Affinity Labeling—a General Method for Labeling the Active Sites of Antibody and Enzyme Molecules*

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A general method, termed affinity labeling, is proposed to achieve the labeling of the active sites of antibody and enzyme molecules. A labeling reagent is utilized with the following main characteristics: (a) it can bind specifically to the particular active site; and (b) it is capable of forming covalent bonds with various amino acid residues. By virtue of the formation of the initial specific and reversible complex between the reagent and the active site, the local concentration of the reagent in the active site is increased, thereby favoring covalent bond formation by the reagent in the site as compared to the rest of the protein molecule. The theoretical basis of the method is examined and thermodynamic conditions for successful labeling are obtained. Experiments are described with the reagent *p*-(arsonic acid)-benzenediazonium fluoborate and pure anti-benzenearsonic acid antibodies. The experiments conform to the predictions of the method and provide evidence that a tyrosine residue in the active site of this antibody is specifically labeled with this reagent.

Considerable success has been achieved in finding reagents which can be covalently attached to some amino acid residue in the active sites of certain enzymes (Balls and Jansen, 1952; Cohen *et al.*, 1959; Barnard and Stein, 1959; Stark *et al.*, 1961; Witter and Tuppy, 1960; Fischer *et al.*, 1959). Once a stable covalent link is thus formed, the attached residue serves as a tag during systematic degradation of the protein for peptide sequence studies. In these instances, advantage has been taken of the fact that most of the enzymes involved have some one uniquely highly reactive residue in their active sites, as for example a serine residue in the case of the hydrolases (Balls and Jansen, 1952; Cohen *et al.*, 1959). Indeed, it may well be that such highly reactive residues are to be expected in the active sites of many enzymes if their catalytic activity involves the formation of covalently-bonded unstable intermediates between the enzyme and fragments of the substrate (*cf.* Koshland, 1959). It should be possible in such cases to find a particular labeling reagent which can form a covalent bond to the unique residue in the enzyme active site, the bond so formed having the additional property of being stable.

The problem of labeling the active sites of antibody molecules is perhaps more difficult because there is no evidence that antibody sites contain any unusually reactive residues. This is of course not too surprising since the usual interaction between an antibody and its specific antigen appears not to involve any covalent bond rupture or synthesis. One method which has been suggested (Pressman and Sternberger, 1951; Singer, 1955; Koshland *et al.*, 1959) has recently been refined experimentally by Pressman and Roholt (1961); the ultimate general usefulness of this method remains to be demonstrated. In this paper we propose a general method, which we term affinity labeling, for attaching covalently bound groups to the active sites of antibody and enzyme molecules, and present experiments with one antibody system, anti-benzenearsonate, which are entirely consistent with the predictions of the method. Another antibody system, anti-2,4-dinitrophenyl, has been studied with similar results, which will be presented elsewhere (Metzger, Wofsy, and Singer, to be published). The discussion of the method and the experiments performed emphasize the application of affinity labeling to antibody active sites, but the generalization to enzyme systems is straightforward. During the course of these studies, reports appeared from several independent laboratories (Baker *et al.*, 1961; Schoellmann and Shaw, 1962; Lawson and Schramm, 1962) of related investigations with enzyme systems.

Elementary Theory of the Method

Consider an antibody (Ab) molecule specific for a particular haptenic determinant (Hp). The reaction of these two generally results in the formation of a non-covalently bonded reversible complex. Let us assume that among the amino acids comprising each active site of a given Hp-specific Ab there is a particular residue Y which can be chemically modified by appropriate reagents. The method does not require prior knowledge of the chemical nature of Y, only that one or more such groups be present in the active site. In addition, there are a number of residues y present on the remainder of the Ab molecule,

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where y may or may not be the same type of residue as Y.

The method of affinity labeling involves the use of a labeling reagent, HpX, with characteristics to be considered in more detail in the Discussion. Here it is sufficient to indicate that the group X should be as small as possible; it should be capable of reacting with preferably more than one kind of amino acid residue to form stable covalent bonds under adequately mild conditions; and it should be attached to Hp by a stable covalent bond, in a position that does not interfere greatly with the formation of the non-covalently bonded reversible complex between HpX and Hp-specific Ab. The central idea of affinity labeling is that by virtue of the formation of this initial non-covalent complex between HpX and the active site of the Hp-specific Ab, the local concentration of X in the active site is increased, and the rate of covalent bond formation between HpX and Y as compared to the rate of reaction of HpX and any other y on the Ab molecule is thereby enhanced.

In order to examine the method in some detail, the following reactions may be considered:

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

$$Y + HpX \xrightarrow{k_4} L \qquad (2)$$

$$y + HpX \xrightarrow{k_5} M \qquad (3)$$

in which the k's are the respective (assumed single-valued) specific rate constants; C is the non-covalent reversible complex between the Ab and HpX; L is the desired covalently labeled irreversible product of the reaction of HpX with the group Y in the active site; and M is the undesired covalently-labeled product of the reaction of HpX with a group y elsewhere on the Ab molecule. Equations (1) and (2) represent two possible paths under a given set of conditions for the reaction of HpX with Y; in equation (1) the reaction proceeds via the initial formation of a specific, non-covalent, reversible complex, whereas in equation (2) the same reaction proceeds via an ordinary bimolecular mechanism. For the purposes of this paper the reaction in equation (2) is assumed to follow second-order kinetics, although in individual instances it might be acid- or base-catalyzed, or otherwise follow more complicated kinetics. The rate of formation of L via initial complex formation is denoted by $\dot{L}_C$, and via the bimolecular path by $\dot{L}_B$; the rate of formation of M is denoted by $\dot{M}$.

