Variability among Anti-*p*-azobenzenearsonate Antibody Preparations as Revealed by Affinity Labeling*

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ABSTRACT: The affinity labeling method as developed by Wofsy and coworkers (Wofsy, L., Metzger, H., and Singer, S. J. (1962), *Biochemistry 1*, 1031) was applied to the antiazobenzenearsonate (anti-R_p) antibody preparations from three individual rabbits. *p*-(Arsonic acid)benzenediazonium fluoroborate was used as the reagent. The extent of labeling and the nature of modified residues varied from one preparation to another, indicating differences in the regions involved in the affinity labeling. Anti-R_p antibody from one rabbit was extensively labeled (0.5 residue/mole) on tyrosine residues only. Anti-R_p antibody from a second rabbit was labeled on both tyrosine (0.14 residue/mole) and probably also on some other residue, perhaps histidine. Anti-R_p antibody from a third rabbit did not show any significant affinity labeling reaction.

The demonstration of the latter population of anti-R_p antibodies suggests that such antibodies, which cannot be affinity labeled by the diazonium salt, may well be present in those anti-R_p preparations which do show affinity labeling, but have not hitherto been detected because of their unreactivity. Sites of those antibodies which were not capable of being affinity labeled were shown to have iodinatable residues, presumably tyrosine, in them. This observation suggests that the affinity labeling reagent may block its own reaction with a potentially reactive residue in the site of some antibodies.

The method of affinity labeling of antibodies (Wofsy *et al.*, 1962) depends on the combination of a hapten with the antibody active site and then a subsequent coupling with residues in or near the active site by a chemically reactive group present on the hapten molecule. This method has been applied in their laboratories to several rabbit antihapten antibodies. The systems they investigated were anti-*p*-azobenzenearsonate antibodies labeled with *p*-(arsonic acid)benzenediazonium fluoroborate (Wofsy *et al.*, 1962), anti-*p*-azophenyltrimethylammonium antibodies labeled with *p*-(trimethylammonium)benzenediazonium fluoroborate (Fenton and Singer, 1965), and antidinitrophenyl antibodies labeled with *p*- and *m*-nitrobenzenediazonium fluoborate (Metzger *et al.*, 1963; Lenard and Singer, 1966; Good *et al.*, 1967). In these systems, it was reported that labeling occurred essentially exclusively on tyrosyl residues of the antibodies. Wofsy *et al.* (1967a,b) recently reported affinity labeling of antibodies against *p*-azophenyl β-lactoside and *p*-azophenyl β-galactoside in which some other residues in addition to tyrosine were labeled. We have found in the work reported here that the residues attacked in the affinity labeling reaction by a diazonium group vary for antibodies from different rabbits, even when these antibodies are directed against the same haptenic group, *p*-azobenzenearsonate (R_p).[1] Most significantly, in one anti-R_p preparation, no affinity labeling was observed. These observations indicate that the region involved in the affinity labeling differs. It seems likely that in various antibody molecules different residues are in a position to react with the affinity labeling reagent; in some molecules no reactive residues are present within coupling range.

Materials and Methods

Rabbit Anti-R_p Antibody and Rabbit Normal γ-Globulin. Rabbit anti-R_p antibodies were obtained from rabbits which were hyperimmunized with *p*-azobenzenearsonate bovine γ-globulin by the method previously described (Nisonoff and Pressman, 1957; Grossberg and Pressman, 1960; Grossberg *et al.*, 1962). Three of the antisera used were from individual rabbits and a fourth was a pooled antiserum from fourteen other rabbits.

The anti-R_p antibodies were specifically purified from the γ-globulin fractions of these antisera. The purification procedure used was that described by Onoue *et al.* (1965). Briefly, this method consists of adsorbing the anti-R_p antibodies onto a conjugate of an insoluble polymer of rabbit serum albumin coupled with R_p groups, eluting the antibodies with 0.3 M *p*-nitrobenzenearsonate, precipitating the antibodies with sodium sulfate and finally dialyzing the solution of dissolved antibody free of hapten. The γ-globulin fraction of normal rabbit serum was prepared by fractionation with

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[1] Abbreviation used in this paper that is not listed in *Biochemistry 5*, 1445 (1966), is: R_p, *p*-azobenzenearsonate.

