Affinity Labeling of the Active Sites of Rabbit Anti-2,4-dinitrophenyl Antibodies with *m*-Nitrobenzenediazonium Fluoroborate*

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ABSTRACT: The method of affinity labeling has previously been shown to be capable of attaching chemical labels with a high degree of specificity to the active sites of antibody molecules. For rabbit antibodies to the 2,4-dinitrophenyl determinant, *m*-nitrobenzenediazonium fluoroborate is a highly specific site-labeling reagent. Quantitative studies with the tritiated form of this reagent have yielded analyses of the relative amount and specificity of labeling of the heavy and light chains of the antibodies. These analyses have shown that all of the label can be accounted for as azotyrosine. The label appears on both heavy and light polypeptide chains and is more than 90% localized to the active sites of the antibodies. The ratio of the label on the two chains remains essentially constant at close to 2:1 on a molar basis, from one batch of rabbit anti-2,4-dinitrophenyl antibodies to the next, and at different levels of labeling. The structural significance of these results is indicated. Finally, the specificity and stability of this label make possible unambiguous identification of peptide fragments originating from the active site regions of the antibodies.

In order to obtain direct chemical information about the active sites of antibody (Ab)[1] molecules, a method called affinity labeling has been developed in this laboratory (Wofsy *et al.*, 1962) for selectively attaching a chemical label covalently to some amino acid residue(s) in the active sites.[2] In this method a labeling reagent is chosen such that it (1) specifically and reversibly combines with the Ab active sites, and (2) contains a reactive functional group capable of forming an irreversible covalent bond with any of a number of amino acid residues. The binding of the reagent by the active site increases the local concentration of the reagent in the site as compared with the concentration in free solution, and thus results in the preferential formation of covalent bonds with amino acid residues within the site as compared with similar residues elsewhere on the protein molecule.

The method has been tested successfully with three different antihapten rabbit Ab systems so far: (1) with anti-*p*-azobenzenearsonate Ab and the labeling reagent *p*-(arsonic acid)benzenediazonium fluoroborate (Wofsy *et al.*, 1962); (2) anti-2,4-dinitrophenyllysyl (anti-DNP) Ab and *p*-nitrobenzenediazonium fluoroborate (PNBDF) (Metzger *et al.*, 1963b); and (3) anti-*p*-azotrimethylphenylammonium Ab and *p*-(trimethylammonium)benzenediazonium difluoorborate (Fenton and Singer, 1965). In each case, remarkably similar results have been obtained. Tyrosine residues on both the heavy (H) and light (L) polypeptide chains of the Ab molecule have been selectively azo derivatized by the specific diazonium reagents, and the evidence is very strong that these residues are present in the Ab active sites (Wofsy *et al.*, 1962; Metzger *et al.*, 1963b; Metzger *et al.*, 1964; Singer and Doolittle, 1966).

The ultimate objective of these studies is to gain structural information about antibody active sites from studies of the labeled peptides released from affinity-labeled Ab by a variety of chemical and enzymic degradative methods. It was decided that the antibodies of choice with which to begin such studies are rabbit anti-DNP. Not only are these among the best characterized anti-hapten Ab known, but they are readily prepared in large quantities. In exploring the chemical degradation of anti-DNP Ab which had been affinity labeled with PNBDF (Metzger *et al.*, 1963b), however, it was found that the azotyrosine derivative so formed was somewhat unstable to the protein degradative treatments subsequently employed, whereas the *meta* analog, MNBDF, not only formed a more stable azotyrosine derivative, but was the more highly specific labeling reagent. In this paper, therefore, studies of the affinity labeling of rabbit anti-DNP Ab with MNBDF are detailed, especially including the use of

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[1] Abbreviations used: Ab, antibody; anti-DNP, antibodies to 2,4-dinitrophenyllysyl; DNPNS, 2-(2,4-dinitrophenylazo)-1-naphthol-3,6-disulfonic acid disodium salt; PNBDF, *p*-nitrobenzenediazonium fluoroborate; MNBDF, *meta* analog of PNBDF; POPOP, 1,4-bis-2′(5′-phenyloxazolyl)benzene; PPO, 2,5-diphenyloxazole.

[2] Similar studies have been independently carried out with enzymes (Baker *et al.*, 1961; Schoellmann and Shaw, 1963; Lawson and Schramm, 1962).

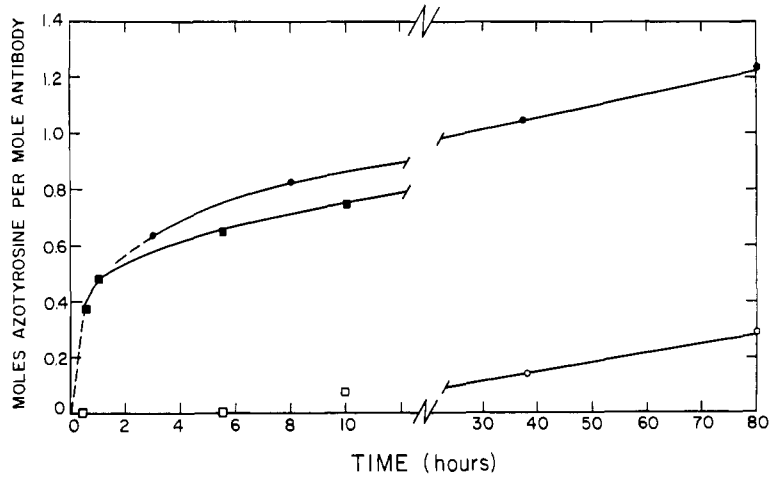


FIGURE 1: A comparison of the rate of reaction of PNBDF and MNBDF with protected and unprotected anti-DNP Ab. (■) PNBDF with unprotected Ab. (□) PNPDF with protected Ab. (●) MNBDF with unprotected Ab. (○) MNBDF with protected Ab.