We may define the coefficient $\dot{L}_C/\dot{M}$ the enhancement. Since in most cases of interest $\dot{L}_B << \dot{L}_C$, the ratio $\int_0^t \dot{L}_C\,dt/\int_0^t \dot{M}\,dt$, where t is the reaction time, will give the average specificity of labeling of Y compared to any other single group y. It is useful to separate the enhancement into two parts:

$$\dot{L}_C/\dot{M} = (\dot{L}_C/\dot{L}_B)(\dot{L}_B/\dot{M}) \qquad (4)$$

and to discuss each part separately.

The coefficient $\dot{L}_C/\dot{L}_B$ may be termed the intrinsic enhancement, the ratio of the rates of formation of L by the route of equation (1) to that of equation (2). It has been shown (Sturtevant *et al.*, 1961; Froese *et al.*, 1962; Day *et al.*, in press) directly in two independent Hp-Ab systems that k_1 is unusually large, attaining values of the order of 10^7–10^8 M^{-1} sec.$^{-1}$ This rapidity of non-covalent complex formation is likely to be generally true with Hp-Ab systems. If the reverse reaction rate constant is given by K_A/k_1, where K_A is the (assumed single-valued) association equilibrium constant for the formation of C, then since most Hp–Ab systems that have so far been studied are characterized by values of K_A of the order of 10^5–10^6 M^{-1}, k_2 will generally be greater than 10 sec.$^{-1}$ Only for exceedingly reactive reagents will k_3 exceed this value. In general, therefore, $k_2 >> k_3$, and the complex C will be in equilibrium with Y and HpX, its concentration given by $K_A[Y]_e[HpX]_e$. It follows that

$$\dot{L}_C/\dot{L}_B = \frac{k_3[C]_e}{k_4[Y]_e[HpX]_e} = \frac{k_3K_A}{k_4} \qquad (5)$$

where the subscript e refers to the equilibrium concentration of a particular substance at any given time during the reaction. Therefore, $\dot{L}_C/\dot{L}_B > 1$ if $k_3/k_4 > 1/K_A$. This inequality is equivalent to

$$\Delta F_3^{\ddagger} - \Delta F_4^{\ddagger} < -\Delta F_A^{0} \qquad (6)$$

where $\Delta F_3^{\ddagger}$ is the free energy of activation required to convert the complex C into the activated complex $C^{\ddagger}$ for the reaction to give L; $\Delta F_4^{\ddagger}$ the free energy of activation to form the (presumed) same activated complex $C^{\ddagger}$ by equation (2); and $\Delta F_A^0 = -RT \ln K_A$. This may be rewritten as

$$(\Delta H_3^{\ddagger} - \Delta H_4^{\ddagger}) - T(\Delta S_3^{\ddagger} - \Delta S_4^{\ddagger}) < -\Delta F_A^{0} \qquad (7)$$

Whenever this inequality is satisfied, the intrinsic enhancement will be favorable, that is, greater than unity.

It may first be noted that in general a factor operating to make $\Delta F_3^{\ddagger} - \Delta F_4^{\ddagger}$ favorably smaller, is the cratic contribution (Gurney, 1953) to the entropy difference $\Delta S_3^{\ddagger} - \Delta S_4^{\ddagger}$. The former activation process is unimolecular. If the latter activation process is assumed to be bimolecular, then because of the choice of standard states corresponding to 1 mole/liter, a term $-R \ln x_0 = R \ln 55.6 = 7.98$ e.u. is contributed to increase $\Delta S_3^{\ddagger} - \Delta S_4^{\ddagger}$, where x_0 is the mole fraction when the concentration is 1 mole/liter and there are 55.6 moles of water in one liter. (Alternatively, this term acts to increase k_3/k_4 by a factor of 55.6.)

Consider the process $C \rightarrow C^{\ddagger}$. Within the complex C, there will be an equilibrium configura-

tion of HpX and of the amino acid residues in the active site such as to determine an equilibrium position of the group X relative to Y. In special cases, this equilibrium position may correspond exactly with a favorable X . . . . Y reaction coordinate, so that only the energy to strain the bonds in the reactants is required to form the activated complex, and $\Delta H_3^{\ddagger} \cong \Delta H_4^{\ddagger}$. In general, however, the equilibrium position of X and Y in the complex C will not correspond to this ideal situation. If the positioning of X and Y within the active site so as to achieve this favorable reaction coordinate requires more energy than $-\Delta F_A^0 + RT \ln 55.6$, the intrinsic enhancement will be less than unity. (For $K_A = 10^6$ at T = 300° A, this corresponds to 10.7 kcal/mole.) If less than this amount of energy is required, then the intrinsic enhancement will be favorable.

