Affinity Labeling of the Active Sites of Anti-2,4-dinitrophenyl Antibodies from Different Species*

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ABSTRACT: Purified antibodies directed to the 2,4-dinitrophenyl (DNP) group from sheep, guinea pigs, and mice have been affinity labeled with the reagent [^{3}H]*m*-nitrobenzenediazonium fluoborate under conditions previously found to be optimal for the specific labeling of the active sites of the analogous rabbit antibodies. The guinea pig antibodies were of two kinds, designated γG_1 and γG_2. In all cases: (1) specific labeling of the active sites was achieved, by the criterion of specific protection of the sites with 2,4-dinitrophenylaminocaproate; (2) the specific label was found on both heavy and light polypeptide chains of the antibodies; and (3) the specific label was azo linked to tyrosine residues on both chains. These results are closely similar to those previously obtained with the rabbit antibodies.

The conclusion that both heavy and light chains contribute to the structure and specificity of the antibody active site is therefore reinforced. In addition, chemical similarities within the active sites of antidinitrophenyl antibodies from a variety of species are indicated.

In the initial papers of this series (Wofsy *et al.*, 1962; Metzger *et al.*, 1963b), it was demonstrated that the method of affinity labeling is capable of covalently attaching a chemical label to one or more residues in the active site of an antibody molecule with a high degree of specificity.[1] Rabbit antibodies (Ab)[2] directed toward three quite different haptenic determinants have been investigated in our laboratories, namely, *p*-azobenzenearsonate (Wofsy *et al.*, 1962), *p*-azotrimethylphenylammonium (Fenton and Singer, 1965), and 2,4-dinitrophenyl (DNP) (Metzger *et al.*, 1963b; Good *et al.*, 1967). The results obtained with all three Ab and their specific affinity labeling reagents have been remarkably similar, and this striking fact, along with other observations, has led us to certain generalizations about the structure of antibody active sites directed to benzenoid haptens (Singer and Doolittle, 1966). On the other hand, rabbit antibody molecules may have some structural features that are unique; for example, the amino acid sequences at the amino-terminal region of rabbit immunoglobulin light chains are significantly different (Doolittle, 1966) from their human and mouse counterparts, which in turn are very similar to one another (Hood *et al.*, 1966). There also appears to be an extra disulfide bridge in rabbit light chains (Crumpton and Wilkinson, 1963; Singer *et al.*, 1968). We therefore undertook to affinity label Ab from several additional species in order to explore the generality of our conclusions.

Since the rabbit system which we have most intensively investigated has been anti-DNP Ab labeled with the tritiated reagent [^{3}H]MNBDF (Good *et al.*, 1967), studies were carried out with this reagent and anti-DNP Ab from sheep, guinea pigs, and mice. The studies with guinea pig Ab are of further interest in that two types, γG_1 and γG_2, from the same serum pool were isolated and labeled. These two types differ in their heavy (H) chains but not in their light (L) chains (Benacerraf *et al.*, 1964; Ovary *et al.*, 1963; Bloch *et al.*, 1963; Thorbecke *et al.*, 1963). The results with all of these preparations support the conclusions that: (1) both H and L chains contribute to the structure and the binding specificity of antibody active sites; and (2) that chemical similarities exist among the active sites of anti-DNP antibody molecules from the different species.

Materials and Methods

Sheep anti-DNP antibodies were obtained from a single animal initially injected subcutaneously in several sites with 46 mg of DNP bovine γ-globulin (Farah *et al.*, 1960) emulsified with Freund's adjuvant and boosted after 4 weeks by intravenous injection

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

[2] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966) are: Ab, antibodies; anti-DNP, antibodies directed to the 2,4-dinitrophenyl group; MNBDF, *m*-nitrobenzenediazonium fluoborate; DNPNS, 2-(2,4-dinitrophenylazo)-1-naphthol-3,6-disulfonic acid, disodium salt.

of 10 mg of the antigen. The animal was bled 7 and 9 days after the booster.[3]

The method of purification of the sheep Ab was the same as that previously used for rabbit anti-DNP Ab (Farah *et al.*, 1960; Metzger *et al.*, 1963b). The amount of residual dinitrophenol in the antibody active sites, as estimated from the optical density at 280 and 360 mμ, was negligible. The purified Ab gave a precipitin line with DNP-bovine γ-globulin but not with bovine γ-globulin itself by Ouchterlony gel diffusion, and migrated as a single peak in the ultracentrifuge with sedimentation constant of approximately 7 S. A preliminary estimation of the equilibrium constant for the specific binding of the sheep Ab and the dye hapten DNPNS (Metzger *et al.*, 1963a) indicated an average value of about 1×10^6 M^{-1}, as compared to a value of around 3×10^7 M^{-1} usually found for rabbit anti-DNP Ab.

