Hektoen, L., and Boor, A. K. (1931), *J. Infect. Diseases 49*, 29.
Johnson, C. A., and Bradley, W. B. (1935), *J. Infect. Diseases 57*, 70.
Moore, D. H., and Reiner, L. (1944), *J. Biol. Chem. 156*, 411.
Rossi-Fanelli, A., and Antonini, E. (1959), *Arch. Biochem. Biophys. 80*, 299, 308.
Rossi-Fanelli, A., Antonini, E., and Caputo, A. (1958), *Biochim. Biophys. Acta 30*, 608.
Rossi-Fanelli, A., Antonini, E., and Caputo, A. (1959a), *Biochim. Biophys. Acta 35*, 93.
Rossi-Fanelli, A., Antonini, E., and Caputo, A. (1959b), *J. Biol. Chem. 234*, 2906.
Smithies, O. (1955), *Biochemical J. 61*, 629.
Tanford, C. (1961), *Physical Chemistry of Macromolecules*, New York, Wiley, p. 358.
Wasserman, E., and Levine, L. (1961), *J. Immunol. 87* 290.

Affinity Labeling of the Active Sites of Antibodies to the 2,4-Dinitrophenyl Hapten*

HENRY METZGER,† LEON WOFSY, AND S. J. SINGER

From the Department of Biology, University of California, San Diego, La Jolla

Received March 19, 1963

The method of affinity labeling provides a general method for attaching a covalently bound label to the active sites of antibody and enzyme molecules. Previous studies with antibodies directed to the benzenearsonate hapten were entirely in accord with the predictions of the method. In the present studies, antibodies to the 2,4-dinitrophenyl (DNP) hapten were investigated. The labeling reagent *p*-nitrophenyldiazonium fluoborate was found to react irreversibly with an excess of the unprotected antibody: (a) at a much more rapid rate than with the antibody whose active sites were protected with an excess of *N*-DNP-ε-aminocaproic acid; (b) to give a product with essentially a pure azotyrosine spectrum; (c) at a rate which was first-order in the concentration of the reversible complex formed initially between the reagent and the antibody sites, in accord with the mechanism proposed for affinity labeling; and (d) to produce a loss of antibody binding sites corresponding closely to the number of azotyrosine groups formed. Related but less extensive studies were carried out with DNP-diazonium and *p*(carboxy)-benzenediazonium fluoborates and with other reagents. The results closely follow the predictions of the method, and provide strong evidence that a tyrosine residue is present in anti-DNP antibody sites. The results also indicate that there is a broad distribution of rates at which the antibody sites are labeled, and therefore that the sites are heterogeneous. These studies provide the basis for an attempt to isolate and analyze labeled peptide fragments from the antibody active sites.

In a previous paper (Wofsy *et al.*, 1962), we proposed and discussed a general method, called affinity labeling, to attach a covalently bound label to the active sites of antibody and enzyme molecules. For an antibody (Ab)[1] specifically directed to a given hapten (Hp) a labeling reagent HpX is used, where X is a chemical group which is constantly attached to Hp and is capable of reacting with one or more kinds of amino acid residues to form stable covalent bonds. By virtue of the initial reversible complex formed between HpX and the active site of the specific Ab, the local concentration of X in the site is greatly increased, thereby favoring covalent bond formation between X and a group in the site.

The proposed mechanism is summarized in equation (1):

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

where Y is a group in the active site, C is the specific reversible complex, L is the desired covalently labeled product, and the k's are (assumed single-valued) specific rate constants for the reactions indicated. Competing with Y for the reagent HpX are a number of residues y elsewhere on the Ab molecule,

$$y + HpX \xrightarrow{k_4} M \qquad (2)$$

which can form the undesired covalently labeled product M. For simplicity, the groups y are here considered identical. If the rate of formation of L, $\dot{L}_c$, is substantially greater than the rate of formation of M, $\dot{M}$, then specific labeling of the active site can be achieved. It was shown that in the highly probable event that the complex C is in equilibrium with Y and HpX, the ratio $\dot{L}_c/\dot{M}$, which is termed the *enhancement*, is given by:

$$\dot{L}_c/\dot{M} = \frac{k_3 K_A [Y]_e}{k_4 [y]} \qquad (3)$$

where $K_A = k_1/k_2$ is the intrinsic association equilibrium constant for the formation of C, $[Y]_e$ is the equilibrium molar concentration of free Y, and $[y]$ is the molar concentration of any single unreacted residue y outside the site.

Experimental studies were also reported in the previous paper with antibodies directed to the benzenearsonate group which strongly supported the proposed mechanism, and which provided substantial evidence that a tyrosine residue was present in antibenzenearsonate Ab active sites. In order to test further the proposed mechanism of affinity labeling, and to explore the generality of its application, we have now carried out studies with antibodies to the DNP hapten, which are described in this paper.

* Presented at the Symposium on Immunochemistry held by the Division of Biological Chemistry at the meeting of the American Chemical Society in Cincinnati, Ohio, January, 1963. The research was supported by Grant E-4246 from the National Institutes of Health, U. S. Public Health Service.

† Helen Hay Whitney Foundation Fellow.

[1] Abbreviations used: Ab, antibody; Hp, hapten; DNP, 2,4-dinitrophenyl; DNPDF, 2,4-dinitrophenyl diazonium fluoborate; PNPDF, *p*-nitrophenyl diazonium fluoborate; CDF, *p*-(carboxyl)benzenediazonium fluoborate; DNPNS, 2-(2,4-dinitrophenylazo)-1-naphthol-3,6-disulfonic acid, disodium salt.

TABLE I
SPECTRAL PROPERTIES OF AZOTYROSINE DERIVATIVES

Compound	pH 6.2		0.15 N NaOH	
	λ_{max}, mμ	ε	λ_{max}, mμ	ε
mono-DNP-azo-*N*-chloracetyl tyrosine	350	18,500	555 360	15,200 13,000
mono-*p*-Nitrophenylazo-*N*-chloracetyl tyrosine	340	20,700	520 345	12,800 13,400

The essential feature of the method has been independently conceived and tested with enzyme systems in other laboratories (Baker *et al.*, 1961; Schoellmann and Shaw, 1962); variants of the method have also been investigated (Lawson and Schramm, 1962; Singh *et al.*, 1962).

REAGENTS AND EQUIPMENT

Proteins.—Pure anti-DNP Ab was prepared from pooled rabbit antisera following closely the procedure for preparation and isolation by Farah *et al.* (1960). Different batches of Ab were routinely 90% or more precipitable by the DNP-bovine γ-globulin antigen. The yields of purified Ab averaged about 20% of the precipitable Ab in the serum pool.

Rabbit normal γ-globulin (fraction II) was obtained from Pentex, Inc., Kankakee, Illinois.

For both anti-DNP Ab and normal γ-globulin the extinction coefficient at 280 mμ of a 1% solution was taken as 14.6, the molecular weight as 160,000, and the nitrogen content as 16.0%.

Diazonium Reagents.—DNPDF was synthesized by the method of Brunton and Suschitzky (1955), mp 198–203° (literature: 208°).

PNPDF was purchased from K&K Laboratories, Jamaica, New York, and was recrystallized twice from methanol at 45°. It melted with decomposition at 158–159° (literature values: 153–155° [Neunhoeffer and Ruske, 1957]).

CDF was prepared as described previously (Wofsy *et al.*, 1962).

Azotyrosine Derivatives.—Mono-DNP-azo-(*N*-chloracetyl)-tyrosine was prepared by adding DNPDF to *N*-chloracetyltyrosine in the mole ratio 1:1.5 in 0.01 M HCl at 0° with vigorous stirring. After about 12 hours' reaction, the mixture was filtered in the cold. The precipitate was washed with dilute HCl, and was then dissolved in methanol. The excess methanol was evaporated in a rotary evaporator and the product was crystallized in the cold. (This compound decomposes if exposed to alkaline *p*H). Mp 225–226°.

Anal. Expected for $C_{17}H_{14}O_8N_5Cl$: C 45.2, H 3.13, N 15.5, Cl 7.85. Found: C 45.0, H 3.10, N 14.9, Cl 7.27.

