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Affinity Labeling of Antibodies to the *p*-Azophenyltrimethylammonium Hapten, and a Comparison of Affinity-Labeled Antibodies of Two Different Specificities*

John W. Fenton, II,† and S. J. Singer‡

ABSTRACT: The active sites of rabbit and mouse antibodies to the *p*-azophenyltrimethylammonium (TMA) antigenic determinant were affinity labeled with the reagent, *p*-(trimethylammonium)benzenediazonium difluoroborate. This reagent specifically labels tyrosyl residues on both the heavy and light polypeptide chains, and by the criteria of affinity labeling these residues and their respective polypeptide chains therefore contribute to the formation of the active sites. The marked similarity of the labeling of tyrosyl residues in the active sites of antibodies directed to the positively charged TMA group, to the neutral 2,4-dinitrophenyl (DNP) group, and to the negatively charged *p*-azobenzenearsonate group, in several species, suggests that a common structural relationship exists among these active sites independent of their specificities and species of origin. Support for this suggestion has been obtained from studies of tryptic peptide fragments prepared from both the heavy and light chains of rabbit and mouse anti-DNP and anti-TMA antibodies. These peptides have been fractionated on calibrated columns of Sephadex in 8 M urea, and the distribution of the radioactive affinity label among the different size peptides has been determined. For a given type of chain and species of origin, the distribution of affinity label among the peptides was characteristic and closely similar for the two antibody specificities. These results suggest that the same local region of the antibody molecule is utilized to form active sites of different specificities.

The method of affinity labeling[1] provides a technique for selectively labeling contact residues within enzyme and antibody (Ab)[2] active sites (Wofsy *et al.*, 1962; Singer, 1967). This method has been applied to a number of Ab with different specificities (Singer and Doolittle, 1966; Singer *et al.*, 1967; Wofsy *et al.*, 1967, 1970). Up to the present time, the

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† U. S. Public Health Service Predoctoral Fellow, 1966–1968. Present address: Division of Laboratories and Research, N. Y. State Department of Health, Albany, N. Y. 12201.

‡ To whom to address correspondence.

[1] Similar labeling methods were independently developed by several investigators (Baker *et al.*, 1961; Lawson and Schramm, 1962; Schoellmann and Shaw, 1963; Wofsy *et al.*, 1962). The subject of active site labeling has recently been reviewed (Baker, 1967; Singer, 1967).

[2] Abbreviations used are: Ab, antibodies; anti-DNP, anti-2,4-dinitrophenyl; anti-TMA, anti-*p*-azophenyltrimethylammonium; BSA, bovine serum albumin; H, heavy and L, light polypeptide chains; PTBDF, *p*-(trimethylammonium)benzenediazonium difluoroborate; MNBDF, *m*-nitrobenzenediazonium fluoroborate.

most detailed structural studies have been performed on affinity-labeled Ab directed to the noncharged 2,4-dinitrophenyl antigenic determinant, elicited in rabbits (Metzger *et al.*, 1963; Good *et al.*, 1967) and mice (Thorpe and Singer, 1969). These studies have established that characteristic tyrosyl residues in the active sites are labeled on both the H and L polypeptide chains of the anti-DNP Ab molecule. In the case of L chains from mouse anti-DNP Ab, the results have also demonstrated that the labeled tyrosine residue is within the variable portion of the L chains (Singer and Thorpe, 1968; Thorpe and Singer, 1969).

In order to examine the nature and extent of structural homologies among Ab of different specificities, the labeling of Ab to the positively charged *p*-azophenyltrimethylammonium (TMA) determinant has been investigated. A preliminary communication (Fenton and Singer, 1965) has reported the affinity labeling of rabbit anti-TMA Ab with the reagent PTBDF at pH 6.0 and compared the labeling results to those obtained for Ab directed to the negatively charged *p*-azobenzenearsonate determinant (Wofsy *et al.*, 1962; Metzger *et al.*, 1964) and for anti-DNP Ab (Metzger *et al.*, 1963; Metzger *et al.*, 1964). The remarkable similarity observed for the labeling of these Ab to differently charged determinants has suggested that Ab active sites share certain common structural regions (Fenton and Singer, 1965; Singer and Doolittle, 1966). The present paper describes the affinity labeling of rabbit and mouse anti-TMA Ab at pH 5.0 under closely similar conditions to those used for the labeling of anti-DNP Ab. In order to explore possible homologies between the affinity-labeled groups of anti-TMA and anti-DNP Ab, tryptic digestions of the H and L chains of affinity-labeled mouse and rabbit anti-TMA and anti-DNP Ab were performed. It was found that the molecular sizes of the labeled tryptic peptides could be effectively discriminated by gel filtration in 8 M urea, and that the distribution of labeled peptides was very similar for the two Ab specificities.

Materials and Methods

Chemicals and Reagents. Crystalline PTBDF (mp 154–156° dec) was prepared as previously described (Traylor and Singer, 1967; Fenton, 1968) and was stored dry at −20° until used. [^{3}H]PTBDF was prepared by Dr. P. S. Traylor at a specific activity of 90 mCi/mmole and was stored frozen in 0.01 N HCl at −20°. Crude trimethylphenylammonium chloride (J. T. Baker Chemical Co.) was recrystallized three times from 2-propanol (mp 246–248° dec) and stored under dry nitrogen.

Spectra of model azo compounds in 0.10 N NaOH were determined by Traylor and Singer (1967) by reacting PTBDF with a 100-fold molar excess of *N*-chloroacetyl-L-tyrosine or *N*-acetyl-L-histidine. On the assumption that the only spectrally significant azo products of the reaction of PTBDF with proteins were azotyrosine and azohistidine, the spectra of PTBDF-modified proteins in 0.10 N NaOH could be analyzed (Tabachnick and Sobotka, 1959) by absorbance measurements at 490 and 420 nm, using the extinction coefficients of Traylor and Singer (1967). Equations 1 and 2 were used to calculate the molar concentrations, m_{AT} and m_{AH}, of azotyrosyl and azohistidyl groups.

$$m_{AT} = (11.18A_{490nm} - 2.74A_{420nm}) \times 10^{-5} \quad (1)$$

$$m_{AH} = (5.14A_{420nm} - 2.93A_{490nm}) \times 10^{-5} \quad (2)$$

Scintillation counting fluid was prepared by dissolving 100 mg of 1,4-di[2-(5-phenyloxazolyl)]benzene (Calbiochem) and 6.0 g of 2,5-diphenyloxazole (Calbiochem) in 325 ml of ethanol plus 675 ml of toluene.