Mouse Anti-DNP Antibodies. Lyophilized purified mouse anti-DNP Ab were kindly donated by Dr. Carmen C. Merryman. These Ab had been raised in randomly bred Swiss Webster mice after intraperitoneal injection of DNP-bovine γ-globulin emulsified with complete Freund's adjuvant. After repeated booster injections, the Ab were isolated from decomplemented ascitic fluid by precipitation with DNP-fibrinogen. Elution of the specific Ab with excess 2,4-dinitrophenol and subsequent removal of the bound dinitrophenol from the Ab were performed essentially by the method of Farah *et al.* (1960). The sample contained both γG_1 and γG_2 Ab (Merryman and Benacerraf, 1963; Nussenzweig *et al.*, 1964) by immunoelectrophoresis developed with antiserum to whole mouse serum. These Ab were not separated for the present labeling studies. Optical density readings at 280 and 360 mμ on the purified mouse anti-DNP preparation after solution in buffer indicated that less than 10% of the active sites contained residual dinitrophenol.

Guinea Pig Anti-DNP Antibodies. Pooled guinea pig anti-DNP serum was obtained as described previously (Benacerraf *et al.*, 1963). Each serum added to the pool had a passive hemolysis titer greater than 3200 and a passive cutaneous anaphylaxis titer greater than 5000.

The anti-DNP Ab was purified by the method of Farah *et al.* (1960) except that two stages were employed. In the first stage, the specific precipitate of anti-DNP Ab and DNP bovine γ-globulin was eluted with 2×10^{-3} M ε-DNP-lysine at pH 8.4 instead of with dinitrophenol, and streptomycin sulfate was not added until the end of the elution period. The yield of specific pseudoglobulin at this stage was 77%. About 1.4 moles of ε-DNP-lysine/mole of Ab remained bound as estimated from the optical densities at 280 and 360 mμ.

In the second stage, in order to remove the bound ε-DNP-lysine, the purified Ab at a concentration of 3.7 mg/ml was dialyzed against four changes of 0.1 M dinitrophenol at pH 7.4 at room temperature for 72 hr. The dinitrophenol was then removed by passage over Dowex 1-X8 resin equilibrated with pH 7.4, 0.001 M phosphate buffer containing 0.15 M NaCl (4-ml bed volume/10 mg of Ab). After precipitation with $(NH_4)_2SO_4$ and dialysis against pH 7.4, 0.01 M phosphate buffer containing 0.15 M NaCl, the antibody yield in this second stage was about 85%. Residual ε-DNP-lysine, estimated from optical densities at 280 and 360 mμ, was about 0.3 mole/mole of Ab.

A number of other methods of removal of antibody-bound ε-DNP-lysine were tried, including exchange with the dye hapten DNPNS at low pH where it binds strongly to the Ab, followed by removal of the dye on Dowex resin at pH 9 where its binding strength is reduced (Metzger *et al.*, 1963a). None of these methods yielded Ab with less than 0.3 mole of residual ε-DNP-lysine/mole.

After removal of as much ε-DNP-lysine as possible, the γG_1 and γG_2 antibodies in the pool were separated from one another by the method of Spalter and Ovary (1965), except that DEAE Sephadex A-25 was used in place of DEAE-cellulose. Adequacy of separation was tested by means of passive cutaneous anaphylaxis and complement fixation titrations (Ovary *et al.*, 1963; Bloch *et al.*, 1963) or by complement fixation alone on the various antibody preparations. Such tests always indicated virtually complete separation of the two antibody types. Both the γG_1 and γG_2 antibodies had the same amount of residual ε-DNP-lysine (0.3–0.4 mole/mole of Ab) and gave a precipitin line against DNP bovine γ-globulin but not against bovine γ-globulin itself when tested by Ouchterlony gel diffusion.