the tritiated reagent, [^{3}H]MNBDF, since these provide the basis for much of the recent work from our laboratories (Choules and Singer, 1966; Lenard and Singer, 1966; Doolittle and Singer, 1965; Singer and Doolittle, 1966) as well as other studies now in progress.

The experimental work reported in this paper is divided into two parts: (1) studies of the affinity labeling characteristics of MNBDF for rabbit anti-DNP Ab including a comparison of the properties of MNBDF and PNBDF as labeling reagents, and (2) radioactive labeling studies with [^{3}H]MNBDF. Preliminary accounts of part of this work have been reported (Singer *et al.*, 1965; Doolittle *et al.*, 1965).

Materials

Proteins. Pure anti-DNP Ab from high-titer pooled rabbit antiserum was prepared essentially by the method of Farah *et al.* (1960) as in our earlier studies (Metzger *et al.*, 1963b). Rabbit normal γ-globulin (fraction II) was obtained from Pentex, Inc., Kankakee, Ill., and was used without further treatment. For both proteins, the extinction coefficient at 280 mμ of a 1% solution around neutral pH was taken to be 14.6 for the whole molecule, 14.5 for the H chains, and 13.2 for the L chains (Utsumi and Karush, 1964). In 1 M propionic acid, the extinction coefficients used were those of Crumpton and Wilkinson (1963) for solutions in 0.01 N HCl: H chain, 13.7; L chain, 11.8. The molecular weight of the whole molecule was taken as 160,000, the H chain at 55,000, and the L chain at 25,000.

Reagents. The preparation and properties of the diazonium reagents and related compounds used in this study are described in the following paper (Traylor and Singer, 1967). 2-(2,4-Dinitrophenylazo)-1-naphthol-3,6-disulfonic acid disodium salt (DNPNS) was obtained from Eastman Organic Chemicals and precipitated three times from water with isopropyl alcohol at pH 5.5. *N*-DNP-ϵ-aminocaproic acid was prepared by the method of Carsten and Eisen (1953). Sodium decyl sulfate was synthesized by a modification of the method of Dreger *et al.* (1944) described by Karush (F. Karush, personal communication). Sodium dodecyl sulfate was obtained from Fisher Scientific Co. and recrystallized from 1-butanol. The following chemicals were used as received: 1,4-bis-2′(5′-phenyloxazolyl)benzene (POPOP), scintillator grade, and 2,5-diphenyloxazole (PPO), scintillator grade, purchased from Calbiochem; 1 M Hyamine hydroxide in methanol, scintillator grade, purchased from Packard. Standard tritiated toluene, ±3%, was obtained from New England Nuclear Corp. All other chemicals were reagent grade.

Experimental Methods and Results

The Reaction of MNBDF with Anti-DNP Ab. Anti-DNP Ab at a concentration of 1.1×10^{-5} M in 0.2 M sodium acetate buffer, pH 5.0, was treated with 2 moles of nontritiated MNBDF/mole of Ab at 0° in the presence (protected) or absence (unprotected) of a molar concentration of *N*-DNP-ϵ-aminocaproate (protector) 13 times that of the Ab. Portions of the protected and unprotected reaction mixtures were removed at various times, protector was added to the unprotected portions, and the reaction was then rapidly quenched by the addition of a 1000-fold excess of resorcinol. Each sample was then precipitated in 75% ethanol in the cold and prepared for spectral analysis as described previously (Wofsy *et al.*, 1962). Protein concentrations were estimated from the absorbance at 280 mμ in 0.02 M phosphate buffer, pH 6.2, containing 0.03 M sodium decyl (or dodecyl) sulfate, while the azo absorption was measured at 490 mμ after making the solution 0.15 N in NaOH. A

blank of unreacted anti-DNP Ab was used to correct the latter readings for the small absorption of the protein at 490 mμ. The results are plotted in Figure 1 in terms of moles of azotyrosine formed per mole of Ab since, as is shown below, the product of the reaction with unprotected Ab is essentially exclusively azotyrosine. The extinction coefficient of the model compound *N*-chloroacetyl-3-(*m*-nitrophenylazo)tyrosine (Traylor and Singer, 1967) was assumed to apply to the protein derivative. Extensive modification of the unprotected Ab samples was observed, but only slight modification of the protected ones under the same conditions. Specificity of the reaction was further demonstrated by the fact that normal rabbit γ-globulin reacted with MNBDF under similar conditions was modified to only a very slight extent.