Immunizing Antigen. High molecular weight hemocyanin was isolated from keyhole limpets (*Megathuria crenulata*) by preparative ultracentrifugation (Campbell *et al.*, 1964) and was stored at 5° as a concentrated solution in 3% NaCl containing 0.1% merthiolate (Eli Lilly & Co.) and 0.01 M sodium phosphate buffer at pH 7.0. Hemocyanin concentrations were determined by using $\epsilon_{278nm} = 2.06$ ml/mg per cm, assuming a nitrogen content of 16%. Nitrogen determinations were made by the Kjeldahl procedure. Solutions of 10 mg/ml of hemocyanin were reacted with a 50-fold molar excess of PTBDF/10^5 g of protein in 0.10 M sodium phosphate buffer at pH 7.0 for 2 hr at 0–5°. The product, TMA-azohemocyanin, was dialyzed against several changes of 1% NaCl at 5°. By spectral analysis, 12 moles of azotyrosyl and 6 moles of azohistidyl groups were formed per 10^5 g of protein.

Precipitating Antigen. The carrier protein used was bovine serum albumin, which was first succinylated in order to increase its activity toward PTBDF and to retard it more effectively on DEAE-Sephadex in the Ab purification procedure (see below). Bovine serum albumin (Pentex, Inc.) was dissolved at a concentration of 50 mg/ml and a fivefold molar excess (assuming 62 free amino groups per albumin molecule) of dry succinic anhydride (Eastman Kodak Co.) was added. The reaction mixture was maintained at pH 8 by the addition of 1 N NaOH for 2 hr at 0–5° (Habeeb *et al.*, 1958; Cherry, 1964). The protein product was dialyzed against several changes of water at 5° and lyophilized. The succinylated BSA was dissolved at a concentration of 50 mg/ml in 0.10 M sodium phosphate buffer at pH 7.0 and was reacted with a twofold molar excess (assuming 20 tyrosyl and 18 histidyl residues per albumin molecule) of PTBDF for 2 hr at 0–5°. This product, TMA-azosuccinylated BSA, was dialyzed against several changes of water at 5° and lyophilized. It was found to contain 20 azotyrosyl and 4 azohistidyl groups per albumin molecule.

Production of Anti-TMA Ab. Mixed bred rabbits were injected in each hind footpad with 0.5 ml of an emulsion made from equal volumes of a 10 mg/ml solution of TMA-azohemocyanin and Freund's complete adjuvant (Difco Laboratories). Six weeks later, the rabbits were injected in the ear vein with 1 ml of a 1.0-mg/ml solution of TMA-azohemocyanin and were routinely exsanguinated on the following week. Sera were pooled and processed by precipitating the globulin fractions three times in half-saturated ammonium sulfate at 5°. These fractions were stored as their slurries at 5° until used in Ab purification.

Swiss-Webster mice were injected intraperitoneally once a week for 4 weeks with 0.1 ml of an emulsion made from equal volumes of a 1.0-mg/ml solution of TMA-azohemocyanin and Freund's complete adjuvant. On the 4th week approximately 10^6 Ehrlich ascites tumor cells were injected intraperitoneally and within 1 week the peritoneal ascites fluids were tapped. These fluids were heparinized to prevent clotting and were centrifuged to remove cellular material. Pooled fluids were further processed and stored as described for rabbit sera.

Anti-TMA Ab titers were determined by quantitative precipitation experiments with TMA-azosuccinylated BSA in 0.10 M sodium phosphate buffer at pH 7.0. The washed precipitates were dissolved in 1.00 ml of 0.10 N NaOH, and their optical absorptions were measured at 290 and 490 nm. The

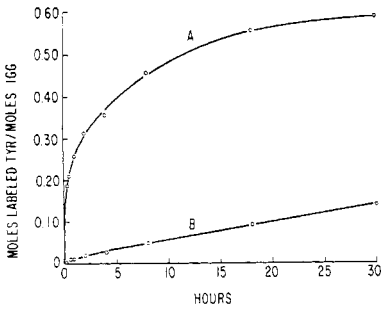

FIGURE 1: Kinetics of the reaction of PTBDF with (A) rabbit anti-TMA Ab, and (B) normal rabbit IgG in 0.20 M sodium acetate buffer (pH 5.0) at 0°. The initial mole ratio of PTBDF to protein was 1.5.

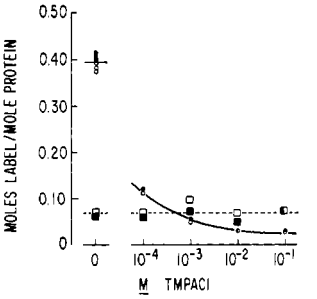

FIGURE 2: The effect of the protector, trimethylphenylammonium chloride (TMPACl), on the reaction of [^{3}H]PTBDF with rabbit anti-TMA Ab (circles) and with normal rabbit IgG (squares). The filled circles and squares represent spectral measurements of the extent of the reaction, while the open points represent radioactivity measurements on the same samples. See text for reaction conditions.

amount of precipitated Ab was obtained by correcting for the 290 nm absorption of the antigen.

Purification of Anti-TMA Ab. Stored globulin fractions were dialyzed free of ammonium sulfate against 1% NaCl at 5°. The amount of TMA-azosuccinylated BSA needed for maximum Ab precipitation was added to the dialyzed globulin fractions in an equal volume of 0.10 M sodium phosphate buffer at pH 7.0. Precipitation was carried out at 37° for 1–2 hr and then at 5° for 6–12 hr. Precipitates were collected by centrifugation and washed three times with 1% NaCl at 5°. The washed precipitates were then dissociated with 0.1–0.5 M trimethylphenylammonium chloride in 0.10 M sodium phosphate buffer at pH 7.0. The resultant mixtures were centrifuged free of small amounts of residual precipitate and the supernatants were applied to a DEAE-Sephadex A-25 (Pharmacia Fine Chemicals, Inc.) column at 23° equilibrated and developed with 0.01 M trimethylphenylammonium chloride in 0.02 M sodium phosphate buffer at pH 7.0. Both the rabbit and mouse Ab eluted as a single peak leaving the antigen firmly bound to the resin. Recovered Ab fractions were exhaustively dialyzed against 1% NaCl at 5° to remove the hapten and were then used directly or stored as slurries in half-saturated ammonium sulfate at 5°.

Affinity-Labeling Experiments. The procedures used were similar to those developed with the anti-DNP Ab system (Good *et al.*, 1967).