Mono-*p*-nitrophenylazo-(*N*-chloracetyl)-tyrosine was prepared by gradually adding PNPDF in 0.01 M HCl to a solution of *N*-chloracetyltyrosine in potassium phosphate buffer, *p*H 5.2, Γ/2 0.1 at 0° with vigorous stirring. The reagents were in the mole ratio 1:1.1. After 7 hours' reaction, the mixture was brought to *p*H 2 and the product was precipitated. After the precipitate was washed with H_2O, it was dissolved at *p*H 9 with 0.1 M NaOH. This precipitation procedure was repeated twice. Finally, the product was recrystallized from aqueous ethanol. Mp (with decomposition) 217–220°.

Anal. Expected for $C_{17}H_{15}O_6N_4Cl$: C 50.2; H 3.72; N 13.8; Cl 8.70. Found: C 49.9; H 3.93; N 13.3; Cl 8.50.

Some pertinent spectral properties of these azotyrosine derivatives are given in Table I.

Other DNP Derivatives.—*N*-DNP-ε-aminocaproic acid was prepared by the method of Carsten and Eisen (1953). DNP-sulfonyl chloride was purchased from K&K Laboratories and was recrystallized three times from chloroform. DNP-sulfonic acid was obtained from Eastman Chemicals, Rochester, New York. It was purified by recrystallization from water three times. The compounds 2,4-dinitrofluorobenzene, 2,4-dinitrochlorobenzene, 3,5-dinitrobenzoyl chloride, and picryl chloride, which were obtained from Eastman, and 2,4-dinitrobenzenesulfenyl chloride and 2,4-dinitrobenzaldehyde, obtained from K&K Laboratories, were used without further purification. DNPNS (Eastman) was purified as described elsewhere (Metzger, Wofsy, and Singer, to be published).

Other Chemicals.—*N*-Chloracetyltyrosine (Mann Research Laboratories, New York, New York) was recrystallized three times from water. Resorcinol (Grade B; California Corp. for Biochemical Research, Los Angeles) was used as purchased. Sodium dodecyl sulfate was obtained from Fisher Scientific Co., and was recrystallized three times from *n*-butanol. All other chemicals were reagent grade.

Equipment.—A Beckman Model G *p*H meter, standardized with Beckman standard buffers, was used for *p*H determinations. Optical density measurements were performed on a Zeiss Model PMQ II spectrophotometer. For the kinetic studies a jacketed cell holder (Zieler Instrument Co.) was attached to an Aminco temperature bath maintained at 4°, and dry air was blown through the cell compartment to eliminate condensation. In these studies a Photovolt Varicord 43 recorder was employed. Complete spectra were recorded on a Cary Model 14R recording spectrophotometer.

EXPERIMENTAL METHODS AND RESULTS

Several different kinds of experiments were performed with anti-DNP Ab and three different reagents, DNPDF, PNPDF, and CDF. It is convenient to consider the experiments with each reagent separately.

Studies with the Reagent DNPDF

(*a*) *Reactivity of the Reagent.*—DNPDF is an exceedingly reactive diazonium compound (Schoutissen, 1933), and correspondingly is unstable in aqueous solutions that are not strongly acidic (Migrdichian, 1957). For the experiments with anti-DNP to be described below, it was necessary to determine at least crudely the stability of DNPDF in a sodium acetate buffer, *p*H 5.0, Γ/2 0.2 at 2–4°. The addition of a 500- to 1000-fold excess of resorcinol (Koltun, 1957) to such a solution instantaneously converts any DNPDF to a monoazo derivative with an absorption maximum at 450 mμ at *p*H 5.0. In this way it was found that no more than about 10% of the DNPDF was lost in 2 minutes under the conditions stated above, while about 60% was lost in 1 hour. The loss was apparently not sensitive to the original concentration of the reagent. In 0.01 N HCl, however, DNPDF was found to be stable for at least several hours at 2–4°.

(*b*) *Reaction of DNPDF with Anti-DNP Ab and Normal γ-Globulin.*—It was found that even at low concentrations (~10^{-6} M) the reaction between DNPDF and anti-DNP Ab at *p*H 5.0 was extremely rapid, being completed a few seconds after mixing. The reaction with normal γ-globulin under comparable conditions was clearly much slower. Because, there-

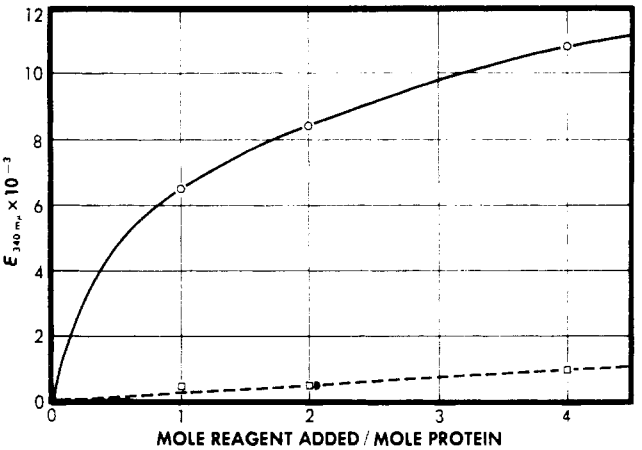

FIG. 1.—A comparison of the reaction of DNPDF with anti-DNP Ab and with normal γ-globulin. All samples were quenched after 5 seconds reaction time, and the proteins were then isolated. The extent of modification is given by the extinction coefficient per mole protein per liter at 340 mμ. ○, unprotected anti-DNP Ab; ●, protected anti-DNP Ab; □, normal γ-globulin. For further details of the experiments see text.

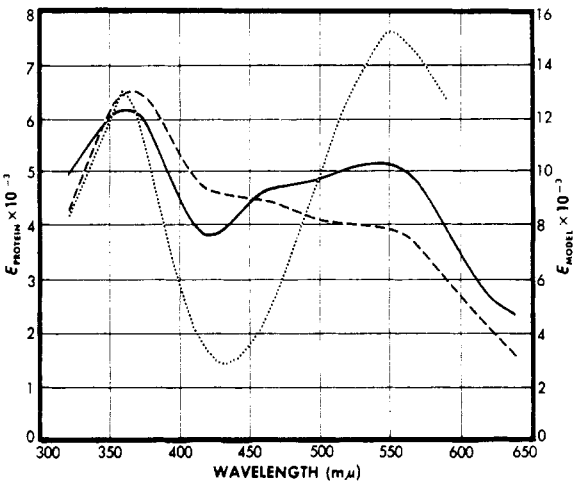

FIG. 2. Spectra in 0.15 M NaOH of: —— unprotected anti-DNP Ab, and - - - protected anti-DNP Ab, both reacted with DNPDF; and .... the model compound mono-DNP-azo-(N-chloroacetyl) tyrosine (right-hand coordinate).

fore, rate studies of the kind reported in our previous paper could not be carried out conveniently, the following alternative experiments were performed.

A stock solution of DNPDF in 0.01 M HCl was freshly prepared for each set of experiments. A suitable dilution of this solution in 0.001 M HCl was prepared and 10 ml of the diluted mixture was rapidly mixed with: (a) 10 ml of 2×10^{-6} M anti-DNP Ab in sodium acetate buffer, *p*H 5.0, Γ/2 0.5 (this sample is referred to as unprotected Ab); (b) solution (a), containing in addition 2×10^{-4} M *N*-DNP-ε-aminocaproic acid (protector) to "block" the active sites of the Ab (this sample is referred to as protected Ab); or (c) 10 ml of 2×10^{-6} M normal rabbit γ-globulin in the same buffer. Experiments were carried out with 1:1, 2:1, and 4:1 mole ratios of DNPDF to protein. After 5 seconds reaction time, a 1000-fold excess of resorcinol in sodium acetate buffer, *p*H 5.0, Γ/2 0.25 was added to quench any remaining reagent. All operations up to this point were carried out at 2–4°.

The resulting azoproteins were processed for spectral analysis in a manner similar to that previously described (Wofsy *et al.*, 1962). First, the protector was added to those samples which did not contain any during the reaction period. The protein derivatives were then denatured and freed from noncovalently bound colored products by precipitation in 75% ethanol and successive washes in ethanol, ether, ethanol-NaCl, and 1% NaCl in H_2O as described. Control samples were prepared in which the protein (either the anti-DNP Ab or the normal γ-globulin), the excess resorcinol, and the protector were first mixed, and the DNPDF was then added, after which the proteins were precipitated and washed as usual. Such controls did not differ spectrally from the untreated protein. Each protein sample was then dissolved in a 0.02 M phosphate buffer, *p*H 6.2, containing 0.5% sodium dodecyl sulfate, and the solution was clarified by centrifugation. The solutions at this point were about 2×10^{-5} M. After the spectra were recorded, sufficient 5 M NaOH was added to make the solution 0.15 M in NaOH and a second set of spectra was obtained. Protein concentrations were determined from the absorbance at 280 mμ of diluted solutions at *p*H 6.2, taking $\epsilon_{280\,m\mu}^{1\%} = 14.6$, since the absorption at this wavelength was only negligibly altered by the presence of a small amount of azo derivative.

