand was also observed (Figure 6). By analogy with the findings on conventional antibodies it is likely that the combining sites on 315 are considerably larger than a nitrophenyl group. This plus the fact that the azo linkage by which the latter group is attached to tyrosine allows for considerable rotational displacement suggests that even if the label is attached within the site as *structurally* defined complete inactivation might not occur. A second possibility is of course that the labeling is occurring within the site as operationally defined but slightly peripheral to it as structurally defined. More detailed knowledge of the combining sites than is obtainable by labeling techniques is required to distinguish between these alternatives. Reexamination of published data on affinity-labeled conventional antibodies (which possess considerable combining site heterogeneity) convinces us that it is impossible to state whether complete or partial inactivation of the sites occurred in those instances (Good *et al.*, 1968; Metzger *et al.*, 1963).

The possibility of double labeling of protein 315 must be considered since the labeled molecules can still bind DNP-caproate, and therefore may have residual affinity for a second molecule of labeling reagent. One of the assumptions in the calculation of the K_A from labeling kinetics is "effective inactivation" of the site by the first label, and significant double labeling would invalidate this assumption. The uniform alteration of all the sites can be inferred from the linearity of the Scatchard plot for the labeled 315 protein (Figure 3). This is strongly against *partial* double labeling of the 315 protein. Specific activity determinations (see Results) showed only 1.2 azotyrosines/pair of light chains or 1 active site. The tryptic peptide contains all of this radioactive label, and therefore any double labeling would

have to occur in this peptide. Further evidence against double labeling therefore is the quantitative composition of the labeled tryptic peptide. Thus 10 nmoles of azotyrosine label, determined spectrally or by radioactive counts, is found in an amount of tryptic peptide producing 10 nmoles each of six other amino acids which are all readily quantitated by amino acid analysis (Table II). If significant double labeling occurred, then quantitation of the peptide by the azotyrosine label would yield nonintegral values lower than unity for the single residues in the peptide.

(3) The failure to obtain the theoretically expected kinetics of labeling at low reagent:combining site ratios is most easily explained by postulating that those nitrophenyl groups already covalently attached to some of the sites can effectively compete for the unlabeled sites with the remaining unreacted reagent. The simultaneously increasing concentration of bound reagent and decreasing concentration of free reagent would explain the progressive fall in the rate of labeling. Consistent with this is the observation that under a variety of affinity-labeling situations progressive precipitation is seen to occur (L. Wofsy, 1969, personal communication; H. Metzger, 1969, unpublished observations). Significantly we have not observed gross precipitation with labeling of the apparently monovalent subunit but have observed it with the polymer. Complex formation can of course still occur in the former instance. At low reagent:combining site ratios the kinetics of labeling resembled those observed with rabbit antibodies. With the latter the rapid fall-off in rate of labeling was ascribed solely to site heterogeneity. It is possible that at least some of the decline in rate was due to the mechanism described above. More efficient labeling, therefore, might be obtained using high reagent: site ratios with conventional antibodies.

Isolation of the Labeled Peptide. We will reserve detailed discussion of the properties of the labeled peptide and its location in the light-chain sequence of protein 315 until our sequence studies on it are complete. Several points can already be made, however. The peptide obtained contains essentially all the label found in the light chains of protein 315. Assuming that these light chains have the same common region sequence previously determined for mouse κ chains, the peptide composition is inconsistent with its being derived from that region. The composition is also inconsistent with the possibility that the labeled tyrosine is $Tyr_{\kappa,86}$. Compelling arguments (albeit based on circumstantial evidence) have been forwarded that that tyrosine was labeled in studies with conventionally raised mouse anti-DNP antibodies (Singer and Thorpe, 1968; Thorpe and Singer, 1969). The composition of our peptide is most consistent with a region of $\sim$30–34 amino acids spanning residues 25–54 (Dayhoff, 1969). The paucity of sequence data available for this region as well as its apparent marked variability makes exact positioning of the peptide impossible without its full sequence.