The important conclusion follows, therefore, that it is not essential to have a perfect and rigid alignment of the groups X and Y within the specific complex C in order to obtain enhancement. Just as long as these groups can come into the proper alignment without the expenditure of more than the indicated (and rather large) energy prior to the formation of the activated complex, a favorable intrinsic enhancement will result. It is difficult at the present time to go beyond this and to define exactly what latitude is permitted to the equilibrium positions of the groups X and Y in the complex C. This will certainly vary for different antibodies. It is pertinent to note here the fact that antibody-hapten specificity is not absolute, but that cross-reactions between structurally related haptens are generally observed (*cf.* Nisonoff and Pressman, 1957). This suggests that some changes in orientation and spatial relationships may be permitted both hapten and amino acid residues in a specific complex without excessive increase in free energy.[1]

With respect to the term $\dot{L}_B/\dot{M}$ in equation (4), the rate of reaction with only one group y is considered because the ultimate experimental objective of these studies is the degradation of the labeled Ab molecule into peptide fragments presumably containing one, or at most a few, groups y.

From equations (1), (2), and (3), it follows that

$$\dot{L}_B/\dot{M} = k_4[Y]_e / k_5[y] \qquad (8)$$

[1] The reactions of related haptens with antibody homologous to the *p*-(*p'*-azophenylazo)-benzoate ion (Nisonoff and Pressman, 1957) provide just one illustration of this effect. If the relative equilibrium constant, K_A, for the binding of benzoate ion to this Ab is taken as 1.0, K_A for 3-nitro,5-chlorobenzoate is 0.07, while for o-methylbenzoate K_A is 0.03. This indicates that the free energy differences involved in accommodating these heterologous haptens, compared to benzoate ion, into the Ab site are relatively small; for $K_A = 0.03$, this free energy difference is only about 2 kcal/mole.

If the groups Y and y are of the same kind chemically, k_4/k_5 is a measure of the relative reactivity of Y and y toward a non-specific small reagent Q-X. Since Ab active sites must be near the external surface of the molecule to be accessible to antigens, there is no reason to expect the reactivity of Y to be much less than that of the average y, and hence k_4/k_5 ought generally in such a case to be of the order of magnitude of unity. If Y and y are chemically different, then only if y is considerably more reactive than Y toward Q-X does any difficulty arise. If this situation obtains in any given system, it can readily be recognized and by additional devices probably can be satisfactorily dealt with.

The term $[Y]_e/[y]$ presents no special difficulties. It can be maximized at a given initial ratio of HpX to Ab by reducing the total Ab concentration, or at a given total Ab concentration by reducing the initial ratio of HpX to Ab, and values of the order of unity for this term can be achieved.

The enhancement is obtained from equations (4), (5), and (8):

$$\dot{L}_C/\dot{M} = \frac{k_3[Y]_e K_A}{k_5[y]} \qquad (9)$$

and, by virtue of the analysis presented, it can be concluded that under suitable conditions it should be possible to find one or more reagents HpX for an Hp-specific Ab for which $\dot{L}_C/\dot{M} > 1$, and with which specific labeling of the active site should be achieved.

It is obvious that the method of affinity labeling can be applied to the labeling of the active sites of enzyme as well as antibodies. The theoretical considerations in this section can be used directly if instead of a reagent HpX one substitutes InX, where In is a specific competitive inhibitor of the enzyme in question.

Materials and Methods

Rabbit Antibody and Rabbit Normal γ-Globulin.—Rabbit antibody to benzenearsonic acid (anti-R Ab) was obtained and specifically purified by methods previously described (Epstein *et al.*, 1956; Pepe and Singer, 1959). Rabbit normal γ-globulin (Fraction II) was purchased from Pentex, Inc.

Chemicals.—*p*-Nitrobenzenearsonic acid (K&K Laboratories, Inc.) was dissolved in water with one equivalent of NaOH, and precipitated as the sodium salt by addition of EtOH. Tyramine hydrochloride, B grade (California Corporation for Biochemical Research), was recrystallized twice from EtOH-Et_2O. Sodium lauryl sulfate (Fisher Scientific Co.) was recrystallized twice from *n*-butanol. The compounds *p*-(arsonic acid)-benzenediazonium fluoborate (RDF) and *p*-(carboxylic acid)-benzenediazonium fluoborate (CDF) were prepared by a procedure based on "Method II" for diazonium fluoborates as described by Roe (1957), with 25% HBF_4 used as

the reaction medium. Elemental analyses yielded the following results: RDF (decomposes 90–95°): calcd. (+4% H_2O): C 22.78, N 8.86, H 2.27; found: C 22.78, N 8.91, H 2.17. CDF (decomposes 95–102°): calcd. (+4% H_2O): C 35.59, N 11.86, H 2.46; found: C 35.59, N 11.94, H 2.46

Reaction of Diazonium Salts with Proteins.—Reagent stock solutions were prepared in water at 0° immediately prior to use. Except where otherwise indicated, 0.10 ml of a solution of 3.03×10^{-4} M diazonium reagent was added, with stirring, to a protein solution at 0° containing 6 mg anti-R or normal γ-globulin in 2.90 ml buffer, 0.17 M borate, and 0.12 M NaCl, *p*H 8.0. Thus reaction solutions were initially 1.25×10^{-5} M in protein and 1.01×10^{-5} M in reagent. (The molecular weight of rabbit γ-globulin was taken as 160,000, $\epsilon^{1\%}_{280\ m\mu} = 14.6$.) In order to investigate the effect on the coupling reaction of first reversibly blocking the Ab active sites by a hapten, sodium *p*-nitrobenzenearsonate (referred to as the protector) was included in some of the protein solutions (prior to reagent addition); in all but specified cases, the concentration of nitrobenzenearsonate used was 6.25×10^{-3} M, a 500-fold molar excess over protein. Anti-R Ab samples with the excess *p*-nitrobenzenearsonate present during the reaction are referred to as protected samples and those without as unprotected. Reactions were terminated at times ranging from 1 minute to 24 hours by the addition of 0.2 ml 0.32 M tyramine (freshly prepared, adjusted to *p*H 8), which rapidly destroyed any unreacted diazonium reagent.