Values for the extinction coefficients at 280 mμ of 1% solutions of rabbit antibodies and their chains at neutral pH (Utsumi and Karush, 1964) were assumed to apply also to their mouse and sheep counterparts.[4] The molecular weights of the different antibodies and their chains were assumed to be the same as for their rabbit counterparts: 160,000 for the whole molecule, 55,000 for the heavy chains, and 25,000 for the light chains. Other materials and reagents were the same as those used in our previous studies (Good *et al.*, 1967).

Affinity Labeling and Analyses. Each of the antibodies was labeled under the conditions normally used for routine preparation of labeled rabbit chains for peptide studies (Good *et al.*, 1967). Antibody at a concentration of $1.1–1.26 \times 10^{-5}$ M was reacted in the presence (protected) or absence (unprotected) of a 13.2-fold excess of the protector, *N*-DNP-ε-aminocaproic acid, with 1.5 moles of [^{3}H]MNBDF/mole of antibody at 0° in pH 5.0, Γ/2, 0.20 M sodium acetate buffer for 6 hr. At the conclusion of the reaction, an equivalent amount

[3] Injection and bleeding of the sheep were performed by Antibodies, Inc., Davis, Calif.

[4] Extinction values for whole guinea pig γG_1 and γG_2 antibodies were determined in this study. They were 3.5% larger and 9.0% smaller, respectively, than the value for the rabbit antibodies. The extinction values of the H and L chains of the guinea pig antibodies were estimated using these proportions of the values for the rabbit antibody chains.

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

Species	Reaction Condn	Whole Antibody	H Chains	L Chains	R	2 H + 2 L
Rabbit[b]	Unprotected	0.66	0.23	0.113	2.0	0.69
	Protected	0.057	0.013	0.007		0.04
Guinea pig γG_1	Unprotected	0.670	0.222	0.059	2.5	0.562
	Protected	0.365	0.117	0.020		0.274
Guinea pig γG_2	Unprotected	0.638	0.214	0.065	3.7	0.558
	Protected	0.171	0.044	0.019		0.126
Sheep	Unprotected	0.832	0.261	0.050	4.8	0.622
	Protected	0.199	0.033	0.003		0.072
Mouse	Unprotected	0.49	0.159	0.087	1.8	0.49
	Protected	0.18				

[a] In moles per mole of Ab by ^{3}H count. [b] From Table I, expt 1, Good *et al.* (1967).

of protector was added to the unprotected sample, and both preparations were dialyzed against 100 volumes of the pH 5.0 acetate buffer overnight at 4°. After dialysis, 2–6-mg aliquots of unprotected and protected Ab were removed, precipitated in 75% ethanol, and washed for spectral analysis and for tritium counting by a modification of the method described previously (Wofsy *et al.*, 1962). Since phosphate and NaCl are incompatible with the solvent system used for tritium counting, 1 ml of ice-cold water was substituted for the last wash of 5 ml of 1% NaCl and the washed proteins were dissolved in 0.03 M sodium decyl sulfate at neutral pH rather than in phosphate–detergent buffer. Subsequent procedures for spectral assays at neutral and alkaline pH, and for counting, were the same as described previously (Wofsy *et al.*, 1962; Metzger *et al.*, 1963b; Good *et al.*, 1967).