A Comparison of MNBDF and PNBDF as Labeling Reagents. An entirely parallel series of experiments to those of the previous section were carried out with nontritiated PNBDF and the same pool of anti-DNP Ab. The only difference in procedure was that the azo absorption of the protein derivatives in alkali were measured at 520 mμ, the absorption maximum of the corresponding model azotyrosine compound (Metzger *et al.*, 1963b). Control experiments showed that both diazonium compounds themselves were essentially completely stab'e under the conditions and for the times employed. The results are plotted in a corresponding manner in Figure 1. After a few tenths of a mole of azotyrosine has formed per mole of Ab, the absolute rate of the MNBDF reaction begins to exceed that of PNBDF, and at large reaction times, substantially more azotyrosine is formed with MNBDF. The protected Ab samples within the experimental error of the spectral assays are equally slightly modified by both reagents. MNBDF, therefore, appears to be a more specific affinity labeling reagent than PNBDF for pooled rabbit anti-DNP Ab, a conclusion also arrived at by a somewhat different method (Lenard and Singer, 1966).

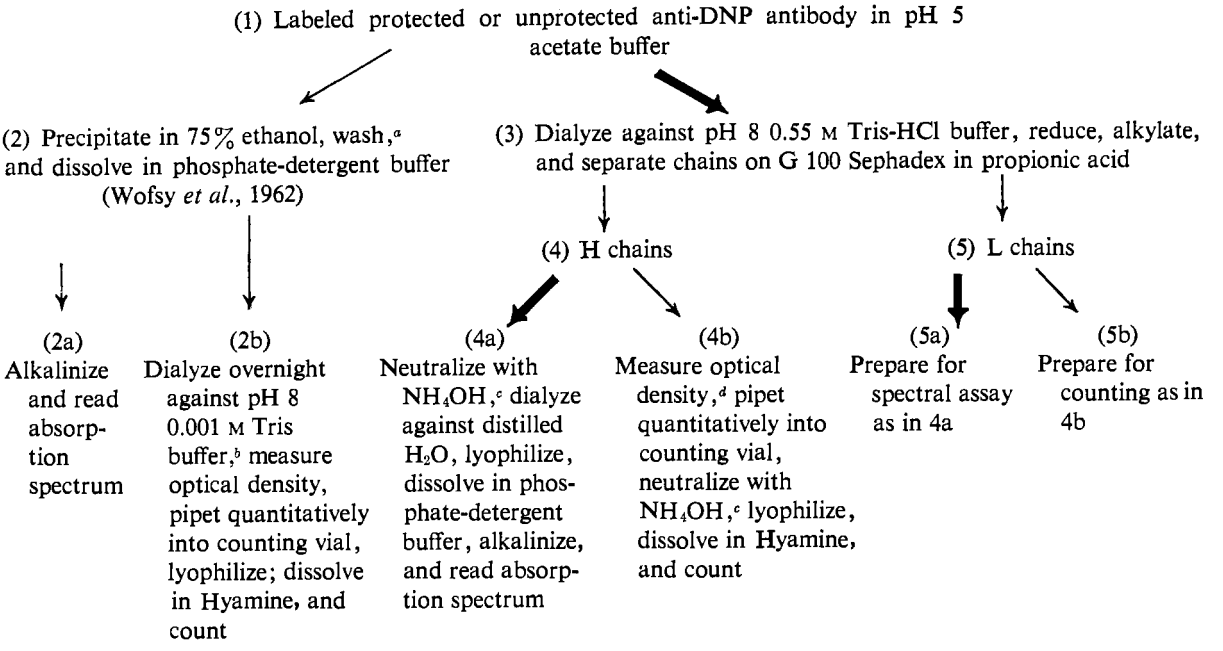
As well as being the more specific labeling reagent, MNBDF was found to form more stable azo bonds than PNBDF to tyrosine residues. Preliminary experiments with the model compounds prepared by the reaction of MNBDF and PNBDF with *N*-chloroacetyltyrosine showed that the former exhibited essentially no spectral change under conditions of mild reduction with mercaptoethanol similar to those used in separation of H and L chains (see below), whereas the azotyrosine derivative of PNBDF showed small but significant changes in the values of both the wavelength of maximum absorption and the extinction coefficient under these conditions. In addition, the azotyrosine derivative of MNBDF was found to exhibit only slight spectral changes after reduction for 45 min at 38° in 0.75 M mercaptoethylamine and 10 M urea at pH 7.5, or after oxidation in performic acid solution for 2.5 hr at −10°.

Labeling Studies with [^{3}H]MNBDF. Because of its greater specificity of labeling and the greater stability of its azotyrosine derivatives under conditions commonly used to prepare antibodies for enzymatic degradation, MNBDF was selected as the labeling reagent of choice for peptide fragmentation studies of anti-DNP Ab. In order to achieve the sensitivity of detection required, tritiated MNBDF was prepared as a labeling reagent (Traylor and Singer, 1967). It was then necessary to show correspondence of the spectral and ^{3}H-labeling results. The following is representative of a number of experiments performed to test this point, and to obtain more quantitative estimates of the specificity of labeling. Anti-DNP Ab at a concentration of 1.31×10^{-5} M was treated with a molar concentration of [^{3}H]MNBDF 1.5 times that of the protein for 6 hr under the conditions stated in the previous sections, with and without the protector present. At the conclusion of the reaction, an equivalent amount of protector was added to the unprotected sample, and both samples were freed of unreacted diazonium reagent by passage over a column of G-25 Sephadex in pH 5.0, 0.2 M ammonium acetate buffer at 4°. Since the procedures for preparing the labeled antibody for spectral assay, tritium counting, and separation into component H and L chains all contain features which were mutually incompatible, the labeled antibody was divided into several aliquots after the Sephadex step and processed in parallel. The details are given in Scheme I.