The kinetics of the specific affinity labeling reaction were investigated with rabbit anti-TMA Ab to determine the optimal reaction conditions for subsequent labeling experiments. To 2.0-ml samples containing 2.4×10^{-5} M purified Ab in 0.20 M sodium acetate buffer at pH 5.0 was added an amount of a stock solution of nonradioactive PTBDF to make the mixture 3.6×10^{-5} M in the reagent. The reactions were carried out at 0°. A parallel set of experiments were carried out using normal rabbit γ-globulin instead of the Ab. The reactions were terminated at various times by precipitating the protein with 6.0 ml of cold ethanol. After centrifugation, the precipitate was dissolved in 0.5–1.0 ml of cold 0.01 N HCl and reprecipitated with cold ethanol. The centrifuged precipitates were then washed with cold ethanol and ether, and were air-dried. The precipitates were then dissolved in 0.10 N NaOH for spectrophotometric analyses described below. The results of these experiments are given in Figure 1.

Another set of experiments, employing [^{3}H]PTBDF, was designed to test the effectiveness of trimethylphenylammonium as a specific protector of the active sites of anti-TMA Ab toward reaction with PTBDF. To 1.0-ml samples of 2.4×10^{-5} M rabbit anti-TMA Ab, or normal rabbit γ-globulin, in 0.20 M sodium acetate buffer at pH 5.0, were added different amounts of trimethylphenylammonium chloride in 1.0 ml of water. To these mixtures [^{3}H]PTBDF was added to give 2.9×10^{-5} M solutions of the labeling reagent. After 8 hr at 0°, the modified protein was precipitated from each sample with 75% ethanol and freed of noncovalently bound impurities as described above. The proteins were analyzed spectrophotometrically and for radioactivity. The results of these protection experiments are shown in Figure 2.

For the experiments to determine the nature and the distribution of the affinity label on the H and L chains of both the rabbit and the mouse anti-TMA Ab, [^{3}H]PTBDF was used in the absence (unprotected) or the presence (protected) of 0.10 M trimethylphenylammonium chloride. Rabbit Ab at a concentration of 2.1×10^{-5} M were reacted in 3.2×10^{-5} M solution of [^{3}H]PTBDF; and similarly mouse Ab and reagent concentrations were 1.7×10^{-5} and 2.5×10^{-5} M, respectively. After 8 hr at 0°, the reactions were quenched by bringing the pH to 4 with acetic acid. Each sample was then dialyzed exhaustively at 5° against 0.20 M sodium acetate buffer, pH 4, then against water, and were lyophilized. Portions of the labeled Ab was analyzed spectrophotometrically and for radioactivity.

For H- and L-chain separations, the rest of the labeled proteins were mildly reduced and alkylated by the procedure of Fleischman *et al.* (1962). The lyophilized Ab was dissolved at 1–2% concentrations in 0.55 M Tris-HCl buffer at pH 8.2, was then reduced in 0.20 M 2-mercaptoethanol at 23° for 1 hr, and subsequently alkylated in 0.22 M iodoacetamide at 0–5° for 1 hr. The reaction mixture was then dialyzed at 5° against 1 M propionic acid prior to application to a Sephadex G-100 (Pharmacia Fine Chemicals, Inc.) column equilibrated and developed with 1 M propionic acid at 5°. In practice, three peaks, designated as A, B, and C, were eluted in that order (Metzger and Mannik, 1964; Good *et al.*, 1967) and were separately pooled. These were dialyzed against water at 5°, lyophilized, and analyzed. Because the yields of peaks A and B were found to be inversely related, the yield of peak A increasing with time of exposure to 1 M propionic acid, the material in peak A was considered to be an aggregated or denatured form of H chains. The values reported for H chains are a weighted average of those found for the material in peaks A and B. The values for L chains correspond to the material in peak C.

Quantitation of Affinity Labeling. For spectrophotometric analyses, dried samples were dissolved in 0.10 N NaOH, and their spectra were determined with a Cary Model 14 recording spectrophotometer. Protein concentrations were then deter-

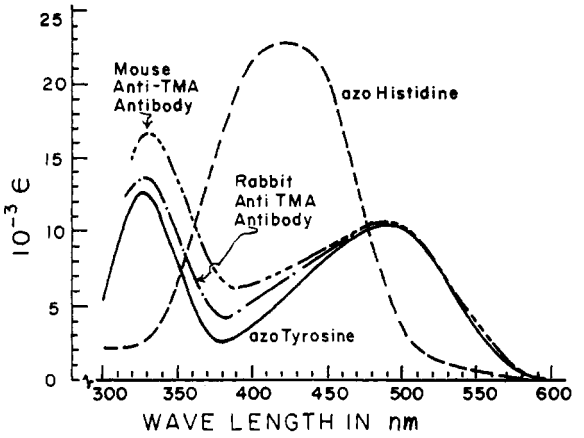

FIGURE 3: Spectra in 0.10 N NaOH of the model compounds formed by the reaction of PTBDF with *N*-chloracetyltyrosine (azotyrosine) and *N*-acetylhistidine (azohistidine); and of whole rabbit and mouse anti-TMA Ab affinity labeled with PTBDF. The ordinate is the molar extinction coefficient of the model compounds. The curves for the labeled Ab are adjusted to have the same ordinate as the curve for the model azotyrosine compound at 490 nm; for the labeled rabbit Ab, A_{490nm} 0.236 at a protein concentration of 8.0 mg/ml, and for the labeled mouse Ab, A_{490} 0.235 at a protein concentration of 10.0 mg/ml.