The main portion of each Ab sample, from which the aliquots for tritium counting had been removed, was prepared for reduction and alkylation by dialysis overnight at 4° against 1 l. of 0.01 M Tris-Cl buffer (pH 8.2), then against 250 ml of 0.55 M Tris-Cl buffer (pH 8.2). Reduction and alkylation were performed essentially by the method of Fleischman *et al.* (1962), using 0.2 M mercaptoethanol as reducing agent for interchain disulfide bonds, and a 10% excess of iodoacetamide to alkylate the liberated SH groups. After reduction and alkylation, the antibody solutions were dialyzed overnight against 1 l. of 1 M propionic acid at 4°. H- and L-chain fractions were then separated on G-100 Sephadex columns in 1 M propionic acid. Pools were made of the peak tubes of the H-chain fraction and of the L-chain fraction. The pools were dialyzed overnight at 4° against several liters of distilled water and then lyophilized. The dried protein was dissolved in 0.03 M sodium decyl sulfate at neutral pH for spectral studies and cleared by low-speed centrifugation. Protein concentrations were estimated from the optical density of an appropriately diluted sample at 280 mμ. The samples were then made 0.15 N in NaOH, and the spectra were again measured. Spectral determinations with a Cary 14 recording spectrophotometer were made as described previously (Good *et al.*, 1967) for all of the antibodies except those of the mouse. Spectra for mouse antibodies were obtained at wavelength intervals of 10 mμ on a Zeiss spectrophotometer using 0.3-cc samples in microcells with 1-cm light path.

Tritium counting was performed on samples lyophilized from the original H- and L-chain pools in propionic acid or on samples lyophilized from the detergent solutions prepared for spectra assay. Details of the counting method were the same as described earlier (Good *et al.*, 1967).

Results

The results of affinity labeling of each of the anti-DNP Ab with [^{3}H]MNBDF are given in Table I. These data are all based upon ^{3}H counts and, therefore, represent the total amount of reagent covalently bound by the Ab. Although the results shown in the table are from single experiments, they appear to be typical, since the results for guinea pig γG_1 Ab have been essentially the same in four different labeling experiments involving two independent Ab pools, and guinea pig γG_2 Ab have reacted in the same way in three different labeling experiments on two pools of Ab. Only one sample was available for sheep and mouse Ab, but repeated experiments on the same sample of sheep Ab gave consistent results.

In all of the systems studied, the whole unprotected Ab were appreciably more highly modified than the corresponding protected Ab. The ratio of the specific activities of unprotected to protected Ab, which is a measure of the specificity of the labeling reaction, varied somewhat among the different Ab. This variation, however, appears to be largely reflected in the

different extents to which the protected samples were modified. For example, the whole protected guinea pig γG_1 Ab was modified about six times as much as protected rabbit Ab. This may reflect the presence on the former Ab molecules of a group (probably not tyrosine, see below) outside the active site which is more reactive than any similar group on the rabbit Ab molecules.

All of the antibodies exhibited similar chromatographic patterns of H- and L-chain fractions on G-100 Sephadex in 1 M propionic acid (Porter and Weir, 1966), with an average of 74% of the absorbance at 280 mμ accounted for by the H-chain fraction and 26% by the L-chain fraction.[5]

In each case, the ^3H label was associated with both the H and L chains, as had previously been found with rabbit anti-DNP Ab (Metzger *et al.*, 1964; Good *et al.*, 1967), and as has been found upon affinity labeling of antisaccharide Ab (Wofsy *et al.*, 1967a,b). Both H and L chains derived from protected Ab samples were always substantially less modified than their counterparts from unprotected Ab.[6]

A measure of the mole ratio, R, of specific affinity labels on the H and L chains may be obtained as: $R = (H_u - H_p)/(L_u - L_p)$, where $H_u - H_p$ is the total moles of MNBDF bound per mole of H chain in the unprotected sample, minus the corresponding quantity for the protected sample, and $L_u - L_p$ is the corresponding difference between the unprotected and protected L chains. The values of R are given in column 6 of Table I. The significant point is that the values are all quite similar, varying not much more than might be due to experimental errors.

Spectral assays were also made with the MNBDF-modified Ab of the nature and amount of the azo linkages formed. In our previous studies with rabbit anti-DNP Ab (Good *et al.*, 1967), it was found that all of the specifically bound ^3H label could be accounted for as azotyrosine linked. In the present studies, two kinds of spectral assays are shown. (1) The *specific spectrum*, the difference spectrum in 0.15 N NaOH of an unprotected sample and its protected analog, was constructed. This is a measure of the extent of *specific* labeling associated with the active sites of the Ab. It was obtained for most of the whole Ab preparations, but not for their chains. (2) The *gross spectrum*, the difference spectrum of a particular unprotected protein preparation in 0.15 N NaOH and in neutral buffer containing detergent, was constructed. This is a measure of the extent of the *total* azo modification, that associated with the active sites plus that elsewhere on the Ab molecules, which results in a change in the near-ultraviolet spectrum of the protein. For comparison, both kinds of difference spectra were also obtained for the model azo compounds formed by the reactions of MNBDF with *N*-chloracetyltyrosine and *N*-acetylhistidine (Good *et al.*, 1967; Traylor and Singer, 1967).