Aliquots (Scheme I, 2 and 2a,b) of protected and unprotected antibody were precipitated in 75% ethanol in the cold and washed as previously described (Wofsy *et al.*, 1962) for spectral analysis and counting. Details of the counting method are given in a later section. The major portion of the labeled antibody (Scheme I, 3) was dialyzed against pH 8.2, 0.55 M Tris-HCl buffer, and then reduced for 1 hr at room temperature in 0.2 M mercaptoethanol and alkylated for 1 hr at 0° by the addition of 0.22 M iodoacetamide according to the method of Fleischman *et al.* (1962). After dialysis overnight against 1 N propionic acid in the cold, the H and L chains were separated by gel filtration on G-100 bead Sephadex. The elution patterns obtained were similar to those reported by Metzger and Mannik (1964), exhibiting three peaks, A, B, and C in the order of their elution. Peaks A and B correspond to the single peak owing to H chains when the separation is carried out on G-75 Sephadex. Presumably peak A is due to aggregates of H chains since it is excluded from the gel, while peak B is monomeric H chains. About 80% of the total protein (absorbance at 280 mμ) was found under peak A plus peak B, and about 20% under the L-chain peak (peak C in this experiment). A pooled H-chain fraction (peak B) and a pooled L-chain fraction (peak C) were processed separately for spectrophotometric measurements and assay of specific radioactivity.

Spectrophotometric assays were carried out directly on washed whole antibody (Scheme I, 2a), lyophilized H chains (Scheme I, 4a), or lyophilized L chains (Scheme I, 5a) dissolved in phosphate-detergent buffer, after making the solutions 0.15 M in NaOH. All spectra shown are corrected for absorption by the appropriate

SCHEME I: Procedure for Comparison of Spectrophotometric and Tritium Assays.



[a] The washing procedure, previously used to remove noncovalently bound material (Wofsy *et al.*, 1962), was found to be necessary prior to counting. Removal of noncovalently bound tritiated diazonium reagent by G-25 Sephadex, exhaustive dialysis, or addition of a large excess of cold diazonium reagent was incomplete since tritium assays on such material always gave values almost 50% higher than the values obtained for washed samples of the same labeled antibody preparation. [b] Phosphate and NaCl (residual from wash) must be removed prior to counting since they are not soluble in the scintillation solvent systems. The detergent is compatible with the scintillation system and remains in the dialysis bag in sufficient concentration to keep the denatured protein in solution if dialysis time is limited to about 15 hr. [c] Neutralization prior to lyophilization improves the precision of the assay. The reason for this is not clear. [d] Extinction must be measured in propionic acid before neutralization since H chains usually precipitate in neutral solution. The extinction coefficients of Crumpton and Wilkinson (1963) for acid solutions are used for calculation of the protein concentration.

unlabeled proteins.

Assays for specific radioactivity were performed on washed whole antibody in pH 8 Tris-detergent buffer (Scheme I, 2b) and on H chains (Scheme I, 4b) and L chains (Scheme I, 5b) in 1 N propionic acid. After measurement of the absorbance at 280 mμ for estimation of protein content, quadruplicate aliquots of each sample were pipetted quantitatively into Wheaton glass vials. Sufficient NH_4OH was added to each vial containing propionic acid solution to render the mixture neutral, after which the samples were frozen on a block of Dry Ice and then lyophilized to dryness in a vacuum desiccator attached to a Virtis freeze-drying apparatus. The dried samples, each containing 0.4–1.0 mg of protein, were dissolved in 0.5 ml of 1 M Hyamine hydroxide in methanol by heating for 10 min at 80°. Counting was performed in a Nuclear-Chicago liquid scintillation counter after addition of 14.5 ml of scintillation fluid (consisting of 4 g of PPO and 50 mg of POPOP/l. of toluene) and 2 hr of temperature equilibration in the counting chamber. Efficiencies were determined directly on each sample by adding a known amount of standard [^{3}H]toluene sufficient to at least double the total count level. From these data and the measured specific activity of [^{3}H]-MNBDF, the moles of label per mole of protein was calculated.

The results are presented in Table I (expt 1) and Figure 2. They may be summarized as follows. (a) The spectra in Figure 2 show a generally close correspondence between the Ab derivatives and the model azotyrosine compound. The fact that the spectra of the labeled isolated chains are somewhat divergent from that of the model compound around 400 mμ may be due to experimental artifact, since the spectrum of the original labeled Ab from which the chains were derived is very similar to that of the model azotyrosine compound in this range. That the label is essentially all in the azotyrosine form is substantiated by the recovery of most of the label as free 3-[^{3}H]-(*m*-nitrophenylazo)tyrosine on pronase digestion of the labeled Ab (R. F. Doolittle and S. J. Singer, unpublished observations). The quantitative agreement between the amount of label attached to the Ab as determined spectrophotometrically as azotyrosine and as measured by total radioactivity is further confirma-