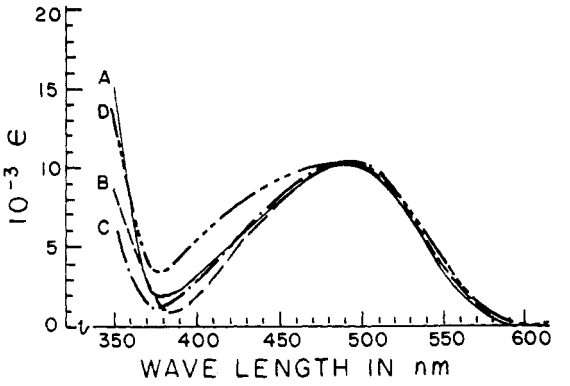

FIGURE 4: Spectra in 0.10 N NaOH of the separated chains of affinity-labeled anti-TMA Ab: (A) rabbit H chain, (B) rabbit L chain, (C) mouse H chain, and (D) mouse L chain. The spectra were adjusted to the same scale as in Figure 3 by normalizing the absorbances at 490 nm to that of the azotyrosine model compound. The actual values of A_{490nm} and protein concentrations in each sample were: (A) 0.228, 11.0 mg/ml; (B) 0.157, 6.2 mg/ml; (C) 0.173, 7.0 mg/ml; (D) 0.112, 6.8 mg/ml, respectively.

mined after dilution into 0.10 M sodium phosphate buffer at pH 7.0 from absorbance measurements at 280 nm. Calculations were based on the values of ϵ_{280nm} = 1.46 ml/mg per cm for rabbit Ab (and assumed for mouse Ab), ϵ_{280} = 1.45 ml/mg per cm for their H chains, and ϵ_{280nm} = 1.32 ml/mg per cm for their L chains (Utsumi and Karush, 1964). The respective molecular weights of 160,000, 55,000, and 25,000 were assumed. Molar difference spectra of labeled Ab or their H and L chains were obtained by subtracting the corresponding spectra of unlabeled Ab or their H and L chains subjected to identical experimental conditions.

For radiometric analyses, three to five replicate 500-μl aliquots of aqueous samples were counted in 15 ml of scintillation counting fluid. All vials were counted to an accuracy of at least ±5% with a Beckman liquid scintillation system and were recounted with the addition of 50 μl of standardized [^{3}H]toluene (New England Nuclear Corp.). The concentrations of labeled groups were calculated from the specific activity of [^{3}H]PTBDF (Traylor and Singer, 1967). Protein concentrations of samples were determined spectrophotometrically, as described.

Performate Oxidation of Affinity-Labeled Chains. For the tryptic peptide studies reported in this paper, the labeled chains were first performate oxidized by the procedure of Hirs *et al.* (1956) using freshly prepared performic acid. This treatment does not affect the affinity label significantly (Doolittle and Singer, 1965). The oxidized chains were lyophilized and stored at 5° until used.

Tryptic Digestions. Trypsin (Worthington, twice recrystallized) was treated with L-(1-tosylamido-2-phenylethyl chloromethyl ketone (Calbiochem) to inhibit chymotryptic activities (Schoellmann and Shaw, 1963; Kostka and Carpenter, 1964). The treated trypsin was added to 1% protein solutions at a weight ratio of 1/50 in 0.1 or 0.2 M NH_4HCO_3 at pH 7.8–8.0 and the digestions were generally allowed to proceed at 40° for 8 hr. Some samples were digested in water; the pH was maintained at 8.0 by the addition of 0.020 N NaOH with a pH stat. For rabbit L chains so digested, 56% of the theoretical NaOH consumption occurred in 1 hr, 68% in 2 hr, and 91% in 8 hr. The digested samples were frozen and lyophilized until used.

Chromatography on Sephadex G-50 in 8 M Urea. Approximately 10 mg of lyophilized tryptic digests of performate-oxidized chains was dissolved in 1 ml of deionized 8 M urea containing a small amount of NH_3. These samples were then applied to a column of Sephadex G-50 fine (90 × 2.4 cm) equilibrated with deionized 8 M urea at pH 8 and operated at 23°. The flow rate was maintained by peristaltic pump at 0.66 g/min, and fractions were collected every 5 min. (Routinely the tubes were weighed before and after collection of fractions; the density of 8 M urea is 1.118 g/ml.) Fractions were analyzed by scintillation counting for ^{3}H content and by absorbance measurements for peptide content at 225 nm. The Sephadex column was calibrated with various peptides and reagents in the range of molecular weights between 400 and 10,000. The compounds used for the molecular weight calibration were as follows: (A) blue dextran (Pharmacia Fine Chemical, Inc.), mol wt ~2 × 10^6 (to indicate the front); (B) [^{3}H]*m*-nitrobenzeneazo-ACTH (prepared by Dr. P. S. Traylor from ACTH kindly supplied by Dr. C. H. Li), mol wt 5.6 × 10^3; (C) [^{3}H]*m*-nitrobenzeneazo-insulin B chain (prepared by reacting performate-oxidized insulin B chain (Mann Research Labs) with [^{3}H]MNBDF), mol wt 3.7 × 10^3; (D) sheep fibrinopeptide A (gift of Dr. R. F. Doolittle), mol wt 1.84 × 10^3; (E) *N*-2,4-dinitrophenylaminocaproyl-α-L-alanyl-(D-alanyl-L-alanyl)$_8$-D-alanine (synthesized by Dr. E. Habicht using the Merrifield solid-state synthesis), mol wt 1.72 × 10^3; (F) [^{3}H]*m*-nitrobenzeneazo-heptapeptide (prepared by reacting L-tyrosyl-L-seryl-L-aspartyl-L-alanyl-L-asparaginyl-L-lysyl-L-arginine with [^{3}H]MNBDF; the heptapeptide was prepared by, and was the gift of, Dr. A. Robinson), mol wt 1.02 × 10^3; (G) α-*N*-chloroacetyl-3-(3′-nitrobenzeneazo)-L-tyrosine (Traylor and Singer, 1967), mol wt 380; and (H) methyl orange (Eastman Kodak Co.), mol wt 327.

Additional Methods. A tryptic peptide fraction from the L chains of rabbit anti-TMA (fraction C in Figure 3a, see below) was analyzed for amino acid composition. The contents of the tubes around the peak maximum were pooled, concentrated, and then freed of urea by ultrafiltration with a Diaflo UM-2 membrane (Amicon Corp.). The peptide was hydrolyzed *in vacuo* in 6 N HCl at 110° for 24 hr. A measured amount of

TABLE I: Anti-TMA Antibodies Treated with [^{3}H]PTBDF.[a]

Species	Reaction Condition	Whole Ab[b] Spectral Azotyr	Whole Ab[b] ^{3}H	H Chains Spectral Azotyr	H Chains ^{3}H	L Chains Spectral Azotyr	L Chains ^{3}H	2 H + 2 L Spectral Azotyr	2 H + 2 L ^{3}H
Rabbit	Unprotected	0.41	0.512	0.099	0.124	0.064	0.068	0.33	0.38
	Protected		0.052		0.014		0.005		0.04
Mouse	Unprotected	0.41	0.399	0.123	0.122	0.045	0.034	0.34	0.31
	Protected		0.070		0.012		0.004		0.03

[a] Data given in mole per mole of Ab. [b] Whole rabbit antibodies were analyzed after ethanol washing procedure to remove noncovalently bound material; whole mouse antibodies were analyzed after dialysis procedure (see text).

radioactivity was applied to the long and short columns of a Beckman Model 120B amino acid analyzer, equipped with a 6.6-cm path-length cuvet. No corrections were made for destruction of amino acids during hydrolysis.