Spectral Assay of Azoproteins.—Spectral analyses to determine the degree and nature of azo-derivatization were performed after denaturation of the azoproteins and an extensive washing procedure to remove any reaction products not covalently bound to protein. The work-up procedure, after termination of the reaction, consisted of the following steps:

(a) To the reaction solutions which contained no protector, 1.88×10^{-2} moles of nitrobenzenearsonate were added; thus, prior to denaturation and washing all solutions contained equal amounts of nitrobenzenearsonate.

(b) Proteins were denatured and precipitated by making the solutions 75% in ethanol.

(c) Precipitates were washed successively by a centrifugation and decanting procedure, with 5 ml ethanol, two 5-ml volumes of ether, a solution of 5 ml ethanol plus 1 ml 1% NaCl (the salt prevented turbidity in the supernatants), and 5 ml 1% NaCl.

(d) The precipitates were dissolved at 37° in 1.5 ml of a 0.5% sodium lauryl sulfate–0.02 M phosphate buffer, *p*H 6.2. Prior to spectral assay, solutions were clarified by centrifugation at 10,000 rpm for 10 minutes.

Spectra of the solutions were recorded with a Cary spectrophotometer between 310 mμ and 600 mμ, first at *p*H 6.2 and then, after addition of sufficient 5 M NaOH, in 0.15 N NaOH. Protein concentrations were determined from optical densities of diluted solutions at 280 mμ, *p*H 6.2, with $\epsilon^{1\%}_{280\ m\mu} = 14.6$, since at this wave length the azo contribution to the absorption of the very lightly modified proteins was negligible. In order to determine the small spectral changes produced in a sample of anti-R Ab by reaction with a diazonium reagent, the background protein spectrum was subtracted. For reasons of accuracy, this was taken as that of the corresponding protected anti-R sample that had been reacted for 1 minute, since the protein samples thus compared were subjected to most nearly identical treatment subsequent to the reaction itself. Tabachnick and Sobotka (1959, 1960) examined the spectra of model compounds formed by the reaction of diazotized arsanilic and *p*-aminobenzoic acids with tyrosine, histidine, and lysine derivatives. The spectra of a given type of compound formed by reaction with either RDF or CDF are almost identical. According to these authors, the extinctions at 460 and 500 mμ of an azo-derivatized protein are not significantly altered by proteolytic digestion, and the assumption is therefore reasonable that the extinction coefficients of the model compounds are essentially the same as the corresponding structures in the azo-protein. It is therefore possible to estimate the amounts of azotyrosine and azohistidine formed by RDF or CDF from the extinctions of a solution of an azo-protein at 460 and 500 mμ (where the contribution of the bis-azo lysine derivative is negligible) by use of an appropriate pair of simultaneous relations.

Results

The reaction of the homologous arsonic diazonium reagent, RDF, is much more rapid with unprotected anti-R Ab than with anti-R Ab protected with *p*-nitrobenzenearsonate (Figs. 1 and 2). Furthermore, the spectra of such protected and unprotected anti-R Ab derivatives differ not only quantitatively but qualitatively as well (Fig. 1). The spectra of protected anti-R Ab derivatives are mixed, that is, the resultant of contributions from azotyrosine, azohistidine, and perhaps other azo structures. Such mixed product spectra are characteristic of the usual reactions of diazonium compounds with proteins (Tabachnick and Sobotka, 1960; Gelewitz *et al.*, 1954). In marked contrast, the spectra of the unprotected anti-R Ab derivatives over the entire range of reaction times are closely similar to the corresponding spectrum of the model compound mono-(*p*-azobenzenearsonic acid) *N*-chloroacetyltyrosine (Tabachnick and Sobotka, 1959), showing maxima (in 0.15 M NaOH) at about 330 and 480 mμ and a minimum near 380 mμ. The number of moles of azotyrosine formed per mole of each unprotected anti-R Ab sample was estimated in two ways: first, by the simultaneous relations of Tabachnick and Sobotka (1960); and, second,

by the use of simply the measured optical density at 480 mμ and the value ϵ_{480} = 11,000 based on the model azotyrosine derivative (Tabachnick and Sobotka, 1959). The results of both methods of calculation are essentially the same. There is within experimental error no azohistidine formed with unprotected anti-R Ab through the first hour of reaction with RDF, and perhaps a trace (<0.03 moles azohistidine per mole protein) after 20 hours.

On the other hand, the spectra of the protected sample and of normal γ-globulin reacted with RDF for comparable times were quite similar (Fig. 1). After 20 hours of reaction time, the former sample contained 0.25 moles azotyrosine and 0.19 moles azohistidine per mole protein, whereas the latter contained 0.19 moles azotyrosine and 0.13 moles azohistidine per mole protein.