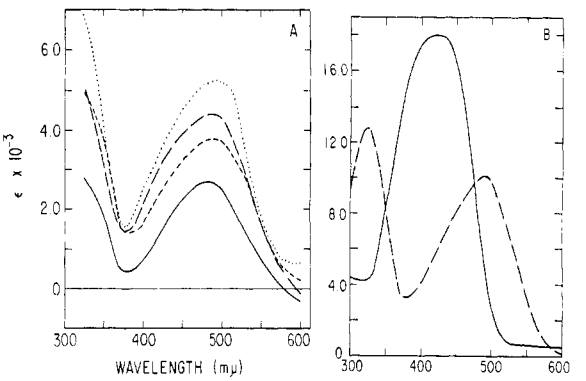

FIGURE 1: Spectral studies. (A) Specific spectra (difference spectra between unprotected and protected samples) in 0.15 N NaOH of [^3H]MNBDF-labeled whole anti-DNP Ab of rabbit (·····), sheep (– – – –), guinea pig γG_1 (——), and guinea pig γG_2 (- - - -). Extinction coefficient per mole of protein. (B) Spectra in 0.15 N NaOH of model azocompounds formed by the reaction of MNBDF with *N*-chloracetyltyrosine (dashed line) and *N*-acetylhistidine (solid line) (Good *et al.*, 1967).

In Figure 1A are shown the *specific spectra* of the whole unprotected Ab of rabbit, guinea pig, and sheep, and for comparison in Figure 1B, the analogous difference spectra of the two model compounds. The correspondence of the specific spectra of the labeled Ab and of the azotyrosine model compound is very close. From these spectra, therefore, using the appropriate extinction coefficient for the azotyrosine model compound, we may calculate the amount of azotyrosine which is *specifically* associated with the active sites of the labeled Ab. These values, in moles of azotyrosine per mole of protein, are given in Table II, column 5.

In Figure 2 are shown the *gross spectra* of (a) labeled unprotected whole Ab, (b) H chains from unprotected Ab, and (c) L chains from unprotected Ab, of the different species. The absorbances were such that, particularly for the lightly modified L-chain preparations, the gross spectra are not accurate below about 400 mμ. In Figure 2D, the analogous difference spectra for the model compounds are shown. The gross spectra of the protein samples are clearly all very similar to the difference spectrum of the azotyrosine model compound.[7] Particularly significant is the fact that the maxima in the gross difference spectra of all the labeled

[5] Under the conditions used, only two peaks were obtained on chromatographic fractionation of the reduced and alkylated Ab, including the rabbit Ab. Larger scale runs employing more concentrated protein solutions and larger columns of G-100 Sephadex usually result in three peaks from a rabbit Ab preparation corresponding to aggregated H chains, dispersed H chains, and L chains, respectively (Metzger and Mannik, 1964; Good *et al.*, 1967).

[6] Insufficient mouse anti-DNP Ab was available in this experiment to study the distribution of the label on H and L chains of protected Ab. In more recent studies, however, we have found that both H and L chains of both γG_1 and γG_2 mouse anti-DNP Ab are specifically labeled with [^3H]MNBDF at tyrosine residues (N. Thorpe and S. J. Singer, unpublished observations).

[7] The elevated spectrum of the labeled mouse L chain seen in Figure 2C may be due to turbidity in the sample. With the small amount of sample available, it was not feasible to centrifuge the sample prior to spectral assay, as was done with the other preparations.

TABLE II: Correlation of ^3H Bound and Azotyrosine Content.