Results

Characterization of Anti-TMA Ab. In an initial study of ten individual rabbits bled at weekly intervals, all ten were found to respond to both the primary and secondary immunizations as described (see Materials and Methods). Despite large individual variations, the overall response was greatest and least variable 1 week following the secondary immunization. The mean Ab titer for these bleedings was 1.94 ± 1.06 mg/ml of sera, and this procedure was adopted without further modification. Similarly, in an initial study of 30 mice, all surviving mice with tumor fluids (25 mice) responded to immunization as detected by capillary ring tests. The Ab titers of pooled tumor fluids from three groups of mice ranged from 0.66 to 1.46 mg per ml of fluid and were therefore comparable to those of rabbit sera.

The Ab purification procedure employed was found to work equally well for both rabbit and mouse Abs. Yields were 30–85% of the original Abs with activities of 50–95% as determined by quantitative precipitation. The rabbit anti-TMA Ab preparation described in the present labeling studies was recovered with an 81% yield and was 82% precipitable. This material sedimented as a single 7S component (Fenton, 1968). The mouse Ab preparation was recovered with a 62% yield and was 79% precipitable.

Specificity of the Affinity-Labeling Reaction. The results of the study of the rates of reaction of PTBDF with rabbit anti-TMA Ab and with normal rabbit γ-globulin are shown in Figure 1. The data are given as moles of azotyrosine formed per mole of protein since, as is discussed below, azotyrosine is essentially the exclusive product formed under these conditions. The kinetics of these reactions are closely parallel to those observed previously in the affinity-labeling of anti-DNP Ab (Metzger *et al.*, 1963; Good *et al.*, 1967). There is a very rapid initial reaction with the specific Ab as compared to normal γ-globulin. By the time that about 0.6 mole of azotyrosine has formed per mole of Ab, however, the apparent overall rates of the reactions with the two proteins are the same. The initial rapid rate of reaction with the antibody reflects the specific affinity labeling of the Ab active sites, and the falling off of reaction rate is attributable to the heterogeneity of these active sites in the population of Ab molecules (Metzger *et al.*, 1963).

Not only does the reaction of PTBDF occur initially much more rapidly with anti-TMA Ab than with normal γ-globulin, but this enhanced rate is eliminated in the presence of the specific protector, trimethylphenylammonium chloride (Figure 2 and Table I). This compound has no effect on the reaction of PTBDF with normal γ-globulin, which proves that its role in the Ab reaction is that of a specific active site protector.

Nature and Distribution of the Affinity-Labeled Residues. The spectra of affinity-labeled whole rabbit and mouse anti-TMA Ab (Figure 3) and of their respective H and L chains (Figure 4), when compared to the spectra of the model azo compounds (Figure 3), show that tyrosine residues are the only groups significantly modified by the specific labeling reactions. From these spectra, and eq 1 and 2, it may be calculated that the ratio of azohistidine to azotyrosine formed was always less than 0.05. This is further supported by the good agreement between the amount of label determined (1) spectrally as azotyrosine and (2) by radioactivity (Table I).

The results of the kinetic experiments (Figure 1) and of the protection experiments (Figure 2) helped to define the optimal conditions for the reaction of PTBDF with anti-TMA Ab for further studies of the affinity-labeled Ab chains. At the concentrations employed, an 8-hr reaction time at 0° was chosen. For the protected sample, the concentration of 0.1 M for the trimethylphenylammonium chloride was selected. Under these circumstances, about 0.4 mole of azotyrosine was formed per mole of Ab, and about 10 times as much ^{3}H was bound to the separated chains of the unprotected Ab compared to the chains of the protected Ab (Table I). The data in Table I show that the affinity label is present on both H and L chains of both rabbit and mouse anti-TMA Ab. The ratio of specific label on H and L chains in the rabbit Ab is a little less than 2, and is about 3 for the mouse Ab.

Tryptic Peptides from Affinity-Labeled Chains. The significance of the tryptic peptide results depends on the characteristics of the chromatographic separations on Sephadex G-50 in 8 M urea. These characteristics were quite reproducible; the elution volume for a particular pure compound was well defined and the peak was usually quite sharp. A smooth logarithmic relationship (Figure 5) between molecular weight and elution volume was obtained in the molecular weight range from 400 to 10,000 (Andrews, 1964). The resolution of peptide fragments by size on this column was much better than was earlier obtained (Doolittle and Singer, 1965) with a Bio-Gel P-10 column using a phenol–acetic acid–water solvent system (Carnegie, 1965).

The tryptic digests of affinity-labeled chains were separated on this column. In Figure 6 are shown the elution profiles of

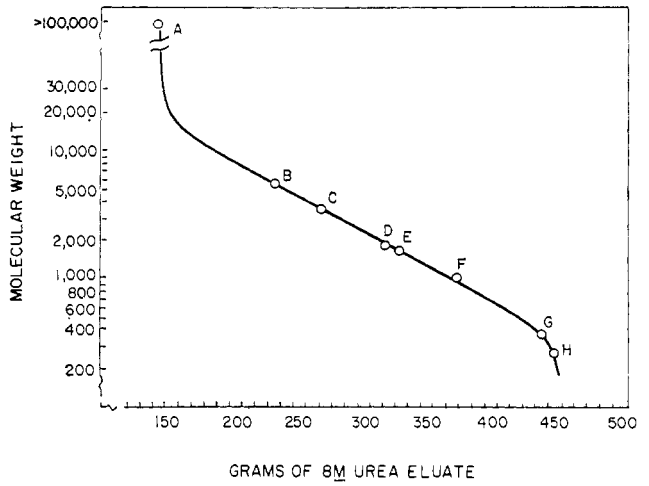

FIGURE 5: Molecular weight calibration of a column of Sephadex G-50 fine developed with 8 M urea. The compounds A–H are described in the text.

1- and 8-hr digests of rabbit anti-TMA L chains. They are essentially the same, indicating that the 8-hr digestion conditions produced a *limit* digest. This was not a *complete* digestion because clearly some only partially hydrolyzed peptides (peaks 1 and A, and probably 2 and B in Figure 7) were present in each digest.