The spectra of normal γ-globulin samples reacted with RDF in the absence or presence of a 1000-fold molar excess of *p*-nitrobenzenearsonate were indistinguishable, which shows that this protecting hapten has no nonspecific effect on the reaction of RDF with proteins. Its effect on the reaction of RDF with anti-R Ab must therefore be entirely due to its capacity to block the Ab active site.

The rates of formation of azotyrosine groups with different protein samples reacted under comparable conditions with either RDF or CDF (Fig. 2) may be summarized as follows:

(a) About 400 minutes of reaction time with RDF is required to form the same amount of azotyrosine with normal γ-globulin that forms in the first one minute with unprotected anti-R Ab.

(b) By contrast, the apparent rates of reaction of the heterologous reagent CDF with unprotected anti-R Ab and normal γ-globulin are very similarly slow.

(c) The rates of reaction of RDF and CDF with protected anti-R Ab and normal γ-globulin are all quite similarly slow.

The products of the 24-hour reaction of the heterologous reagent CDF with protected and unprotected anti-R Ab exhibited slightly different spectra (Fig. 3). Although in both cases the spectra were mixed, *i.e.*, due to a combination of azotyrosine, azohistidine, and perhaps other groups, the unprotected anti-R Ab derivative appeared to contain somewhat more azotyrosine (0.18 mole/mole protein) than the comparable protected one (0.13 mole/mole protein).

Discussion

The theory presented in this paper indicates that under suitable circumstances it should be possible by the method of affinity labeling to attach a labeling group to the active site of an antibody or an enzyme molecule. That the active site has actually been labeled in any particular experimental system is, however, a difficult proposition to prove, mainly because of the

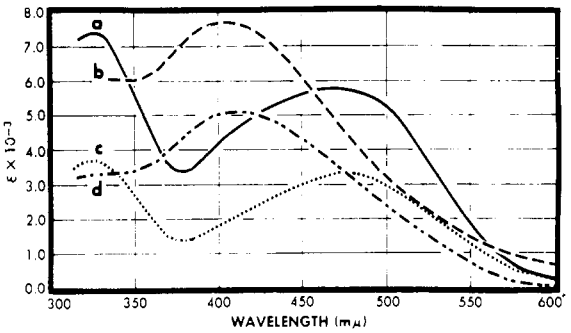

FIG. 1.—Spectra in 0.15 N NaOH of rabbit anti-R Ab and rabbit normal γ-globulin after reaction with RDF. All reactions were performed at *p*H 8, 5°, protein concentration = 1.25 × 10^{-5} M, reagent concentration = 1.01 × 10^{-5} M. *a*, Unprotected anti-R Ab, after 20 hours. *b*, Protected anti-R Ab (reacted in presence of 6.25 × 10^{-3} M *p*-nitrobenzenearsonate), after 20 hours. *c*, Unprotected anti-R Ab, after 1 hour. *d*, γ-Globulin, after 20 hours.

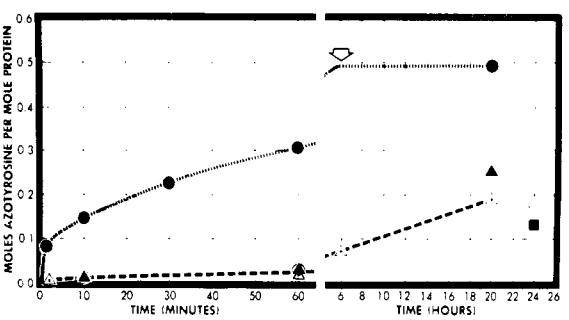

FIG. 2.—Rate of formation of monoazotyrosine residues in the reaction of rabbit anti-R Ab and rabbit normal γ-globulin with RDF or CDF: ●—●, unprotected anti-R Ab with RDF; ▲—▲, protected anti-R with RDF; △—△, γ-globulin with RDF; O—O, unprotected anti-R with CDF; ■—■, protected anti-R with CDF; shaded triangles, γ-globulin with CDF. The arrow indicates the fact, verified in separate experiments, that no further modification of unprotected anti-R Ab with RDF is detected after 6 hours.

ambiguities which are at present inherent in the concept of the active site itself. It is useful, therefore, to discuss in two stages the experimental results presented in this paper. In the first stage, we inquire what the evidence is that the reaction of RDF and unprotected anti-R Ab does indeed proceed by the mechanism of equation (1), *i.e.*, by way of an initial non-covalent specific complex; and in the second stage, what the evidence is that the active site of anti-R Ab has actually been successfully labeled.