The extinction coefficients (per mole protein per liter) of the protein derivatives at 360 mμ at *p*H 6.2 may be used as a measure of the extent of the reaction with DNPDF, since the spectra of all the protein derivatives at *p*H 6.2 were qualitatively similar with a peak of absorption at 360 mμ. The data plotted in Figure 1 show that in 5 seconds reaction time DNPDF reacted much more extensively with unprotected anti-DNP Ab than with protected Ab or normal γ-globulin.

Furthermore, although the spectra of the various protein derivatives at *p*H 6.2 were roughly similar, marked differences appeared in 0.15 M NaOH. In Figure 2 the alkaline spectrum of an unprotected Ab sample which had been reacted with 2 moles DNPDF per mole protein for 5 seconds is compared with that of a protected Ab sample reacted for 18 hours, and with that of the model compound DNP-azo(*N*-chloracetyl)-tyrosine.[2] The model compound shows a distinctive bimodal spectrum in alkali, characteristic of other azotyrosine derivatives (Tabachnick and Sobotka, 1959). The spectrum of the unprotected Ab sample corresponds more closely to the model than does that of the protected Ab sample, but does not coincide with it. These spectral results are considered further in the discussion.

Studies with the Reagent PNPDF

The great reactivity and the instability of DNPDF made it desirable to turn to a more tractable reagent for extended study. PNPDF was found to be considerably less reactive and quite stable at *p*H 5.0.

(*a*) *Rate Enhancement and Spectral Studies.*—A fresh stock solution of 1.64×10^{-4} M PNPDF in 0.001 M HCl was prepared, and 0.5 ml was added to 0.5 ml of (a) 1.09×10^{-4} M anti-DNP Ab in sodium acetate buffer, *p*H 5.0, Γ/2 0.5 (unprotected Ab); (b) solution (a) containing in addition 8×10^{-4} M *N*-DNP-ε-aminocaproic acid (protected Ab); (c) 1.09×10^{-4} M normal rabbit γ-globulin in the same buffer; and (d)

[2] The alkaline spectrum of the DNP-azo-(*N*-chloracetyl)-tyrosine was recorded immediately after the solution was made 0.15 M in NaOH. While the DNP-azoderivative decomposes in alkaline solution, the rate of this decomposition is not rapid enough to result in any detectable spectral alteration in samples assayed over an interval of a few minutes.

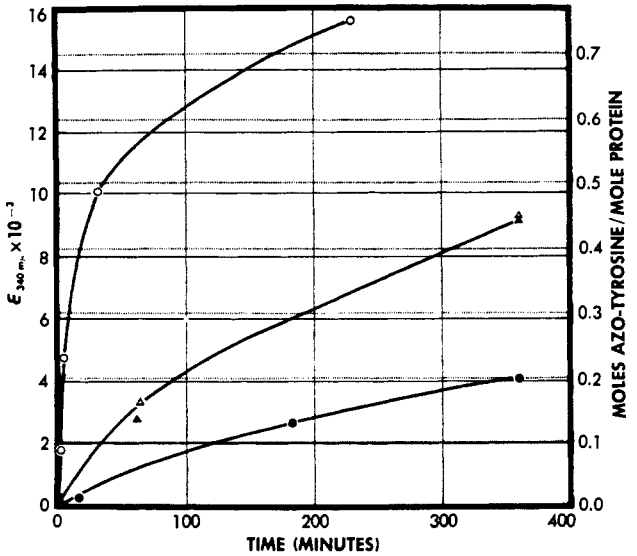

FIG. 3.—A comparison of the rate of reaction of PNPDF with anti-DNP Ab and normal γ-globulin. Absorbance measurements made at *p*H 6.2. O, unprotected Ab; ●, protected Ab; △, normal γ-globulin without protector; ▲, normal γ-globulin with protector. Right-hand coordinate applies only to unprotected Ab.

solution (c) containing in addition the same amount of protector as in solution (b). Different batches of these solutions were treated at various times with excess resorcinol and the remaining reagent was rapidly quenched. All operations were carried out at 2–4°.

The protein samples were than prepared for spectral assay at *p*H 6.2 and in 0.15 M NaOH exactly as described for the DNPDF-treated samples. In Figure 3 the extinction coefficient (per mole protein) at *p*H 6.2 at 340 mμ (the wavelength of the azoderivative absorption peak at this *p*H, see Figure 4A) is plotted as a function of the reaction time. At initial stages of the reaction the unprotected Ab acquired absorbance at 340 mμ at a rate about 100 times faster than the protected Ab. It is noteworthy that the rate of modification of normal γ-globulin was unaffected by the presence of the protector,[3] which demonstrates that the effect of the protector in the specific Ab system is entirely due to its interaction with the active sites.

The alkaline spectra of these protein derivatives exhibited distinctive differences, as shown in Figure 4B. In this figure, the spectra of unprotected Ab (5-minute reaction time), protected antibody (360-minute reaction time), and normal γ-globulin (62-minute reaction time) are compared along with corresponding spectrum of a 1.56×10^{-5} M solution of the model compound *p*-nitrophenylazo-(*N*-chloracetyl)-tyrosine. A striking similarity exists between the spectra of the unprotected Ab derivative and the model compound; and these spectra are markedly different from those of the protected Ab and normal γ-globulin derivatives. On the basis of this evidence that the reaction of PNPDF and unprotected Ab produces primarily, if not exclusively, azotyrosine groups, and on the assumption that the extinction coefficient of such protein-bound azotyrosines is that of the model compound (Tabachnick and Sobotka, 1959, 1960), the rate data shown in Figure 3 for the *unprotected Ab* may be expressed in terms of azotyrosine

[3] The rate of modification of normal γ-globulin appears to be greater than that of protected Ab. A possible explanation is that in its preparation the normal γ-globulin may be denatured to some extent and some groups on the protein made more reactive in the process.

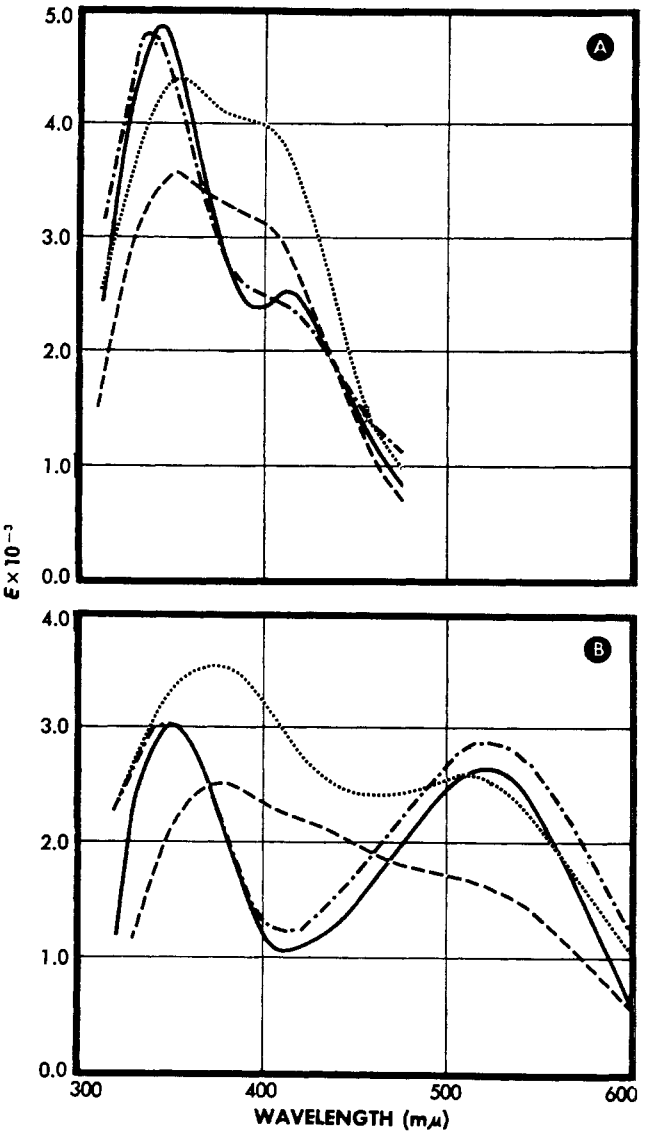

FIG. 4.—Spectra: A, at *p*H 6.2, and B, in 0.15 M NaOH; of the reaction products of PNPDF with: ——, uuprotected anti-DNP Ab, ... protected anti-DNP Ab, - - - normal γ-globulin and -·-·-· *p*-nitrophenylazo-(*N*-chloracetyl)-tyrosine. The extinction coefficients for the protein samples are per mole protein.