The optical density and radioactivity profiles of the 8-hr digests of different affinity-labeled chains are compared in Figure 7. Owing to a lack of material, only radioactivity profiles could be determined for the digests of L chains of mouse anti-TMA Ab. These are shown in Figure 8 along with comparable profiles for the L chains of mouse anti-DNP Ab.

In general, the optical density and radioactivity profiles of a given digest were quite different, as would be expected if the affinity label were localized to characteristic tyrosine residues within the chain. For a given type of chain and species of origin, not only were the optical density profiles very similar for the two Ab specificities, but the radioactivity profiles were similar as well (compare, *e.g.*, Figure 7a,c, 7b,d, and the two curves in Figure 8). The significance of these results is considered below.

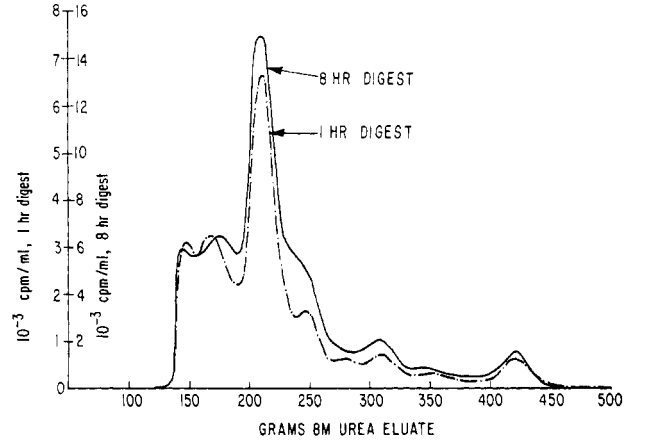

FIGURE 6: Separation of tryptic peptides of performate-oxidized L chains from affinity-labeled rabbit anti-TMA Ab, on the Sephadex G-50 column in 8 M urea after 1- and 8-hr digestion. The elution profiles of radioactivity of the affinity label are shown.

TABLE II: Amino Acid Composition of a Major Tryptic Peptide from Performate-Oxidized L Chains of Affinity-Labeled Rabbit Anti-TMA Ab.[a]

Amino Acid Residue	Moles of Residue[b]	
Lysine	2.00	2
Histidine	0.19	0
Arginine	0.00	0
Cysteic acid	1.89	2
Methionine sulfoxide	0.32	<0.5
Aspartic acid	7.79	8
Threonine	9.90	10
Serine	8.00	8
Glutamic acid	7.66	8
Proline	2.98	3
Glycine	9.07	9
Alanine	6.04	6
Valine	6.23	6
Isoleucine	2.33	2
Leucine	3.35	3
Tyrosine	4.33	4
Phenylalanine	2.85	3
Total Residues	74.9	74

[a] Fraction used corresponded to fraction C in Figure 7a. Sample analyzed is described in section on Materials and Methods. No corrections for destruction of residues was made, and no tryptophan analysis was carried out. [b] Calculated per 2.0 moles of lysine. The molecular weight of the peptide calculated in this manner corresponds to that found by gel filtration (7692 *vs.* 7600). The specific radioactivity of this isolated peptide fraction corresponded to 0.034 mole of affinity label per mole of peptide of mol wt 7692, in fair agreement with the specific activity of the original affinity-labeled L chain, 0.045 mole/mole of chain. That this peptide fragment contains only one residue of lysine is ruled out by these considerations.

In the case of rabbit L chains, the major peak of radioactivity (peak C) and of absorbance (peak 3, Figure 7a,c) coincided. This fraction was isolated from an experiment similar to that of Figure 7a, and was subjected to amino acid analysis (Table II).

Discussion

Specificity of Affinity Labeling of the Active Sites of Anti-TMA Ab. The evidence that the reaction of PTBDF with both rabbit and mouse anti-TMA Ab is largely specific for the Ab active sites is: (1) the rapid initial reaction with specific Ab as compared to normal γ-globulin; (2) the attachment of six to ten times as much radioactive label on unprotected as compared to protected Ab (Table I); and (3) the much more extensive reaction of PTBDF than of the nonspecific reagent *p*-(arsonic acid)benzenediazonium fluoroborate with anti-TMA Ab (Fenton and Singer, 1965). These results, together with the generally accepted conclusion that Ab active sites are much larger than PTBDF, so that the diazonium group of the reagent must fall within the active site when PTBDF becomes bound to the Ab, establish that specific labeling of the active sites has been achieved (Singer and Doolittle, 1966).

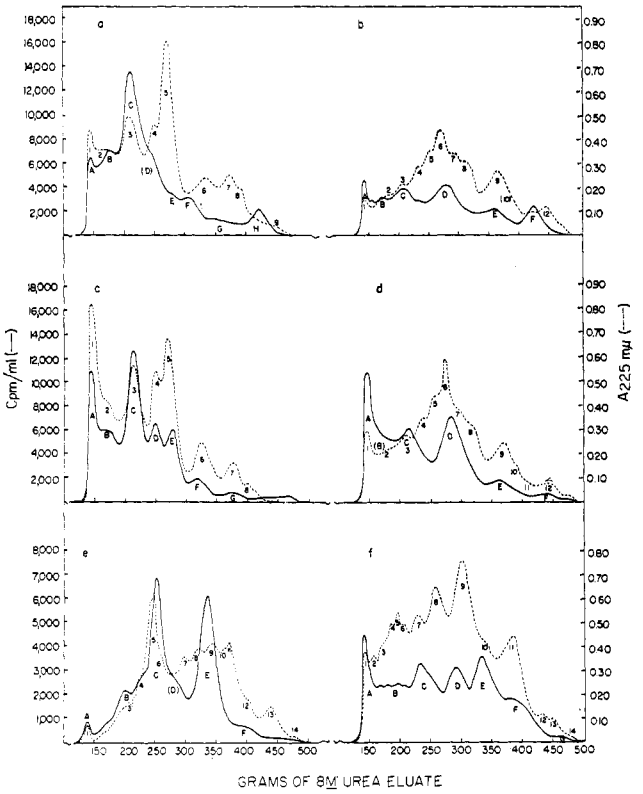

FIGURE 7: Comparisons of the profiles of absorbance at 225 mμ (peptide concentration) and of radioactivity of the affinity labels, of tryptic digests of performate-oxidized chains chromatographed on G-50 Sephadex in 8 M urea. (a) L chains of rabbit anti-TMA Ab; (b) H chains of rabbit anti-TMA Ab; (c) L chains of rabbit anti-DNP Ab; (d) H chains of rabbit anti-DNP Ab; (e) L chains of mouse anti-DNP Ab; and (f) H chains of mouse anti-DNP Ab. Upper case letters on the figure correspond to peaks of affinity label distribution, and numerals to peaks of peptide distribution.