The Mechanism of Reaction of RDF and Anti-R Ab.—The following items are pertinent to the discussion of this mechanism:

(a) The initial rate of formation of azotyrosine derivative in the reaction of RDF with anti-R Ab is about 200 times greater than with normal γ-globulin. Let us assume that there is only one tyrosine in each of the two active sites

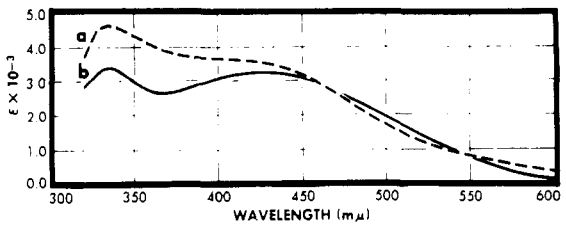

FIG. 3.—Spectra in 0.15 N NaOH of unprotected anti-R Ab, *a*, and protected anti-R Ab, *b*, after reaction with CDF for 24 hours.

of an anti-R Ab molecule with which RDF reacts. On the other hand, there are about 60 tyrosine residues on a normal γ-globulin molecule (Smith *et al.*, 1955). If this difference is taken into account, therefore, the initial value of the enhancement [equation (9)] is 400 × 60/2, or about 10^4. Such a large enhancement is explicable only by the mechanism of initial reversible complex formation, according to the theory proposed above. The only alternative explanation, that there is a unique extraordinarily reactive tyrosine residue on the anti-R molecule (either within or outside of the active site) that is not present on normal γ-globulin, is ruled out by the fact that the closely similar but heterologous reagent CDF shows no such enhancement of reaction rate with anti-R Ab.

(b) On the other hand, the difference in reaction rates of RDF and CDF with anti-R Ab is entirely consistent with the concepts of affinity labeling. According to equation (9), the enhancement should be correlated with K_A, the equilibrium constant for the formation of the non-covalent reversible complex C. Direct measurements of K_A for RDF and CDF with anti-R Ab are difficult to make because of the covalent-bond-forming capacities of these haptens. For our purposes, however, estimates of K_A values may be obtained from studies on related systems. The hapten *p*-(*p'*-hydroxyphenylazo)-benezenearsonic acid (Eisen and Karush, 1949) binds to anti-R Ab with $K_A = 3.5 \times 10^5$, and the influence of different para-substituents on benzenearsonic acid on the binding to anti-R Ab has been shown to be relatively small (Pressman *et al.*, 1942). A value of $K_A = 10^5$ for the reversible combination of RDF and anti-R Ab is therefore very likely to be correct within a factor of about 10. From the results of studies of hapten inhibition of precipitation (Nisonoff and Pressman, 1957; Pressman *et al.*, 1942),[2] it can be inferred that K_A for the binding of a heterologous benzoate Hp to anti-R Ab should be no greater than 10^{-2} times that of the corresponding homologous benzenearsonate Hp. It is clear that a reduction of K_A by as large a factor as 10^2 would, other things being equal, reduce the initial enhancement of about 10^4 observed for the covalent reaction of RDF with anti-R Ab to a value of about 10^2 for the corresponding reaction of CDF [equation (9)]. Such a relatively smaller value of enhancement would not be readily detectable as a significant increase in over-all rate of reaction (Fig. 2), because of the competing effects of the many non-specific groups on the Ab molecule. On the other hand, the small spectral difference between comparable protected and unprotected anti-R Ab samples reacted with CDF (Fig. 3) suggests that some small enhancement is indeed detected by this more sensitive type of observation.

(c) The presence of an excess of the hapten *p*-nitrobenzenearsonate largely eliminates the enhanced rate of reaction of RDF and anti-R Ab. On the other hand, this hapten has no effect on the reaction of RDF with normal γ-globulin. The effect of *p*-nitrobenzenearsonate in the former case must therefore be due to its specific interaction with the active sites of anti-R Ab. This is precisely what is to be expected if RDF must first bind reversibly to the active site before it reacts to form a covalent bond with the Ab. The effect of the protecting hapten is to lower the concentration $[Y]_e$ in equation (9).

Baker *et al.* (1961) have recently reported investigations which bear a strong relationship to those presented in this paper but which have a different ultimate objective. These authors sought to find irreversible inhibitors of enzymes which "fit the active site of an enzyme reversibly, then become irreversibly bound by alkylation of the enzyme adjacent to the active site." Much of the chemistry involved is formally similar, but our concern has been primarily to label the active sites. Of particular interest here are studies they performed which provide strong and independent kinetic evidence for a reaction mechanism involving the formation of a specific reversible complex between a reagent and the active site of an enzyme prior to the formation of an irreversible covalent bond. It was found that the two compounds, 4-(iodoacetamido)-salicylic acid and 4-(iodoacetamido)-benzoic acid, irreversibly inactivated the enzyme glutamic dehydrogenase. The ratio of the rates of irreversible inactivation of the enzyme by the two compounds was closely similar to the ratio of their reversible association constants with the enzyme, as expected from equation (9).

The Nature of the Groups on Anti-R Ab Molecules Labeled with RDF.—From the discussion of reaction mechanism just given, it follows that the formation of the irreversible covalent bond between RDF and unprotected anti-R Ab must occur while the reagent is bound to the Ab active site. The only remaining question is whether

[2] The results of Nisonoff and Pressman (1957) actually compare the binding of benzoate and *p*-arsanilate ion haptens to anti-*p*-(*p'*-azophenylazo)-benzoate antibody, and show that K_A for the latter hapten is less than 0.01 the value for the former. It is assumed that the relative binding of these haptens to anti-R antibody would, to a first approximation, be correspondingly different (Landsteiner, 1945).

the bond is formed with a group actually in the active site or one very close to, but not part of, the site. The evidence is substantial that the reaction is specifically with a tyrosine residue in the active site.