Protein	Total ^3H Bound	Sp ^3H Label[a]	Total Azotyrosine from Gross Spectrum	Sp Azotyrosine from Sp Spectrum
Rabbit I				
Whole Ab	0.66	0.60		0.59
H chain	0.23	0.22		0.16
L chain	0.11	0.11		0.09
2 H + 2 L	0.69	0.65		0.49
Rabbit II				
Whole Ab			0.55	0.54
H chain			0.16	
L chain			0.06	
2 H + 2 L			0.43	
Guinea pig γG_1				
Whole Ab	0.67	0.31	0.36	0.29
H chain	0.22	0.11	0.12	
L chain	0.06	0.04	0.04	
2 H + 2 L	0.56	0.29	0.32	
Guinea pig γG_2				
Whole Ab	0.64	0.47	0.44	0.39
H chain	0.21	0.17	0.17	
L chain	0.07	0.05	0.05	
2 H + 2 L	0.56	0.43	0.44	
Sheep				
Whole Ab	0.83	0.63	0.51	0.45
H chain	0.26	0.23	0.19	
L chain	0.05	0.05	0.04	
2 H + 2 L	0.62	0.55	0.45	
Mouse				
H chain	0.16		0.15	
L chain	0.09		0.10	
2 H + 2 L	0.49		0.49	

[a] Calculated as values for a protected sample minus that for its unprotected sample, from Table I. [b] Moles per mole of Ab.

Ab and their chains are all close to 490 mμ, corresponding precisely to that of the azotyrosine model compound. As little as 10% azohistidine in the total amount of azoderivative on the Ab preparations would have been revealed by a noticeable broadening of the peak and a shift toward lower wavelengths in the maxima of the gross spectra. Only with sheep L chains is there a suggestion of this effect, but the total extent of modification of the sheep L chains was the smallest of all the L chains, and the spectrum was correspondingly less certain.

As a first approximation, therefore, the gross spectra of the protein samples allow us to calculate the *total* amount of azotyrosine formed, both within and without the active sites, using the appropriate difference extinction coefficients of the azotyrosine model compound. These values, in moles of azotyrosine per mole of protein, are listed in Table II, column 4.

Discussion

To establish that the affinity-labeling reaction exhibits specificity for the Ab active sites, the main criterion employed in this paper is the marked reduction in the extent of modification of the Ab when the labeling reaction is carried out in the presence of a specific protector of the active sites. With rabbit anti-DNP Ab, it has also previously been shown that affinity labeling of the Ab with MNBDF produces a stoichiometric inactivation of the active sites (Good *et al.*, 1967). Furthermore, the small size of the diazonium reagent compared to the generally accepted size of an Ab active site (Kabat, 1962; Eisen and Siskind, 1964), and the kinetic demonstration with rabbit anti-DNP Ab (Metzger *et al.*, 1963b) that the affinity-labeling reagent must be reversibly bound to the active site in order to form the specific covalent bond to the Ab,

provide additional strong arguments that the Ab residues which are affinity labeled are contact residues in the active sites. This question has been discussed more fully elsewhere (Singer and Doolittle, 1966; Singer, 1968).

The results obtained with the sheep, guinea pig, and mouse anti-DNP Ab are closely similar to those obtained with the rabbit Ab. The unprotected Ab in each case irreversibly bound much more ^3H than the protected Ab. Furthermore, the amount of ^3H attached to both H and L chains of the sheep and guinea pig Ab[6] were substantially greater for the unprotected than for the protected samples, indicating that the affinity label in the active sites is associated with both the H and L chains (Metzger *et al.*, 1964; Good *et al.*, 1967).

From spectral observations, the amino acid residues to which the ^3H becomes attached can be ascertained. In the case of rabbit anti-DNP Ab, we have earlier shown (Good *et al.*, 1967) that essentially all of the ^3H specifically bound to the whole Ab and to the H and L chains can be accounted for as azo linked to tyrosine residues. In this paper, the *specific* spectra obtained with whole sheep and guinea pig γG_1 and γG_2 Ab likewise indicate that the amount of azotyrosine formed within the active sites is within experimental error equal to the amount of ^3H specifically bound to the active sites. In addition, the *gross* spectra of the labeled H and L chains of sheep, guinea pig γG_2, and mouse Ab indicate that, under the conditions used with these affinity-labeling reactions, the total amount of ^3H bound could be accounted for by tyrosine residues having been predominantly, if not exclusively, modified. It follows, therefore, that tyrosine residues play an important role as contact amino acids in all of these Ab active sites.[8]