The 315 protein subunit appears to be univalent (Eisen *et al.*, 1968; Metzger and Potter, 1968). It might be supposed that this is due to a structural asymmetry in the protein. Because the labeling studies directly implicate the light chains as contributing to the combining sites, it might be further supposed that all the light chains in this protein are not identical. Evidence against this is that a full complement of light chains appears under a single peak by gel filtration and that the labeled chains are indistinguishable from unlabeled chains by disc electrophoresis (Metzger and Potter, 1968). In addition both our estimate of the number of intrachain disulfides and of the total amino acid composition is consistent with two intact light chains per mole of monomer. More convincing would be the isolation of a peptide sequence from the unlabeled chains identical with that of the labeled peptide and in equivalent amounts. Experiments in this direction are under way.

Acknowledgments

We wish to thank Mr. George Poy and M. John Lee for skillful technical assistance.

Added in Proof

Sequence data on the amino- and carboxy-terminal ends of the light chains (E. J. Goetzl and H. Metzger, submitted for publication) as well as serologic data (F. R. McIntire personal communication) now make it clear that the 315 light chains are of the λ type. Sequence data on several Balb/c λ chains (M. Weigert, personal communication) and on the labeled peptide unequivocally show that the labeled peptide comes from the region indicated in the text.

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Sea Urchin Satellite Deoxyribonucleic Acid. Its Large-Scale Isolation and Hybridization with Homologous Ribosomal Ribonucleic Acid*

James B. Patterson and Darrel W. Stafford

ABSTRACT: A satellite deoxyribonucleic acid (DNA) has been isolated from sperm of the sea urchin *Lytechinus variegatus*. This satellite DNA has a density of 1.722 g/cc as compared with a density of 1.695 g/cc for main band DNA.

The satellite was isolated by selective denaturation of main band DNA followed by separation of the native and denatured DNA in a polyethylene glycol–dextran two-phase system. The satellite was purified 700-fold by this step alone. Preparative CsCl density gradient centrifugation further purified the satellite. The yield of satellite DNA was 45% of the total amount determined present in the purified whole sperm DNA. The sedimentation coefficient was 23.3 S, corresponding to a molecular weight of about 1×10^7 daltons. The satellite was shown to contain sequences which hybridize with homologous ribosomal ribonucleic acid.

The first evidence that the genes coding for rRNA could be physically separated from the rest of the genome in *Xenopus laevis* was presented by Birnstiel and coworkers (1966). There has subsequently been much work on the characterization of this (Birnstiel *et al.*, 1968; Brown and Dawid, 1968; Brown and Weber, 1968a,b; Brown *et al.*, 1967) and other similar satellite DNAs (Lima-de-Faria *et al.*, 1969; Matsudo and Siegel, 1967; Skinner, 1969; Stafford and Guild, 1969). Much of the work has been hampered by the difficulty and expense of obtaining sufficient satellite DNA.

We recently reported the presence of a G + C rich satellite DNA from sperm of the sea urchin *Lytechinus variegatus*. The satellite DNA was partially purified (Stafford and Guild, 1969) by thermal chromatography on hydroxylapatite (Miyazawa and Thomas, 1965) and it was shown that the G + C rich fraction contained sequences specific for rRNA. However, we could not show that the sequences specific for rRNA were definitely contained in the satellite DNA.

We report here a method for large-scale purification of the satellite DNA and show that it does contain sequences specific for rRNA.

Materials and Methods

Reagents. Nitrocellulose membrane filters (Type B-6, 25 mm diameter) were purchased from Carl Schleicher & Schuell Co. Dextran T500 was purchased from Pharmacia. Polyethylene glycol (Carbowax 6000) was purchased from Union Carbide Chemicals. Cesium chloride (optical grade) was purchased from Harshaw Chemical Co. Scintillation vials were purchased from Packard. Kieselguhr (Hyflo Supercel) was purchased from Johns Manville Products Corp. Isoamyl alcohol and toluene were purchased from Allied Chemical. Chloroform, phenol, and 95% sodium dodecyl sulfate were purchased from Matheson, Coleman & Bell. Pronase

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