(a) The only significant product of the enhanced reaction of RDF and unprotected anti-R Ab, according to direct spectral evidence, is the *p*-(arsonic acid)-benzeneazotyrosyl group. On the other hand, if the active site is protected with *p*-nitrobenzenearsonate, the reaction of RDF with anti-R Ab produces a variety of products, and the reaction rates are much reduced.

About 0.8 mole RDF was added per mole of unprotected anti-R Ab, and, according to spectral analysis, about 0.5 mole, or better than 60% of the reagent, was recovered after 6 hours of reaction time as protein azotyrosine residues.

(b) The diazonium group is small and therefore the effective reaction sphere of the RDF reagent, while bound to the active site of the anti-R Ab molecule, encompasses the active site and little more.

There is some independent evidence that a tyrosine residue is critically involved in the active sites of anti-R Ab. Pressman and Sternberger (1951) showed that anti-R Ab was inactivated by iodination and that the extent of this inactivation could be reduced by the presence of a specific Hp protector. Subsequent studies (Koshland *et al.*, 1959; Pressman and Roholt, 1961) of the specific incorporation of radioactive iodine into unprotected as compared to protected anti-R Ab lend weight to the suggestion that the effect of iodination in this case is to iodinate a tyrosine residue in the Ab active site. Another finding which is consistent with tyrosine involvement in anti-R active sites comes from the studies of Epstein and Singer (1958) on the variation with *p*H of the equilibrium constant of the reaction between a bivalent benzenearsonic acid hapten and anti-R Ab. The *p*H effect could be quantitatively accounted for as due to the ionization of a single group with *p*K of about 9.9 in each Ab active site. This *p*K value is characteristic only of lysine ϵ-NH_2 and tyrosine OH groups, and recent chemical evidence (Wofsy and Singer, in press) now indicates that a lysine ϵ-NH_2 group cannot be critically involved in anti-R active sites.

Conclusive proof that labeling of the active site has occurred, however, can only result from the accumulated weight of a great many different types of experiments. A next stage in these studies obviously involves the isolation and characterization of peptide fragment(s) bearing the *p*-benzenearsonic acid–azotyrosine residue. If the same fragment(s) are labeled with use of a variety of other HpX reagents specific for rabbit anti-R Ab, and if significant correlations are found in the composition and sequence of such fragments from among rabbit antibodies of different specificities and from among antibodies of the same specificity but produced in different animals, then the proof would be substantially stronger. Such studies are contemplated or in progress. Rabbit antibody to the 2,4-dinitrophenyl (DNP) hapten (Farah *et al.*, 1960) has been reacted with specific diazonium reagents with results similar to those described with anti-R Ab in this paper. Furthermore, the diazonium and several other HpX-type labeling reagents were found to be effective in *specifically* inactivating anti-DNP Ab.[3] This investigation will be reported separately (Metzger, Wofsy, and Singer, to be published).

The Choice of Reagent HpX.—Having discussed the particular experimental system studied in this paper, we now consider further some general aspects of the method of affinity labeling. A vital factor is the reagent HpX.

(a) *The Group X.*—It is important to realize that the reactivity of the group X can vary within wide limits. Even a highly reactive group may be used, which may ordinarily react quite rapidly with groups y on a protein molecule [*i.e.*, k_5 in equation (9) may be large], because, for a given value of K_A, the enhancement [equation (9)] depends on a ratio of rate constants k_3/k_5 and not on the absolute value of either. Indeed, to increase the probability of forming a labeled product in the active site, the group X should preferably be one which reacts at appreciable rates under mild conditions with as wide a range as possible of amino acid residues. Thus, some group Y in the site should be sufficiently favorably situated to satisfy the inequality in equation (6). This factor strongly favors highly reactive reagents.

(b) *The Group Hp.*—The HpX reagent most nearly homologous to the particular Ab will likely exhibit the largest K_A value for the formation of the reversible complex with that Ab. On the other hand, it is conceivable that a reagent Hp'X, with a heterologous but cross-reacting group Hp', might react with the Ab with a larger value of k_3/k_5 [equation (9)] than the reagent HpX. Being less strongly bound, and therefore presumably less well fitted to the Ab active site, the reagent Hp'X might have more positional free-

[3] No data are presented in the text concerning the activity of anti-R Ab after reaction with RDF. In early experiments carried out under somewhat different conditions and with 2:1 and 4:1 mole ratios of RDF to Ab, a loss of 25–40% of precipitating capacity of the Ab for the antigen benzenearsonic acid-azo-bovine γ-globulin was observed. These experiments were not pursued, however, because a quantitative interpretation of such results is rendered ambiguous by at least two factors: (a) the modification affects Ab solubility; and (b) the reaction with RDF attaches haptenic groups to the Ab (thereby making anti-R Ab simultaneously an R-antigen) which may affect the Ab assay. The latter factor would also operate in equilibrium dialysis assays of Ab activity. These considerations will be amplified elsewhere.

dom within the site than the homologous reagent HpX, and hence might react more readily with the group Y in the site. In other words, the parameters K_A and k_3/k_5, other things being equal, may vary inversely in such systems; the resultant enhancement could conceivably be larger for the heterologous reagent Hp'X than for HpX.