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

		Whole Ab			H Chains		L Chains		2H + 2L	
Expt	Reaction Condn	Spectral Azotyrosine	^{3}H	Ab Binding Sites Lost	Spectral Azotyrosine	^{3}H	Spectral Azotyrosine	^{3}H	Spectral Azotyrosine	^{3}H
1	Unprotected	0.58	0.66	—	0.23	0.23	0.11	0.113	0.68	0.69
	Protected	—	0.057	—	—	0.013	—	0.007	—	0.04
2	Unprotected	0.61	0.48	0.50 ± 0.05	—	0.128	—	0.068	—	0.39
3	Unprotected	—	0.0244	—	—	0.0069	—	0.0024	—	0.019
	Protected	—	0.0053	—	—	0.0026	—	0.0004	—	0.006

[a] The ^{3}H values are slightly different from those previously presented for some of these same experiments in Doolittle and Singer (1965) and Singer and Doolittle (1966). Small corrections to the specific activity of the [^{3}H]MNBDF reagent have been required. Data given in moles per mole of Ab.

tion of the exclusively azotyrosine nature of the label. (b) The low level of radioactivity of the protected Ab and its component chains as compared to the level of the corresponding unprotected proteins indicates that the Ab active sites are labeled with a high degree of specificity. Over 90% of the label attached to H and L chains must be associated with the specific active site regions of both chains. (c) The ratio of specific label on H and L chains is approximately 2:1 on a molar basis, similar to the distribution of label found with PNBDF (Metzger *et al.*, 1964). In 25 other labeling experiments performed under the same conditions over a period of 2 years, a similar ratio was found.[3] These experiments included 23 different pools of rabbit anti-DNP and five different preparations of MNBDF, both tritiated and untritiated. The average ratio of label on the H chain to label on the L chain was 2.4:1, with extremes of 1.6:1 to 3.3:1.

An experiment was next conducted to determine whether the ratio of labeling on H and L chains was a function of the over-all extent of labeling. To this end, a much lower extent of labeling was achieved utilizing a procedure closely similar to that of Scheme I, except that (1) only 0.036 mole of [^{3}H]MNBDF was added/mole of anti-DNP; (2) the reaction time was 40 min; and (3) no spectral analyses were performed. The results are shown in Table I, expt 3. Within the more limited accuracy of the measurements, the ratio of H to L label on the unprotected samples was 2.9:1.

An additional test was carried out of the specificity with which [^{3}H]MNBDF reacts with the active sites of anti-DNP Ab. Each site so reacted should no longer be capable of reversibly binding a specifi DNP-hapten, and the moles of reversible binding sites lost should correspond to the moles of label attached to the Ab. For this purpose another sample of anti-DNP Ab was labeled with [^{3}H]MNBDF at a molar concentration 1.5 times that of the protein for 6 hr in the pH 5.0 acetate buffer at 0°. After removing excess diazonium reagent by dialyzing against 500 volumes of the acetate buffer, the labeled antibody was dialyzed against several changes of pH 7.4, $\Gamma/2$ 0.2 phosphate buffer (Metzger *et al.*, 1963b). Samples of the labeled antibody and a sample of unlabeled antibody from the same anti-DNP lot were then titrated with the dye hapten, DNPNS, under standard conditions (Metzger *et al.*, 1963a,b). DNPNS undergoes a change in the pK of its naphtholic hydroxyl group from about 6.5 to 9.0 on specific reversible binding to anti-DNP antibody (Wofsy, 1961; Sturtevant *et al.*, 1961). At pH 7.4, binding results in a profound spectral shift from blue (free dye) to red (bound dye), and it is thus possible to calculate the concentration of bound and free hapten from the measured optical densities of the DNPNS-antibody solution at 486 and 590 mμ (Metzger *et al.*, 1963a). The results of titration of 1.2×10^{-5} M solutions of labeled and unlabeled antibody with a 4.8×10^{-4} M solution of DNPNS are given in Figure 3. The plateau value of r (moles of DNPNS bound per mole of protein) in hapten excess is taken as equal to the number of binding sites per Ab molecule. The number of sites lost as measured by DNPNS binding was equal to the number of sites labeled as measured by specific radioactivity (Figure 3 and Table I, expt 2).

In view of the incompleteness and kinetic heterogeneity (Figure 1) (Metzger *et al.*, 1963b) with which MNBDF or PNBDF labels anti-DNP Ab sites, and the differing geometries of the two reagents, it appeared possible that each reagent might label a somewhat different segment of the population of anti-DNP Ab sites. If so, they might exhibit at least partially

[3] Preparative runs for production of labeled heavy and light chains for peptide studies were monitored by counting 50-μl aliquots of the pooled H and L chains on filter paper strips. Calculations were based on an average efficiency of 12.8%. Although this method is less precise than the Hyamine method, the results are comparable.