formation. For the other protein derivatives the absorption at 340 mμ is clearly due to the simultaneous modification of several kinds of residues, including tyrosine, histidine, and lysine (Tabachnick and Sobotka, 1959, 1960); in the absence of spectra for all the corresponding model compounds, the rate of azotyrosine formation cannot be quantitatively determined from the data, as was possible with the antibenzenearsonate system (Wofsy *et al.*, 1962).

(*b*) *Kinetic Order Studies.*—The experiments just described made it feasible to attempt a more direct kinetic test of the mechanism proposed in equation (1). According to this mechanism, the rate of azotyrosine formation in the reaction of PNPDF and unprotected Ab should be first order with respect to the concentration of the complex C; whereas if the reaction were a bimolecular one, such as expressed in equation (2), the rate should be first order with respect to both the Ab and the PNPDF concentrations. To circumvent kinetic complications which are considered in the discussion, the experiments were carried out in the following manner. Three solutions, I, II, and III,

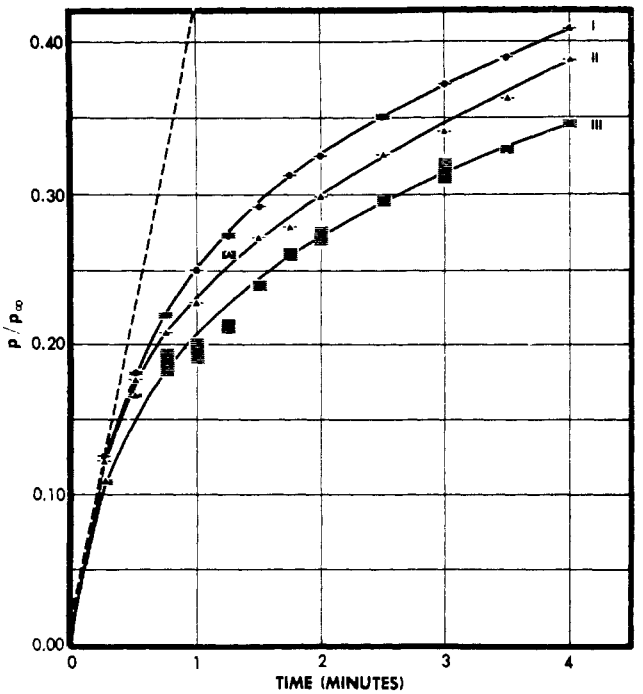

FIG. 5.—The fractional extent of the reaction of PNPDF with anti-DNP Ab as a function of time, at a fixed initial ratio of reactants but at three different initial total concentrations (see text). The dashed line is the theoretical curve for experiment I if the system were homogeneous and the kinetics followed equation (6). The bars indicate the agreement between duplicate experiments.

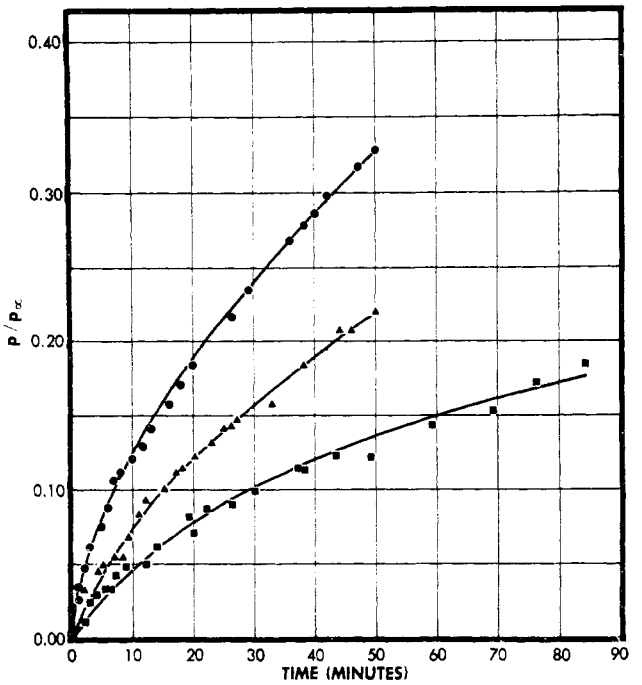

FIG. 6.—The fractional extent of the reaction of PNPDF with normal γ-globulin as a function of time, at a fixed initial ratio of reactants but at three different total concentrations which were in the ratio 4(●): 2(▲): 1(■) (see text).

were studied in which the *ratio* of the initial molar concentrations of PNPDF and Ab was kept constant (at 1:2.2, in substantial Ab excess), but the *total* concentration was in the proportion 4:2:1, respectively. The most concentrated of the solutions contained initially 2.11×10^{-4} M anti-DNP Ab (and, correspondingly, 0.98×10^{-4} M PNPDF). The sodium acetate buffer, *p*H 5.0, $\Gamma/2$ 0.2 was the reaction medium.

A thermostated cell-holder fitted to the Zeiss spectrophotometer was maintained at 4°. A 1.0-ml portion of an Ab solution was allowed to come to temperature equilibrium in a cell fitted with a small Teflon plunger. At time zero, 0.02 ml of a chilled solution of the appropriate concentration of PNPDF was added with agitation of the plunger, and the rapid increase in absorbance at 390 mμ was recorded. (The wavelength 390 mμ was chosen because it is well outside the range of significant reagent absorption.)

For several purposes, it is useful to reproduce the results in terms of the fractional extent of reaction, p/p_∞. For a given experiment, p_∞ is the absorbance at 390 mμ calculated for the complete conversion of the known initial amount of PNPDF to azotyrosine groups (using $\epsilon_{390\ m\mu}$ for the model azotyrosine compound at *p*H 6.2), and p is the observed absorbance at time t. Portions of these curves at early stages of the reaction only are shown in Figure 5. (After 1 week at room temperature, all three solutions exhibited p/p_∞ values of 0.9.) Since the individual curves do not follow simple kinetics, meaningful relative rates of reaction were obtained as follows: It was found that the times required to attain any given value of p/p_∞ between 0.2 and 0.4 were within experimental error in the constant ratio 1.6_4:1.3_2:1.00 for the curves III, II, and I, respectively. In view of the different total concentrations in these solutions, the relative rates of reaction were therefore as $1.0/1.6_4$: $2.0/1.3_2$:4.0/1.00, or as 1.0:2.6:6.6, for experiments III, II, and I, respectively.