It should also be noted that an enhancement as large as the value of about 10^4 observed in the RDF-anti-R Ab system implies a specificity of labeling of a putative active peptide fragment of about 10^4 compared to any other peptide fragment. Since, with a radioactive HpX reagent, a much smaller specificity of labeling could easily be detected in peptide fragmentation studies, an appreciable decrease in K_A, and hence an appreciable degree of heterology in Hp', may be tolerable in a given system.

These considerations indicate that wide latitude in the choice of reagent HpX is permitted by the method of affinity labeling.

Other Aspects of Affinity Labeling.—The concepts of affinity labeling should be applicable in other directions in addition to that so far discussed.

(a) *Mapping of Protein Structure.*—By means of a series of reagents Hp-t-X in which the length and configuration of a "tail" t separating Hp and X are systematically varied, it may be possible to label groups outside of the Ab active site in a systematic manner. The distances and orientations of such groups from the active site may conceivably be "mapped" in this manner (Lawson and Schramm, 1962).

(b) *Heavy Atom Isomorphous Replacements.*—For x-ray crystallographic studies of proteins (Kendrew *et al.*, 1961), it is at present essential to prepare various isomorphous crystalline preparations of a particular protein containing heavy atoms in various fixed positions in the crystal. Affinity labeling of a protein enzyme may be produced with a reagent InX (see Theoretical section), where In is a specific competitive inhibitor of the enzyme containing a heavy metal atom. With a value of the enhancement of the order of 10^4, a highly specific and highly localized attachment of the heavy atom to the protein could be achieved, and, if no significant conformational change of the protein resulted, a suitable derivative might be prepared.

(c) *Model Systems for Enzyme-Substrate Interactions.*—Strong analogies exist between the process of affinity labeling and the normal functioning of hydrolase enzymes (*cf.* Koshland, 1959). In both cases, the initial rapid formation of a specific reversible complex is followed by the formation of a covalent bond between the small molecule and a group in the active site of the protein molecule. In fact, an Hp-specific Ab would be a specific enzyme for the substrate HpX if the covalent bond formed between HpX and Y were sufficiently labile to be hydrolyzed at an adequately rapid rate, and the group Y were regenerated. In this sense, affinity labeling is a simpler process than hydrolase function. Insight into the latter process may be gained by detailed studies of the kinetics of affinity labeling and of the effects of variations in Hp structure and in the geometry of the group X not only on K_A, but particularly on $\Delta H_3^{\ddagger}$ and $\Delta S_3^{\ddagger}$ [equation (7)]. In another connection, it may eventually prove possible to prepare a specific enzyme catalyst for a given reaction of the substance Hp'X by utilizing an Ab specific to a suitably designed antigenic determinant, Hp.

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Interaction of Alkaline Phosphatase of *E. coli* with Metal Ions and Chelating Agents*

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The chelating agent 1,10-phenanthroline inhibits *E. coli* alkaline phosphatase by removal of zinc; the inhibition is reversed on addition of Zn^{++} ions. Dialysis of the enzyme against the chelating agent results in an inactive zinc-free apophosphatase to which activity is completely restored on addition of Zn^{++} ions. Among a number of other metal ions tested Co^{++} and Hg^{++} restore activity, though only partially. Much higher concentrations of Mg^{++} ions bring about a similar effect, probably by a different mechanism. Cd^{++}, Co^{++}, Pb^{++}, and Cu^{++} ions inhibit the enzyme, presumably by displacement of the native Zn^{++} ion. Be^{++} ions also inhibit the alkaline phosphatase of *E. coli* as has been observed with other phosphatases. Activity of the native enzyme is enhanced by increasing ionic strength.

Purified preparations of *E. coli* alkaline phosphatase contain about 2 gram atoms of zinc per mole of enzyme protein (Plocke *et al.*, 1962). The purified enzyme is inhibited by a number of agents which form stable complexes with zinc ions in solution. The effectiveness of these inhibitors is closely related to the magnitudes of the stability constants of their zinc complexes. Zinc, therefore, seems to be involved in the action of the enzyme.

The present report considers the mechanism by which one of these agents, 1,10-phenanthroline, inactivates the enzyme through removal of zinc. The zinc-free apophosphatase can be reactivated completely by the addition of Zn^{++}, and partially by Co^{++} and Hg^{++} ions.

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METHODS AND MATERIALS

Alkaline phosphatase of E. coli was prepared as previously described (Plocke *et al.*, 1962).

Protein concentration was determined by the absorbancy at 278 mμ, a molar absorbancy index (a_M) of 5.6×10^4 being used (Plocke *et al.*, 1962).

All metals were determined by emission spectrography (Vallee and Hoch, 1955); zinc was also measured by means of the dithizone method (Vallee and Gibson, 1948).

Enzymatic activity was determined in 1 M Tris, *p*H 8.0, at 25°, with 10^{-3} M *p*-nitrophenyl phosphate (Garen and Levinthal, 1960; Plocke *et al.*, 1962). Where specified, 0.02 M veronal buffer was substituted for 1 M Tris.

Reagents.—The method of cleaning glassware and the preparation of metal-free water has been described previously (Thiers, 1957). Tris (Sigma 121) and sodium diethylbarbiturate (veronal) were dissolved in metal-free distilled water, adjusted to neutral *p*H with HCl, and extracted with dithizone in carbon tetrachloride to remove contaminating metal ions. Excess dithizone was removed by extractions with c.p. carbon tetrachloride. The extracted solutions were adjusted