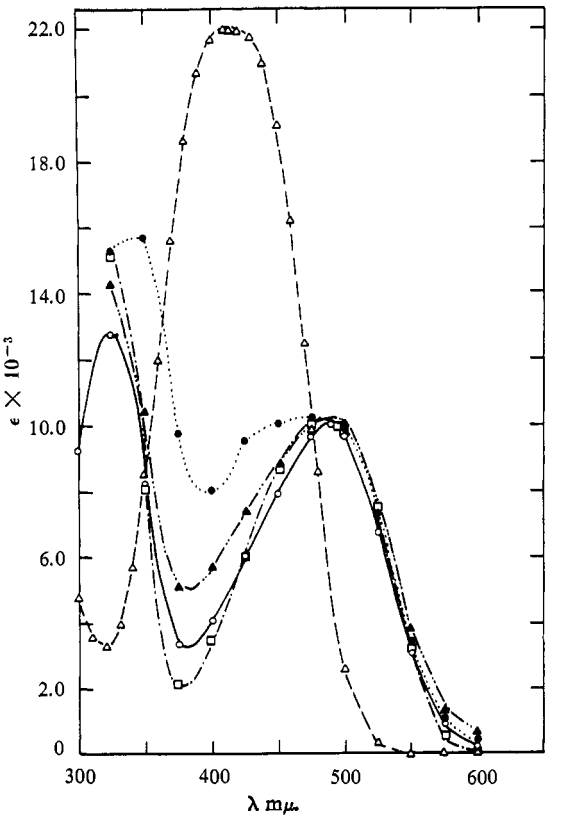


FIGURE 2: Spectra in 0.15 M NaOH of: (a) *m*-nitrophenylazo-*N*-chloracetyltyrosine (O—O), (b) *m*-nitrophenylazo-*N*-acetylhistidine (Δ- - -Δ), (c) unprotected anti-DNP Ab treated with MNBDF for 6 hr (□—·—□), (d) H-chain fraction of c (●· · · · ·●), and (e) L chain fraction of c (▲—· ·—▲). The values of ϵ refer to curves a and b. Curves c–e have been adjusted to the same scale. For whole Ab, $A_{490\,m\mu} = 0.076$ at a concentration of 1.88 mg of protein/ml; for the H-chain fraction, $A_{490\,m\mu} = 0.190$ at a concentration of 4.28 mg of protein/ml; and for the L-chain fraction, $A_{490\,m\mu} = 0.197$ at a concentration of 3.54 mg of protein/ml (see Traylor and Singer, 1967).

additive, rather than competitive, labeling if used in sequence.

This possibility was tested in a preliminary experiment in which the initial labeling was carried out with MNBDF, followed by reaction with PNBDF. A suitable antibody pool was divided into three parts. Two were labeled for 6 hr at pH 5, 0°, with 2 moles of [^{3}H]-MNBDF/mole of protein, while the third was labeled for 45 min under the same conditions with [^{3}H]PNBDF. (Each experiment was accompanied by a protected control.) At the conclusion of the initial labeling period, an aliquot from each pool was pipetted into a tube containing a 1000-fold excess of resorcinol. This mixture was then precipitated in 75% ethanol, washed, and counted as described in the previous section. In the second stage of the experiment, another aliquot of reagent sufficient to give a molar concentration of fresh reagent two times the residual protein concentration was added to each reaction mixture. The times used for the second stage were the same as in the first stage, *i.e.*, 6 hr for [^{3}H]MNBDF and 45 min for [^{3}H]PNBDF. At the conclusion of the experiment, the reactions were terminated by the addition of a 1000-fold excess of resorcinol and processed for counting as before. In Table II, the results of sequential labeling with [^{3}H]-MNBDF followed by [^{3}H]PNBDF are compared with the results for two successive labeling periods for each of the single reagents. The significant findings are (a) that the amount of label (0.09 mole/mole of protein) attached by the reaction of PNBDF after an initial labeling with MNBDF is not larger than the amount of label (0.15 mole/mole of protein) attached by the same dose of PNBDF after an initial labeling with PNBDF; and (b) that the total amount of label (0.84 mole/mole of protein) attached by the sequential reaction of MNBDF and PNBDF is close to the mean value ((1.09 + 0.54)/2 = 0.82 mole/mole of protein) of the amounts attached by two successive doses of either reagent alone. Therefore, the reactions of MNBDF and PNBDF with anti-DNP Ab sites appear to be largely competitive rather than additive.

TABLE II: Sequential Labeling of Anti-DNP Ab with [^{3}H]MNBDF and [^{3}H]PNBDF.

Reagent Sequence		Label Attained (moles of label/mole of protein)		
		Initial Stage	Final Stage	Difference
[^{3}H]MNBDF– [^{3}H]MNBDF	Protected	0.095	0.205	0.11
	Unprotected	0.75	1.09	0.34
[^{3}H]PNBDF– [^{3}H]PNBDF	Protected	0.043	0.070	0.03
	Unprotected	0.39	0.54	0.15
[^{3}H]MNBDF– [^{3}H]PNBDF	Protected	0.095	0.111[a]	0.016
	Unprotected	0.75	0.84[a]	0.09

[a] Determined by difference, based on specific activity of [^{3}H]PNBDF and total disintegrations per minute less disintegrations per minute due to [^{3}H]MNBDF from initial labeling period.