Even more striking, however, is the fact that the ratio of specific azotyrosine label on the H and L chains is nearly the same among the different anti-DNP Ab. We have elsewhere (Singer and Doolittle, 1966; Singer *et al.*, 1968) stressed the relative constancy of this ratio. From a kinetic point of view, this constancy is entirely nonrandom. That is, given a heterogeneous population of anti-DNP antibody molecules in any one species (let alone the populations from different species), one would expect that this ratio of H- to L-chain label might vary by many orders of magnitude if the active sites contained tyrosine residues which were in random steric positions within different sites. The constancy of the ratio of label on H and L chains strongly suggests, on the contrary, that the tyrosine residues which become labeled are structurally unique and constant features in different active sites. As corollaries, we (Singer and Doolittle, 1966) postulated

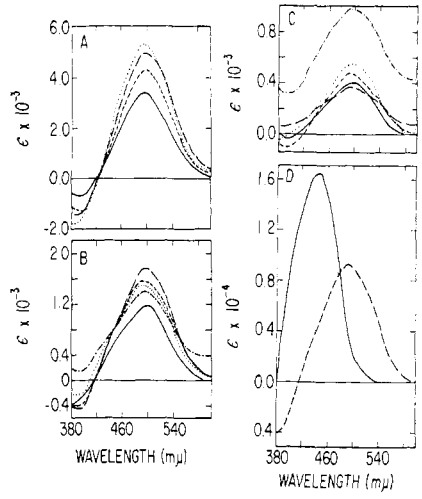

FIGURE 2: Gross spectra (difference spectra between 0.15 N NaOH and neutral detergent buffer) of [^3H]MNBDF-labeled anti-DNP Ab and their chains: (A) whole Ab, (B) H chains, and (C) L chains. The value of ϵ is given per mole of the appropriate protein. The species are denoted as follows: rabbit (·····), sheep (– – – –), guinea pig γG_1 (——), guinea pig γG_2 (– – – – –), and mouse (— — — — —). (D) Analogous difference spectra for the model azocompounds formed by the reaction of MNBDF with *N*-chloracetyltyrosine (dashed line) and *N*-acetylhistidine (solid line).

that: (1) the labeled tyrosine residue on rabbit anti-DNP L chains occurs at a characteristic position in the amino acid sequence of the L chains, and likewise for the labeled tyrosine on H chains; and (2) that the positions and orientations of the unique H and L tyrosines relative to each other are closely similar in different active sites. These postulates provide an explanation for the constancy of the labeling results, for they suggest that the affinity-labeling reagent, when reversibly bound to any active site in the population, has an essentially fixed probability of reacting either with the H- or the L-chain tyrosine in each site.

We have recently demonstrated (N. O. Thorpe and S. J. Singer, to be published; Singer *et al.*, 1968) that the first of these two postulates appears to be a fact. With rabbit anti-DNP Ab, the labeled tyrosine on H chains occurs predominantly in a dipeptidyl sequence, -threonyl-tyrosyl-, whereas that on L chains is in the sequence -valyl-tyrosyl-. Further studies are in progress to localize these residues within the chain sequences, but there is good evidence that they occur within the variable segments of their respective chains (Doolittle and Singer, 1965; Singer and Doolittle, 1966).

We therefore suggest from the results presented in this paper that the tyrosines which become affinity labeled on the H and L chains of anti-DNP Ab from sheep, guinea pigs, and mice are structurally homologous to their counterparts in rabbit anti-DNP active sites. These residues have presumably been conserved throughout the evolutionary time span of the species involved. It would be of great interest to determine whether similar results are obtained upon affinity-labeling anti-DNP Ab from evolutionarily much more primitive organisms.

[8] Only in the cases of sheep and guinea pig γG_1 and γG_2 whole Ab, and of guinea pig γG_1 H chain, did the total amount of ^3H bound exceed beyond experimental error the total amount of azotyrosine calculated from the gross spectrum. With the three whole Ab, some unreacted [^3H]MNBDF may have been strongly noncovalently bound (or weakly covalently bound) and subsequently removed by the procedures involved in isolating their H and L chains.

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