The rates of reaction of PNPDF with normal γ-globulin and with *N*-chloracetyltyrosine in the sodium acetate buffer at 4° were investigated in a parallel manner, at a constant ratio of reagents, and at different total concentrations varied by multiples of 2. With normal γ-globulin, the most concentrated of the three solutions studied contained initially 1.30×10^{-4} M protein and 0.98×10^{-4} M PNPDF; in the other case, the corresponding concentrations were 5.65×10^{-2} M *N*-chloroacetyltyrosine and 3×10^{-4} M PNPDF. Relative rates were obtained by the same procedure just described for the reaction with Ab. For the normal γ-globulin experiments, p_∞ was taken as the absorbance at 390 mμ of the reacted samples after remaining 7 days at 5°. The kinetic curves are shown in Figure 6, and the relative rates are collected in Table II. For each of the three reacting systems, the relative rate of reaction of the most dilute solution studied was taken as 1.0. It is clear that the dependence of reaction rate on concentration was much less marked in the reaction of PNPDF with the Ab than

TABLE II

EFFECT OF TOTAL CONCENTRATION ON RELATIVE RATES OF REACTION WITH PNPDF

Relative Total Concentrations	Relative Rates 1	2	4
Expected: for bimolecular mechanism	1.0	4	16
Found: (a) for *N*-chloracetyltyrosine	1.0	5.0	17
(b) for γ-globulin	1.0	4.0	16
Expected: for unimolecular mechanism			
(a) $K_A = 5 \times 10^3$	1.0	3.0	7.9
(b) $K_A = 1 \times 10^4$	1.0	2.6	6.2
(c) $K_A = 5 \times 10^4$	1.0	2.2	4.7
Found: for anti-DNP	1.0	2.6	6.6

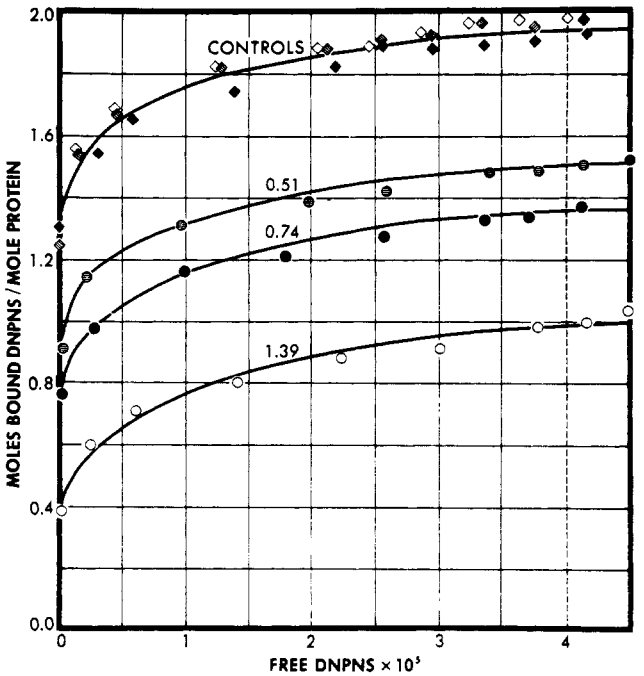

FIG. 7.—The determination of the residual Ab activity of PNPDF-labeled anti-DNP Ab, by titration with the hapten DNPNS. The numbers above the curves give the moles of azotyrosine formed per mole protein in the labeling reaction. The controls include: ◇ and ◆, duplicate samples of the unreacted anti-DNP Ab; and ◈, a sample of unreacted anti-DNP that was treated with *p*-nitroaniline, then dialyzed, and titrated with DNPNS. (This control shows that any PNPDF which remained reversibly bound to the Ab sites after the labeling reaction would have been removed by dialysis and would not have interfered with the DNPNS titration.)

with normal γ-globulin or *N*-chloracetyltyrosine. In the latter two cases, a 4-fold change in total concentration produced the 16-fold change in rate expected of a simple bimolecular reaction mechanism.[4] The interpretation of the results with anti-DNP is considered in the discussion.

(*c*) *Activity Studies.*—It was of interest to determine whether and to what extent the enhancement reaction between PNPDF and anti-DNP Ab produced a loss of Ab binding capacity; such a correlated loss of activity might be expected if the labeling reaction were indeed occurring in the active site. To a 1.25×10^{-5} M solution of anti-DNP Ab in sodium acetate buffer, *p*H 5.0, Γ/2 0.2 at 2–4°, was added either two or three times the molar quantity of PNPDF. In addition to two control samples which contained only unreacted antibody, another control which contained in addition 7.75×10^{-5} M *p*-nitroaniline was studied simultaneously. The reactions were effectively terminated at various times by exhaustive dialysis against the sodium acetate buffer, and the samples were then dialyzed against a potassium phosphate buffer, *p*H 7.4, Γ/2 = 0.2. The protein concentrations were determined from $\epsilon_{280\ m\mu}$, and the extent of azotyrosine formation from the value $\epsilon_{340\ m\mu} = 1.98 \times 10^4$ at *p*H 7.4 for the model compound *p*-(nitrophenylazo)-*N*-chloracetyltyrosine.

To determine the residual Ab activity of these samples, they were titrated spectrophotometrically with the hapten DNPNS. Upon specific reversible binding to anti-DNP Ab, DNPNS undergoes a *pK* shift of its naphtholic OH group from about 6.5 to 9.0 (Wofsy, 1961; Sturtevant *et al.*, 1961). Thus the specific binding of DNPNS at *p*H 7.4 is accompanied by a profound spectral change associated with the protonation of the OH group. This provides an accurate and simple method for assaying anti-DNP Ab activity, the details of which are given elsewhere (Metzger, Wofsy, and Singer, to be published). The titration data, obtained at protein concentrations of 1.2×10^{-5} M, are shown in Figure 7. The limiting values of *r* (where *r* = moles DNPNS bound/mole protein) reached on the plateaus of the curves are taken as equal to the residual number of binding sites per Ab molecule, and are listed in Table III.

Studies with the Reagent CDF

It was of interest to investigate the reaction of anti-DNP Ab with a diazonium reagent whose specificity for the Ab-active sites is relatively low. Some rate enhancement and spectral studies were therefore carried out with the reagent CDF and anti-DNP Ab. This diazonium compound is considerably less reactive than either DNPDF or PNPDF, and its reactions were investigated at a higher *p*H, in a buffer containing 0.17 M sodium borate and 0.12 M NaCl, *p*H 8.0 (Wofsy *et al.*, 1962). Solutions containing initially 9.3×10^{-5} M Ab, with or without 8.9×10^{-4} M *N*-DNP-ε-aminocaproic acid, and 8.6×10^{-5} M CDF were allowed to react at 4°. The reactions were quenched at appropriate times by the addition of a 1000-fold excess of resorcinol, and the protein derivatives were prepared for spectral assay as described above for comparable studies with the reagent PNPDF. The alkaline spectra of these derivatives showed that both azotyrosine and azohisitidine groups had formed on the unprotected as well as the protected anti-DNP Ab. The amounts of these groups were estimated from absorbance measurements in 0.15 N NaOH at 460 and 500 mμ, according to the procedure of Tabachnick and

[4] Diazonium reactions have been shown to follow second-order kinetics (Zollinger, 1952, 1953). The specific second-order rate constant (k_5, equation [2]) for the reaction of PNPDF and *N*-chloracetyltyrosine calculated from our results is 1.2×10^{-2} M^{-1} sec^{-1} under these conditions.

TABLE III
EFFECT OF LABELING WITH PNPDF ON ACTIVITY OF ANTI-DNP AB

	Labeling Conditions			Extent of Modification	Activity	
No.	Protein (conc.)	Reagent (conc.)	Length of Reaction	Moles Azotyrosine per Mole Protein	"*r*"	Moles Sites Lost
1	1.25×10^{-5}	None	—	0	1.90	—
2	1.25×10^{-5}	None	—	0	1.96	—
3	1.25×10^{-5}	None	—	0	1.95	—
4	1.25×10^{-5}	2.5×10^{-5}	60 min	0.51	1.50	0.44
5	1.25×10^{-5}	2.5×10^{-5}	405 min	0.74	1.36	0.58
6	1.25×10^{-5}	3.75×10^{-5}	2880 min	1.39	1.02	0.92

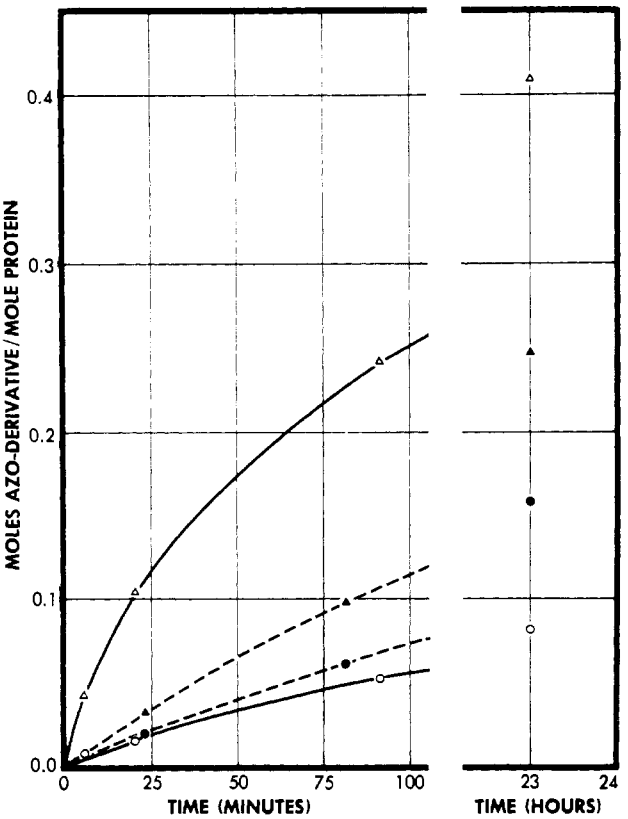

FIG. 8.—The rates of reaction of CDF with anti-DNP Ab. △, unprotected Ab, azotyrosine formation; ○, unprotected Ab, azohistidine formation; ▲, protected Ab, azotyrosine formation; ●, azohistidine formation. See text for details of experiments.