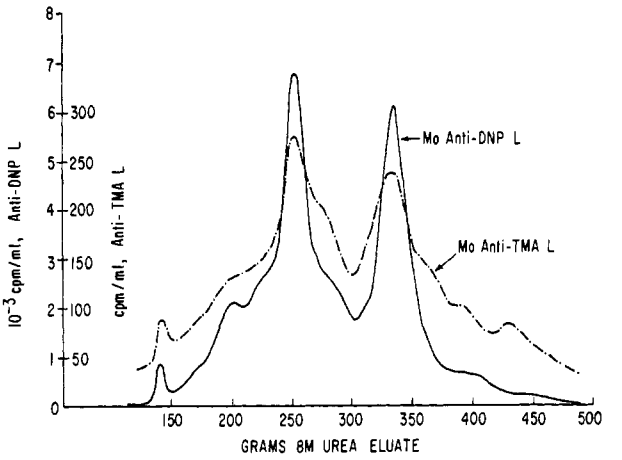

FIGURE 8: Comparisons of affinity-labeled (radioactivity) profiles of tryptic digests of the performate-oxidized chains of the L chains of mouse anti-TMA and anti-DNP Ab, chromatographed on G-50 Sephadex in 8 M urea.

The spectral and radioactivity results indicate that tyrosine residues are essentially the only groups with which PTBDF reacts in the Ab active sites. These tyrosine residues are on both the H and L chains of both rabbit and mouse anti-TMA Ab. Therefore both chains contribute to the formation of their respective Ab active sites.

It is remarkable that both qualitatively and quantitatively, the affinity-labeling results with rabbit and mouse anti-TMA Ab and with rabbit and mouse anti-DNP Ab (Good *et al.*, 1967; Thorpe and Singer, 1969) are so similar. On the other hand, not only are the Ab of the two specificities noncross-reactive, but the reagents used to affinity label each specific Ab are, of course, different. Yet in each case tyrosine residues on both the H and L chains are the exclusive groups which are labeled, and the ratio of label on H and L chains is remarkably uniform, ranging between about 2 and 4. These striking similarities must reflect some chemical and structural similarities among the active sites of the Ab of both specificities, as well as of both species of origin. The characteristics of the tryptic fragments of the affinity-labeled Ab permit a further exploration of these similarities.

Tryptic Peptides from Affinity-Labeled Chains. For typical homogeneous proteins, tryptic cleavages at lysyl and arginyl peptide bonds generally result in well-defined peptide fragments. For Ab chains, however, the situation is more complex because the Ab is molecularly heterogeneous (*cf.* review by Edelman and Gall, 1969). In any one species, there may be several classes of both H and L chains present in its immunoglobulins, each with its own characteristic lysyl and arginyl bonds. Furthermore, for any one class of chains, the variable regions may show variations in the positions of some of the lysyl and arginyl residues. Although such considerations suggest that tryptic peptide fragmentation might not be very useful in the structural characterization of Ab chains, we were encouraged by the findings that the affinity-labeled tyrosine residues were characteristic tyrosines, the residue from any one type of chain having predominantly one amino acid as its amino-terminal neighbor (Singer and Thorpe, 1968; Thorpe and Singer, 1969). Furthermore, it was possible to show conclusively in the case of L chains from affinity-labeled mouse anti-DNP Ab that the characteristic tyrosine residue was in the variable portion of the chain.

We therefore anticipated that if there were a characteristic tyrosine residue which was affinity labeled in all the types of chains in any one chain preparation, the labeled tryptic fragments arising from the variable regions might be heterogeneous but might exhibit a limited and characteristic *pattern of heterogeneity*. Furthermore, if the labeled tyrosines on anti-TMA and anti-DNP Ab were indeed structurally homologous to one another, then very similar patterns of heterogeneity might be observed for their labeled tryptic fragments. This expectation has been reasonably well borne out by the results obtained.

In the case of tryptic digests of rabbit L chains, for example, the affinity label was found predominantly in a particular peptide fraction, whose behavior corresponded to peptides of molecular weight about 7600 (peak C in Figure 7a,c), from *both* anti-TMA and anti-DNP Ab. This peak coincided with a peak of optical density (peak 3, Figure 7a,c) which was, however, not the major peak of optical density in the digests. When this fraction was isolated and analyzed (Table II), it appeared to have a fairly characteristic amino acid composition; *e.g.*, it contained little or no histidine or arginine, and two moles of lysine and cysteine per mole of peptide of mol wt 7600. The results suggest the presence of one lysyl bond resistant to tryptic cleavage. We suggest that this peptide fraction arises from a particular portion of the variable region of rabbit L chains containing the affinity-labeled tyrosine residue (or residues). When sufficient amino acid sequence data for rabbit L chains become available (Hood *et al.*, 1969; Jaton *et al.*, 1970),

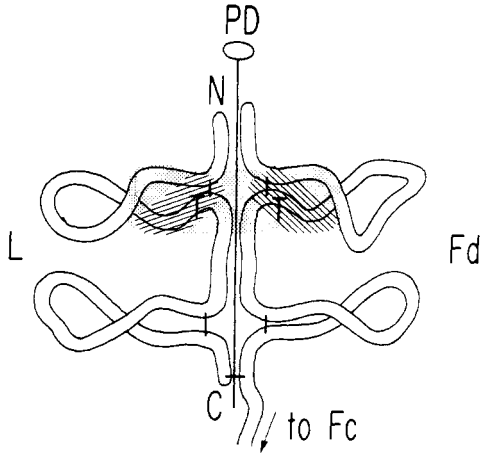

FIGURE 9: A schematic representation of the structure of an Fab fragment of an Ab molecule, after Singer and Thorpe (1968). PD represents the pseudodyad axis of symmetry relating the L chain and the Fd piece of the H chain in the Fab fragment. N and C are the amino terminus and carboxyl terminus of the L chain. The bars between chains and chain segments represent the inter- and intrachain disulfide bridges. T denotes the positions of the two tyrosine residues that are affinity labeled; it is proposed that these L- and H-chain tyrosines are homologous to one another in their respective chain sequences, and related by the pseudodyad axis. The active-site region of the fragment is schematically defined by the shaded region, the striped portion of which represents the zone within the active site which it is proposed is conserved among sites of different specificities, and the stippled portion of which represents the zone which varies in composition among sites of different specificities (see text for details). This structure is schematically represented in two dimensions, but must of course ultimately be translated into three.

we predict there will be found within the variable segments of these chains a peptide region which (1) is peptide bonded at its amino terminus to a lysine or arginine residue; (2) has another lysine residue about 75 residues further along the chain; (3) contains two cysteine residues and one lysyl bond (perhaps adjacent to a proline residue) which is resistant to trypsin, but contains no other internal arginine, lysine, or histidine residues; and (4) contains a tyrosine residue which is homologous to tyrosine-86 of mouse and human L chains (Singer and Thorpe, 1968), with one of the two cysteines being the invariant cysteine-88 and with a valine residue at position 85 (Thorpe and Singer, 1969).