In order to provide a reference state for the reactions of MNBDF and PNBDF with Ab, some kinetic experiments were carried out of the reactions between these reagents and *N*-chloroacetyltyrosine. A Cary 14 recording spectrophotometer fitted with a thermostated cell holder was used. Reactions at concentrations of diazonium reagents of about 5×10^{-5} M in a 50- to 100-fold molar excess of the tyrosine derivative were carried out at 25.30 ± 0.02° in the acetate buffer, pH 5.00, used in our Ab experiments. Pseudo-first-

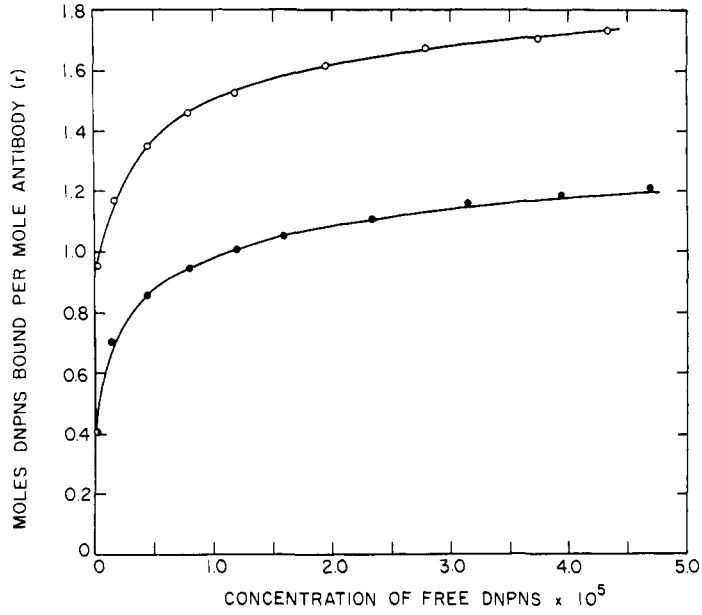


FIGURE 3: The titration of residual Ab activity with the hapten DNPNS: (O—O) unlabeled anti-DNP Ab; (●—●) anti-DNP Ab from the same pool containing 0.5 mole of azotyrosine/mole of Ab.

order rate constants were determined, from which apparent second-order rate constants, k_s, were derived, using the stoichiometric concentration of *N*-chloroacetyltyrosine. The values of k_s were 0.110 and 0.119 M^{-1} sec^{-1} for the reactions of MNBDF and PNBDF, respectively. Under these model reaction conditions, PNBDF is, therefore, slightly the more reactive reagent, although in the labeling reaction with Ab, it is MNBDF which is more reactive.

Discussion

The Specificity of the Labeling Reaction. The evidence is very strong that the reaction of [^{3}H]MNBDF is highly specific for the active sites of the anti-DNP Ab. The results may be summarized as follows: (a) the reaction occurs at a rapid rate only with the homologous Ab, and at much slower rates with normal γ-globulin; (b) the enhancement in rate is eliminated by the presence of the specific protector of the active sites, DNP-aminocaproate; (c) a number of reversible binding sites which is equal to the number of moles of label attached per mole of the Ab is lost; (d) a single kind of residue, tyrosine, is labeled in the reaction; and (e) the size of the anti-DNP Ab site is large enough (Eisen and Siskind, 1964) that the diazonium group of MNBDF is well within the site when the reagent is specifically bound to the Ab. These operational criteria at the level of the whole labeled protein molecule are exactly analogous to those which were used in the well-known case of the labeling of the active sites of esterase enzymes with diisopropyl fluorophosphate and similar compounds (*cf.* Cohen *et al.*, 1959). This specificity for the active sites extends to both the H- and L-chain labels, since specific protection of the sites markedly and equally diminishes both labels (Table II).

With the information presented in this paper as a starting point, studies have been initiated (Doolittle and Singer, 1965) on the labeled peptide fragments from the H and L chains of [^{3}H]MNBDF-labeled anti-DNP Ab. The results given in Table I, expt 1, show that over 90% of the label on both chains is specific to the active sites. Therefore, the distribution of radioactivity is a direct measure of the distribution of peptide fragments from the active sites.

The specificity of the labeling reaction with MNBDF is greater than with PNBDF, as indicated both by the results of Figure 1 and by the independent experiments of Lenard and Singer (1966). The over-all rate of the MNBDF reaction is greater in spite of the fact that PNBDF is slightly more reactive toward *N*-chloroacetyltyrosine under essentially the same conditions. While the effects are real, there is not enough information available to interpret their significance. In view of the heterogeneity of the anti-DNP Ab, one possibility is that the two reagents react with a somewhat different, if overlapping, population of active sites. This possibility does not appear to be important, according to the results of the sequential labeling experiments of Table II. Another possibility is that MNBDF exhibits a higher average reversible binding constant, K_A, for anti-DNP sites than does PNBDF; the rate of affinity labeling is directly proportional to K_A (Wofsy *et al.*, 1962), other things being equal. Estimates of K_A for PNBDF and anti-DNP Ab have been made from a detailed analysis of kinetic experiments (Metzger *et al.*, 1963b) but were not carried out with MNBDF.

A further possibility is that the stereochemistry of the two reversibly bound reagents in the active sites is different; *i.e.*, the equilibrium positions and orientations of the diazonium groups of these reagents relative to the tyrosine residues is different. On the other hand, it is noteworthy that with both reagents, the ratio of label on the H- and L-chain tyrosines in the sites is about the same.