Sobotka (1959, 1960) which we utilized previously (Wofsy *et al.*, 1962). The data, shown in Figure 8, reveal that the initial rate of azotyrosine formation with unprotected Ab is enhanced about 5-fold over the rate with protected Ab. On the other hand, the initial rates of azohisitidine formation appear to be about the same.

Studies with Other Reagents

At an early stage in these investigations, a preliminary survey was made of the effect of several DNP reagents on the activity of anti-DNP Ab. To a solution of 1.7×10^{-5} M anti-DNP Ab in a buffer containing 0.06 M sodium borate, 0.04 M NaCl, *p*H 8.0, was added five times the molar quantity of the reagent to be tested. After 2 hours at room temperature the samples were dialyzed extensively against a potassium phosphate–NaCl buffer, *p*H 7.4, Γ/2 0.17. In order to remove any reversibly but tightly bound reagent, the samples were then dialyzed against 100 volumes of a 1×10^{-3} M solution of DNPNS in the potassium phosphate–NaCl buffer. This process exchanged any noncovalently bound reagent for DNPNS. After removal of the major portion of the free DNPNS by further dialysis, the residual Ab-bound DNPNS was estimated spectrophotometrically, as discussed above. At the time these experiments were carried out this assay of anti-DNP Ab activity was not as refined as it has since become (Metzger, Wofsy, and Singer, to be published), and the resultant data are only qualitative. However, a loss of Ab binding capacity of 20% or more, compared to unreacted anti-DNP controls treated with DNPNS in a parallel manner, was readily detectable and is considered significant. With several of the reagents which produced a loss of Ab activity, a second set of experiments was carried out including a control sample to which was first added a 10-fold excess of the protector *N*-DNP-ε-aminocaproic acid over reagent. The results are given in Table IV.

TABLE IV
SURVEY OF INACTIVATION OF ANTI-DNP AB BY VARIOUS REAGENTS

Reagent	Inactivation Effect[a]	Inactivation Effect with Protector
2,4-Dinitrobenzenesulfonyl chloride	+	0
3,5-Dinitrobenzoyl chloride	+	0
Picryl chloride	+	—
2,4-Dinitrofluorobenzene	±	—
2,4-Dinitrochlorobenzene	0	—
2,4-Dinitrobenzenesulfonic acid	0	—
2,4-Dinitrobenzenesulfenyl chloride	0	—
2,4-Dinitrobenzaldehyde	0	—
CDF	+	0
p-Trimethylanilinium diazonium fluoroborate	0	—

[a] A + effect indicates 20% or greater inactivation as measured by DNPNS binding; see text.

The reagents 2,4-dinitrobenzenesulfonyl chloride, 3,5-dinitrobenzoylchloride, and picryl chloride produced 20% or greater inactivation of the Ab. This inactivation was largely eliminated by the protector where it was used, and was therefore specific. In several of those instances where a reagent produced little or no loss of Ab activity, the conditions of reaction may have been unfavorable, and no modification of the protein may have been produced. Further studies of these and related reagents are clearly indicated.

DISCUSSION

Mechanism of the Reactions of DNPDF, PNPDF, and CDF with Unprotected Anti-DNP Ab

The results are first examined for the evidence they provide concerning the proposed mechanism of affinity labeling (equation 1).

(*a*) *Rate Enhancement Studies.*—Consider first the data obtained with the reagent PNPDF (Figure 3). It has already been noted that at *initial* stages of the reaction unprotected anti-DNP Ab acquires absorbance at 340 mμ at a rate about 100 times faster than protected Ab. However, in the case of the unprotected Ab, this absorbance is essentially entirely attributable to azotyrosine formation, whereas with protected Ab, azohistidine and azolysine as well as azotyrosine formation must contribute appreciably (Figure 4B). The ratio of initial rates of *azotyrosine* formation for the unprotected and protected Ab is therefore larger than 100; a reasonable figure is about 200. An estimate of the initial value of the enhancement (equation 3) may be obtained as follows. It is concluded later in the discussion that the azotyrosine which forms with unprotected anti-DNP Ab is in the Ab-active site; since there are some sixty tyrosine residues on the whole Ab γ-globulin molecule (Smith *et al.*, 1955) and two active sites, then the ratio of initial rates must be multiplied by a factor of about 60/2. In addition, for a K_A value of 1×10^4 (see below), $[y]/[Y]_t$ is close to 4 at the reagent concentrations employed. Combining these factors, we estimate the initial enhancement to be of the order of magnitude

of 10^4. Such a large enhancement can be explained satisfactorily only by the formation of a reversible complex as an intermediate according to equation (1).

Studies of the initial rates of reaction of DNPDF with anti-DNP Ab were unfortunately not feasible with methods available. Since the specific reaction was completed by 5 seconds, the data in Figure 1 reflect an *average* enhancement of the rate of the specific reaction, rather than its initial value. In all cases so far studied (Figures 3 and 8; and Figure 2 in Wofsy *et al.*, 1962) the average enhancement is between one and two orders of magnitude smaller than the initial value, for important reasons to be considered in detail. The fact, therefore, that the *average* rate of reaction of DNPDF with unprotected Ab appeared to be roughly twenty times that with protected Ab (Figure 1) suggests that the initial value of the enhancement (equation 3) is at least as large, and perhaps considerably larger, for DNPDF than for PNPDF, with anti-DNP Ab.

If the large enhancements observed with the reagents DNPDF and PNPDF were due simply to the presence of an especially reactive tyrosine residue on the anti-DNP molecule, one would expect any nonspecific diazonium reagent to react equally rapidly with the Ab. If, on the other hand, a specific reversible complex forms as an obligatory intermediate, then diazonium reagents of low Hp specificity for the anti-DNP–active sites should show little or no enhancement. It is therefore consistent with the mechanism of equation (1) that the reagent CDF, in its reaction with anti-DNP Ab, exhibits an enhancement of azotyrosine formation only about 0.02 that of PNPDF. The fact that the enhancement is not completely eliminated with CDF is not unreasonable, since some substantial cross reaction between the sterically similar carboxylate ion and nitro groups has been observed in other studies (Nisonoff and Pressman, 1957). Furthermore, the reagent *p*-trimethylanilinium diazonium fluoborate, which must have essentially no cross reactivity for anti-DNP Ab sites, is ineffective in inactivating the Ab as compared to the reagent CDF (Table IV).

Of vital significance to the proposed mechanism is the elimination of the enhanced reaction in every case by the presence of a protecting hapten. The predicted function of the protector is to decrease $[Y]_e$ in equation (3). In this connection, it may be noted that as little as a 5 M excess of the DNP-aminocaproic acid protector over PNPDF reagent was sufficient for the purpose, because of the much larger value of the association equilibrium constant, $\sim 10^8$, of the protector and anti-DNP Ab (Velick *et al.*, 1960) than for the reversible complex formed between PNPDF and the Ab (see below).

(*b*) *Kinetic Order Studies.*—The dependence upon concentration of the relative reaction rates of PNPDF with anti-DNP Ab is clearly less marked than would be expected for a simple second-order reaction (Figures 5 and 6, and Table II). In fact, it can be demonstrated that the concentration dependence is entirely accounted for by the unimolecular mechanism proposed in equation (1).