For the purposes of this paper, however, because this large labeled fragment contains 4 tyrosine residues, and the experiments reported herein do not discriminate among them, no very precise test can be made of the possible homology between the affinity-labeled tyrosines on rabbit L chains of the two different Ab specificities. More useful, however, are the results obtained with mouse L chains (Figure 8). For both Ab specificities, the affinity label is found principally in two sharp fractions, corresponding to molecular weights of about 4500 and 1700, whereas the unlabeled tryptic peptides are distributed broadly over many different size classes (Figure 7e). It may be significant that these two size classes correspond to the predicted sizes of tryptic peptides including tyrosine-86 from two human Bence–Jones proteins. The larger-sized fraction corresponds in size to the peptide fragment of the protein Roy (Hilschmann, 1967a) which would result from tryptic cleavages at arginine-61 and lysine-103 to release a 42-amino acid peptide of mol wt 4777, while the smaller sized fraction correlates with a similar fragment from protein Cum (Hilschmann, 1967b) cleaved at arginines-77 and -91 to yield a 14 amino acid peptide of mol wt 1710. The mouse L chains of the two Ab might contain proteins corresponding to both Roy and Cum. (On the other hand, the majority of Bence–Jones protein sequences predict fragments containing from 25 to 37 amino acid residues including tyrosine-86.) It should be emphasized, however, that although the precise structural origins of these labeled peptide fragments from mouse L chains cannot be established from these data, the close correspondence of the *pattern of heterogeneity* of the labeled peptides from both Ab specificities seems highly significant, and suggests a structural homology between the tyrosine residues which are affinity labeled on the L chains of the two Ab specificities. In the case of the L chains of mouse anti-DNP Ab, the labeled tyrosine residue is characterized by having an aspartic acid or asparagine residue as its predominant amino-terminal neighbor (Thorpe and Singer, 1969), and the discrete size distribution of the labeled tryptic peptides most probably reflects the discreteness of the tyrosine residue which is affinity labeled. The conclusion, therefore, is that the labeled residue on the L chains of the mouse anti-TMA Ab is equally discrete and characteristic, since the labeled tryptic peptides show essentially the same size distribution as for anti-DNP Ab. A more direct demonstration of this homology would require detailed sequence studies of these labeled chains, a not-too-promising approach in view of the Ab heterogeneity.

The results just discussed clearly imply that very similar local regions of different immunoglobulin molecules are utilized to form the active sites of Ab of two different specificities. This conclusion may apply to a broad range of Ab of different specificities (Fenton and Singer, 1965; Singer and Doolittle, 1966; Wofsy *et al.*, 1967; Kennel and Singer, 1970), in view of the recurring observation that tyrosine residues on both H and L chains become affinity labeled in these systems. Singer and Thorpe (1968) have presented a model of the Ab active site which can be used to explore this hypothesis in somewhat more detail. In this model (Figure 9), the active site is formed by the apposition of homologous regions of the H- and L-variable segments. We suggest (see Figure 8 in Singer and Doolittle, 1966) that within the active site two overlapping zones are present. One zone would consist of certain *invariant* (or nearly invariant) *residues within the variable segments* of both chains, and would include the tyrosine residues that become affinity labeled; this zone might be essentially the same from one Ab specificity to another. The other zone would contain some of the *highly variable residues* within the variable segments of the chains, and might be involved in determining the subtle differences in specific binding affinity from one site to another. It is of considerable interest in this connection that one highly variable portion of the variable region sequence of Bence–Jones L chains occurs a few residues to the carboxyl side of cysteine-88 (*cf.* review by Edelman and Gall, 1969); this highly variable region might therefore be situated in the vicinity of the conserved residue tyrosine-86 in the three-dimensional structure of the molecule. Further affinity-labeling studies may show whether this hypothesis is correct, and may establish which residues carry out these several functions.

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Heparan Sulfates of Cultured Cells. I. Membrane-Associated and Cell-Sap Species in Chinese Hamster Cells*

Paul M. Kraemer

ABSTRACT: Heparan sulfate has been isolated and identified from three cellular fractions of Chinese hamster cells (line CHO) grown in suspension culture: as a cell surface component removable with trypsin under conditions preventing irreversible cell damage; as a free, directly acid-soluble component of the "cell-sap"; and as a part of the residual acid precipitate that became acid soluble following papain digestion. Heparan sulfate from the latter cell fraction could be divided into two portions: a minor portion that labeled rapidly and a major portion that labeled slowly. These two forms also differed slightly in apparent molecular size and charge.

All four forms of cellular heparan sulfate were identified by cellulose acetate electrophoresis, by depolymerization by direct nitrous acid treatment, and by comparisons with authentic bovine heparan sulfate. The isolated forms differed in both *N*- and *O*-sulfate composition and may differ also in residual peptide content. It is proposed that the various forms represent different maturation and/or storage forms of a single metabolic system.

We have previously reported (Kraemer, 1968) the isolation of a glycosaminoglycan with the characteristics of heparan sulfate from Chinese hamster cells grown in suspension culture. The same glycosaminoglycan material was isolated quantitatively and qualitatively from either the direct trichloroacetic acid soluble fraction of whole cells or the trichloroacetic acid soluble fraction of the "cell-sap" (*i.e.*, supernatant fluid of freeze–thaw broken cells after 105,000*g*, 60 min). The glycosaminoglycan was identified as heparan sulfate on the basis of sugar composition, presence of *N*-sulfate groups, resistance to degradation by testicular hyaluroni-

* From the Biomedical Research Group, Los Alamos Scientific Laboratory, University of California, Los Alamos, New Mexico 87544. *Received May 21, 1970.* This work was performed under the auspices of the U. S. Atomic Energy Commission.