The Occurrence of Tyrosine Residues in the Ab Active Sites. The question arises, are all or only some anti-DNP active sites capable of being affinity labeled at tyrosine residues? The over-all rate of modification of the specific Ab by MNBDF falls to a value only slightly greater than that of protected Ab after about one-half of the Ab active sites have reacted (Figure 1). This does not necessarily mean, however, that the remaining sites do not contain tyrosine residues. It may mean only that the *enhanced* rate of reaction at tyrosine residues in the remaining Ab sites is not sufficiently large to be evident above the background of nonspecific reactions occurring at the many other reactive amino acid residues outside the site. Both theory and experiment (Wofsy *et al.*, 1962; Metzger *et al.*, 1963b) indicate that the heterogeneity in the rate of affinity labeling is at least partially due to the heterogeneity of K_A values of the Ab for the labeling reagent. Our results show that among those sites which become labeled, the first 0.03 mole exhibits the same ratio of H:L-chain tyrosine labeling as the first 0.60 mole of sites/mole of Ab (Table I). This argues for a remarkable structural similarity among these sites (see next section) in spite of the heterogeneity of their K_A values. It is entirely possible, therefore, that this structural similarity (in particular, the presence of tyrosine residues) applies to all rabbit anti-DNP sites, but that some of the sites exhibit small K_A values and correspondingly small rates of labeling. On the other hand, we cannot at this time eliminate the possibility that some active sites not containing tyrosine residues are present in the anti-DNP population.

On the Structure of Ab Active Sites. It is a highly significant fact that MNBDF and PNBDF form specific azotyrosine labels on both H and L chains. What is more striking, however, is the essential constancy of the ratio, approximately 2:1 on a mole basis, of label on the two chains. Among 23 independently prepared anti-DNP Ab pools labeled with [^{3}H]MNBDF, the extremes found for this ratio were 1.6:1 and 3.3:1. At two levels of total label differing by a factor of 20, the ratio was essentially unchanged (Table I). In two independent rabbit Ab systems labeled with their specific diazonium reagents (Metzger *et al.*, 1964; Fenton and Singer, 1965) very similar results were obtained. In view of the fact that strong steric restrictions operate in affinity labeling reactions (*cf.* Singer and Doolittle, 1966; Singer, 1967), this constancy in the pattern of labeling is an entirely nonrandom result. It can reasonably be explained only by postulating a strong structural similarity among different Ab active sites.

These data taken together have formed the basis for the proposal recently made (Singer and Doolittle, 1966) that both H and L chains contain limited characteristic regions within them that are utilized to form the active sites of many, if not all, rabbit Ab molecules. These characteristic regions include the tyrosine residues that become labeled. From one L chain to another, and from one H chain to another in an Ab molecular population, differences in amino acid composition and sequence occur in these characteristic regions in the vicinity of the tyrosine residues (Hilschmann and Craig, 1965; Doolittle and Singer, 1965). The further proposal was made and supported that the H and L chains are related gene products, *i.e.*, their structural genes have arisen from some common ancestral gene during evolution. As related gene products, H and L chains might be expected to be chemically and functionally similar but not identical. It was suggested, therefore, that the characteristic region of one H chain and the nonidentical but homologous characteristic region of one L chain come together to form one active site of an intact Ab molecule.

On the other hand, given the chemical heterogeneity of these characteristic regions, and the resulting binding heterogeneity of the Ab, it is surprising that a constant ratio of H:L-chain labeling is found among the different Ab sites. This suggests that the characteristic homologous regions of H and L chains might somehow be matched in any one Ab molecule. In other words, the pairing of H and L chains might be not at all random, but might be restricted by close chemical homology of the two chains in these active site regions. That restrictions do in fact exist on the pairing of H and L chains is supported by a number of studies on the recombination of H and L chains; in particular, those of Roholt *et al.* (1965) and Hong and Nisonoff (1966). The latter authors studied the recombination of H and L chains isolated from two different fractions of anti-DNP Ab from the same rabbit. H and L chains from a given fraction were more effective than heterologous pairs in reconstituting the binding activity. What might be the biosynthetic and selective origins of such H- and L-chain pairing is obscure at the present time, but is clearly of great importance to theories of Ab formation.

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The Preparation and Properties of Some Tritiated Diazonium Salts and Related Compounds*

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ABSTRACT: Several tritiated diazonium salts of high specific activity and radiochemical purity have been investigated. The methods used in the preparation and characterization of these compounds and their azo derivatives are presented in detail. These compounds have been effective in the specific labeling of antibody active sites, and should be of general use in connection with protein structure studies.

In the course of several years' studies from this laboratory on the affinity labeling of antibody active sites, several diazonium salts and their derivatives, both nonradioactive and tritiated to high specific activities, have been prepared and their properties investigated. Diazonium compounds are ordinarily quite unstable and, for most biochemical applications, have been prepared *in situ* from their corresponding amines and then used immediately. For our purposes, these conventional procedures were not satisfactory; the preparation of stable highly radioactive diazonium compounds was required to obtain quantitative and reproducible labeling results. Fluoroborate salts of diazonium compounds have commonly been used as stable reagents in organic chemistry, and two of these in nonradioactive form have been employed in our earlier studies (Wofsy *et al.*, 1962; Metzger *et al.*, 1963). In this paper, our experience with three tritiated diazonium fluoroborates and their derivatives is described not only as a reference for our own investigations (*cf.* Good *et al.*, 1967), but also in the hope that these

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