For this purpose, it is assumed that all Ab sites are equivalent and there is one tyrosine residue per Ab site which is capable of undergoing reaction. The initial rate, $\dot{L}_o$, of a labeling reaction according to equation (1) should be given by

$$\dot{L}_o = k_3 C_o \qquad (4)$$

where C_o is the equilibrium molar concentration of the reversible complex which forms immediately upon mixing HpX and Ab. C_o is determined from

$$C_o = K_A(HpX_t - C_o)(Y_t - C_o) \qquad (5)$$

where HpX_t and Y_t are the molar concentrations of reagent and of Ab sites, respectively, that are originally mixed together.

Because the relative rates of the three experiments with PNPDF and Ab were found to be essentially independent of the fractional extent of the reaction, they may be taken to a good first approximation as *initial* relative rates. These initial relative rates can be accounted for (Table II) by a reaction following the mechanism of equation (1), if a K_A value of about 1.0×10^4 is used to determine the initial concentration (C_o, equation 5) of reversible complex formed between PNPDF and anti-DNP Ab in equation (4). An independent determination of this K_A has not been made, but the above value (in sodium acetate buffer, *p*H 5.0, at 4°) is entirely reasonable in view of the value of $K_A = 7.5 \times 10^3$ given by Eisen (1962) for the association of the similar compound *p*-nitroaniline with anti-DNP Ab at *p*H 7.5 and 32°. (It should be noted [Table II] that K_A values of 5×10^3 or 5×10^4 give calculated relative rates which are significantly different from the observed rates.)

In summary, the evidence presented in this paper together with that previously reported for the anti-benzenearsonate Ab system (Wofsy *et al.*, 1962) provides very strong support for the mechanism proposed for affinity labeling. We turn next to consider the evidence that the active site of anti-DNP Ab has indeed been labeled by these diazonium reagents.

Active Site Labeling of Anti-DNP Ab

(*a*) *The Reagent PNPDF.*—Essentially the sole product of the reaction of PNPDF and an excess of unprotected anti-DNP Ab, according to the spectral evidence of Figure 4, is the (*p*-nitrophenylazo)-tyrosine group. The mechanism studies just discussed demonstrate that this covalent bond must form *while the reagent is reversibly bound to the Ab active site.* This tyrosine residue must therefore be either part of the active site or exceedingly close to it.

That the tyrosine residue is indeed in the Ab site is strongly suggested by the nearly complete correlation which exists between the number of azotyrosine groups formed by PNPDF and the number of active sites lost per mole of Ab (Table III).[5] What small discrepancy exists between these numbers at larger degrees of azo formation is most likely related to Ab heterogeneity (see below).

(*b*) *The Reagents CDF and DNPDF.*—In the reaction of CDF with anti-DNP Ab, azotyrosine groups are formed at a significantly enhanced rate with unpro-

[5] Two reservations may arise about the significance of this inactivation assay. One is whether the *p*-nitrophenylazo groups on the labeled Ab might function as hapten groups, and compete with DNPNS in the reversible binding to active anti-DNP sites. However, since DNPNS binds with a K_A about 300 times larger (Metzger, Wofsy, and Singer, to be published) than do *p*-nitrophenyl haptens to anti-DNP Ab, such competition cannot be significant. The second is whether the observed inactivation may be due to nonspecific structural changes produced in the Ab molecule by the azo modification. It is exceedingly unlikely that significant structural changes could occur at such small degrees of modification, however. Consistent with this conclusion are the facts that substantial modification of anti-DNP (Table IV) and anti-benzenearsonate Ab (Wofsy, unpublished observations) by *p*-trimethylanilinium diazonium fluoborate did not produce any significant Ab inactivation.

tected as compared to protected Ab, which is consistent with the conclusion that a tyrosine residue is present in the Ab-active sites. That azohisitidine groups are also formed with unprotected Ab can be explained as due to the fact that the value of the enhancement is sufficiently small that some CDF reacts with groups outside the Ab site as well. That reaction in the site occurs is further indicated by the specific inactivation of anti-DNP which is produced by CDF (Table IV).

The alkaline spectrum of the product of the reaction of DNPDF with unprotected anti-DNP Ab shows the same maximum and minimum wavelengths as that of the model compound DNP-azo-(*N*-chloracetyl)-tyrosine (Figure 2). Since azohistidine and azolysine derivatives generally exhibit markedly different alkaline spectra from that of the corresponding azotyrosine compound, this evidence together with the data of Figure 1 indicate that in this reaction as well azotyrosine groups are formed at a greatly enhanced rate compared to protected Ab. On the other hand, the Ab product spectrum is not superimposable on that of the model compound. By contrast, the corresponding PNPDF derivatives of anti-DNP and *N*-chloracetyltyrosine exhibit closely similar spectra (Figure 4B). The explanation for the spectral difference in the DNPDF case is not clear. It cannot be due to the reaction of DNPDF with residues other than tyrosine (such as histidine) *outside* the Ab active sites because the enhancement is so large. That is, groups outside the site react much too slowly. Therefore, if some enhanced reaction is indeed occurring with histidine or other residues, these must along with tyrosine also be within the active sites. On the other hand, the product of the reaction with unprotected Ab may be exclusively azotyrosine groups, but such groups incorporated into the Ab molecule may not exhibit the same spectrum in alkali as the corresponding model compound. In the case of *p*-(arsonic acid)-azotyrosine groups, there is evidence (Tabachnick and Sobotka, 1959, 1960) that incorporation into a protein does not produce any marked spectral effect, and the corresponding assumption has been made in this paper about *p*-nitrophenylazotyrosine groups, but this may not apply to DNP-azotyrosine especially when it is localized in the specific Ab site.[6]

The evidence thus presented for the involvement of a tyrosine residue in anti-DNP Ab–active sites is as good as the present methodology of protein chemistry can provide. On the other hand, the concept of the active site, while clear enough in its gross aspects, is not capable of precise definition at the present time. In the case of the hydrolase enzymes (cf. Cohen *et al.*, 1959), the involvement of a serine residue in the active sites is most convincingly demonstrated by its ubiquity, and by the close relationship of peptide sequences around the serine, among the different enzymes. In this connection, it is exceedingly interesting that a tyrosine residue has been demonstrated (Wofsy *et al.*, 1962) in active sites of anti-benzenearsonate Ab by closely similar experiments to those presented in this paper. Experiments on the inactivation of Ab of several specificities by reaction with iodine (Grossberg *et al.*, 1962) have been interpreted to indicate the presence of tyrosine residues in the active sites of these Ab. The possibility is suggested that antibenzenearsonate and anti-DNP Ab, and perhaps rabbit antibodies of other specificities as well, have characteristic related peptide sequences involving a tyrosine residue in their active sites. Peptide isolation and sequence studies of labeled fragments which will explore this possibility are under way.

[6] It is possible, for example, that the OH group of the DNP-azo-tyrosine (formed in the labeling reaction and localized in the active site of the anti-DNP Ab) undergoes a marked pK shift such as is exhibited by DNPNS when bound to the active site; and that even in 0.15 M NaOH, the OH group is incompletely ionized. This could account for the observed spectrum of the DNPDF-labeled Ab.

The Heterogeneity of Ab-Active Sites

Several lines of evidence obtained in the course of these studies strongly suggest that anti-DNP Ab sites are not all identical.

(a) The kinetics of the labeling reactions reflect this heterogeneity. It can generally be assumed, according to kinetic arguments presented elsewhere (Wofsy *et al.*, 1962), that the complex C in equation (1) remains in equilibrium with Y and HpX through the course of a labeling reaction. For a homogeneous system reacting by this mechanism, the integrated rate expression for the concentration of L (the labeled product) is:

$$\frac{1}{K_A\sqrt{-q}}\ln\left(\frac{2x-\alpha}{2x-\beta}\right)\left(\frac{2C_o-\beta}{2C_o-\alpha}\right)-\ln\left(\frac{x^2-Sx+P}{C_o^2-SC_o+P}\right)=k_3t$$

$$L = x - K_A\,(Y_i - x)(HpX_i - x) \quad (6)$$

where $\alpha = S + \sqrt{-q}$, $\beta = S - \sqrt{-q}$, $q = 4P - S^2$, $S = Y_i + HpX_i$, $P = (Y_i)(HpX_i)$, t is the time after mixing of the reactants at the initial concentrations Y_i and HpX_i, and C_o is the initial equilibrium molar concentration of reversible complex (equation 5) that forms immediately after mixing, that is, effectively at zero time. For purposes of illustration this relation may be applied to the reaction of PNPDF with anti-DNP Ab and the theoretical time-course of the reaction of solution I in Figure 5 may be calculated. The observed initial rate of the reaction, utilizing equations (4) and (6) and the value $K_A = 1 \times 10^4$ (determined from the concentration dependence of the reaction rate, Table II) leads to the value $k_3 = 1.2 \times 10^{-2}$ sec^{-1}. With these values for k_3 and K_A and equation (6), the theoretical curve (dotted line) in Figure 5 is obtained. For that portion of the experiment shown in the figure, the rate of formation of L should have been almost constant if the system were indeed homogeneous. (Even if K_A were an order of magnitude smaller than 1×10^4, this conclusion would still hold.) The observed rate, on the contrary, fell off sharply even by the time p/p_∞ was 0.2, at which time only about 5% of the Ab sites were converted, and the reaction was far from completed. A similar marked decrease in rate at early stages of the labeling reaction was observed in the antibenzenearsonate Ab system (Wofsy *et al.*, 1962). Such kinetic heterogeneity can only be due to a heterogeneity of K_A or k_3 values, or both, characterizing the population of Ab sites in both the anti-DNP and antibenzenearsonate systems.

(b) The incompleteness of the labeling reaction which we have often observed is a related reflection of this kinetic heterogeneity. For example, after 2880 minutes of reaction of PNPDF with anti-DNP Ab at a mole ratio of reagent to Ab sites of 1.5 (last line of Table III) only about 0.7 mole of azotyrosine is produced per mole of Ab sites. From the initial rate of the reaction, one would have expected the reaction to have gone to completion, i.e., more nearly 1.0 mole azotyrosine to have formed. Similarly, in the antibenzenearsonate system (Wofsy *et al.*, 1962) with the Ab sites in considerable excess only 60% of the labeling reagent was recovered as protein azotyrosine residues.

(In both cases, the reagent was quite stable under the reaction conditions employed.)

Furthermore, in the reaction of PNPDF with anti-DNP Ab (Table III), the ratio of the number of Ab sites inactivated to the number of azotyrosine groups formed decreased with increasing extent of the specific labeling reaction. This ratio was unity at small degrees of labeling but fell to less than 0.7 at higher degrees. For a homogeneous system, this ratio should have remained unity.

It is probably significant in this connection that in independent studies of the affinity labeling of the enzyme chymotrypsin (Schoellmann and Shaw, 1962), 1 mole of labeling reagent was found to completely inactivate 1 mole of enzyme. The inference is that the active sites of this enzyme are much more homogeneous than those of the two hapten-specific Ab systems which we have so far studied.

The Ab heterogeneity thus revealed in affinity labeling experiments is not too surprising in view of the heterogeneity of equilibrium constants, K_A, which has often been observed to characterize the reversible association of haptens and specific Ab, even with Ab obtained from individual rabbits (cf. Eisen, 1962; Day *et al.*, 1962; Nisonoff and Pressman, 1958). The question arises: do those Ab sites which are most rapidly labeled with a HpX reagent show the largest K_A values for the reversible reaction with Hp? There is good evidence that this is the case with anti-DNP Ab reacted with PNPDF. This may be seen qualitatively from the data in Figure 7. The reversible binding of DNPNS to unlabeled anti-DNP Ab was so strong that when half the Ab sites were bound to DNPNS the equilibrium concentration of free DNPNS was too small to detect. On the other hand, for the sample of Ab which had about half the sites labeled (lowest curve, Figure 7), when half the remaining sites were titrated with DNPNS an appreciable concentration of free DNPNS was present at equilibrium. The remaining unlabeled sites in this sample were therefore not able to bind DNPNS as strongly; we estimate from other data not reproduced here that such sites were characterized by an average K_A at least 20-fold smaller than for the original unlabeled Ab.

Whether this Ab heterogeneity is the result of differences in amino acid composition and sequences within the active sites of Ab molecules *of a given specificity* or to different spatial arrangements of the same amino acid sequences should be answered by sequence studies of labeled peptides which are in progress.

The Method of Affinity Labeling

The evidence that the method of affinity labeling has enabled the active sites of two independent Ab systems, anti-DNP and antibenzenearsonate, to be labeled with an initial enhancement of the order of 10^4, together with related and independent studies in other laboratories (Baker *et al.*, 1961; Schoellmann and Shaw, 1962), suggests that the method will prove of general use with Ab and enzyme systems.

Although our own studies have dealt mainly with HpX reagents where X was the diazonium group (which has the advantage of producing colored reaction products), a considerable variety of other X groups might be useful. This is suggested by the preliminary observations recorded in Table IV for the anti-DNP Ab system. In addition, halomethyl ketones have been used successfully as specific reagents for enzyme systems (Baker *et al.*, 1961; Schoellmann and Shaw, 1962; Lawson and Schramm, 1962).

A further aspect of the versatility of the method is illustrated by the fact that PNPDF is an excellent labeling reagent for anti-DNP Ab sites, despite the much smaller affinity of the *p*-nitrophenyl group compared to the homologous DNP group for anti-DNP Ab. Even the reagent CDF exhibits sufficient enhancement in the labeling reaction with anti-DNP Ab sites to be potentially useful in peptide sequence studies. In favorable cases, therefore, a considerable reduction in the specificity of an HpX reagent for an Ab site may still permit a usefully large degree of specific labeling to occur. In particular, it may be possible to use the same HpX reagent to label Ab sites of several different specificities.

Acknowledgment

We are indebted to Mrs. Margie Adams for assistance in the preparation and characterization of the compounds used in this study.

References

Baker, B. R., Lee, W. W., Tong, E., and Ross, L. O. (1961), *J. Am. Chem. Soc. 83*, 3713.

Bruton, J. C., and Suschitzky, H. (1955), *J. Chem. Soc. 1955*, 1035.

Carsten, M. E., and Eisen, H. N. (1953), *J. Am. Chem. Soc. 75*, 4451.

Cohen, J. A., Oosterbaan, R. A., Jansz, H. S., and Berends, F. (1959), *J. Cell. Comp. Physiol. 54*, (Suppl. 1), 231.

Day, L. A., Sturtevant, J. M., and Singer, S. J. (1962), *J. Am. Chem. Soc. 84*, 3768.

Eisen, H. N. (1962), *Federation Proc. 21*, 721.

Farah, F. S., Kern, M., and Eisen, H. N. (1960), *J. Exp. Med. 112*, 1195.

Grossberg, A. L., Radzimski, G., and Pressman, D. (1962), *Biochemistry 1*, 391.

Koltun, W. L. (1957), *J. Am. Chem. Soc. 79*, 5681.

Lawson, W. B., and Schramm, H. J. (1962), *J. Am. Chem. Soc. 84*, 2017.

Migrdichian, V. (1957), Organic Synthesis, Vol. II, New York, Reinhold, p. 1511.

Neunhoeffer, O., and Ruske, W. (1957), *Ann. 610*, 143.

Nisonoff, A., and Pressman, D. (1957), *J. Am. Chem. Soc. 79*, 1616.

Nisonoff, A., and Pressman, D. (1958), *J. Immunol. 80*, 417.

Schoellmann, G., and Shaw, E. (1962), *Biochem. Biophys. Res. Commun. 7*, 36.

Schoutissen, H. A. J. (1933), *J. Am. Chem. Soc. 55*, 4541.

Singh, A., Thornton, E. R., and Westheimer, F. H. (1962), *J. Biol. Chem. 237*, PC 3006.

Smith, E. L., McFadden, M. L., Stockwell, A., and Buettner-Janusch, V. (1955), *J. Biol. Chem. 214*, 197.

Sturtevant, J. M., Wofsy, L., and Singer, S. J. (1961), *Science 134*, 1434.

Tabachnick, M., and Sobotka, H. (1959), *J. Biol. Chem. 234*, 1726.

Tabachnick, M., and Sobotka, H. (1960), *J. Biol. Chem. 235*, 1051.

Velick, S. F., Parker, C. W., and Eisen, H. N. (1960), *Proc. Nat. Acad. Sci. U. S. 46*, 1470.

Wofsy, L. (1961), Ph.D. dissertation, Yale University.

Wofsy, L., Metzger, H., and Singer, S. J. (1962), *Biochemistry 1*, 1031.

Zollinger, Hch. (1952), *Chem. Rev. 51*, 347.

Zollinger, Hch. (1953), *Helv. Chim. Acta 36*, 1723.