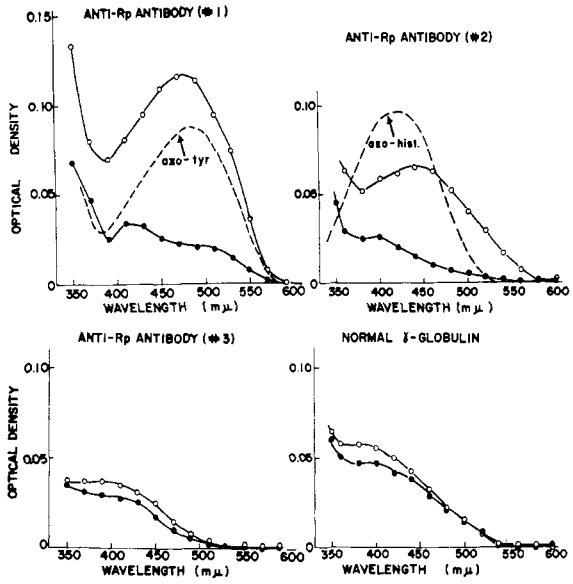

FIGURE 1: Absorption spectra of normal rabbit γ-globulin and of specifically purified anti-R_p antibody preparations from individual rabbits following reaction with *p*-(arsonate)-benzenediazonium fluoroborate, in the absence (○—○) and in the presence (●—●) of 0.01 M *p*-nitrobenzenearsonate. Protein concentration is 2×10^{-6} M. Reference spectra for 8×10^{-6} M mono(*p*-azobenzenearsonate)chloroacetyltyrosine (azo-Tyr) and 4×10^{-6} M mono(*p*-azobenzenearsonate)-acetylhistidine (azo-His) are included. All spectra were determined on solutions 0.1 N in NaOH. Other details are in the text.

sodium sulfate according to the method of Kekwick (1940).

Chemicals. *p*-(Arsonic acid)benzenediazonium fluoborate was prepared by the procedure based on method II for diazonium fluoborates described by Roe (1957). ^{125}I-Labeled *p*-iodobenzenearsonate used for equilibrium dialysis was prepared from the unlabeled compound by an exchange reaction, as previously described (Nisonoff and Pressman, 1958).

Buffers. Borate buffer was made by adding 1 N NaOH to a solution of 12.4 g of H_3BO_3 and 8.1 g of NaCl to raise the pH to 8.0 or 8.5, then bringing the total volume to 1000 ml with water. A stock solution of Tris buffer was prepared by adding 9.33 ml of 6 N HCl to a solution of 12.1 g of Tris and 5.9 g of NaCl, then bringing the total volume to 100 ml with water. Prior to use, 2 ml of this stock solution was diluted to 100 ml with 0.9% saline and the pH was adjusted to 8.0 or 8.5 with 2 N NaOH. Glycine buffer used for iodination was prepared by adding 2 N NaOH to a solution of 7.5 g of glycine to adjust the pH to 9.0 and bringing the total volume to 100 ml with water.

Affinity Labeling Reaction. Solutions of *p*-(arsonic acid)benzenediazonium fluoroborate were prepared in water at 4° immediately prior to use. Except where otherwise described, 0.05 ml of a solution of 4×10^{-4} M diazonium salt was added, with stirring, to a protein solution containing 3.2 mg (2×10^{-5} mmole) of anti-R_p antibody in 1 ml of Tris buffer or borate–saline buffer at pH 8.5. For investigation of the effect of hapten on the coupling reaction, sodium *p*-nitrobenzenearsonate, buffered at pH 8.5, was added to an antibody solution at a final concentration of 1×10^{-2} M before the addition of coupling reagent. The reactions were carried out at 4° with constant stirring. At the end of the reaction time, the coupling reactions were stopped by the addition of 0.1 ml of 0.02 M phenol (pH 9.0), which rapidly coupled with any unreacted diazonium reagent.

Estimation of Azoproteins. To estimate the degree and nature of azo derivatization, azoproteins were treated as described by Wofsy *et al.* (1962). After ethanol denaturation of the azoproteins formed, any reaction products not covalently bound to proteins were removed by extensive washings with ethanol, ether, 5 ml of ethanol plus 1 ml of 1% sodium chloride, and 1% sodium chloride. After successive washings with these solvents, precipitates were dissolved in 0.5 ml of 0.1 N sodium hydroxide.

The spectra of the azoproteins were determined between 350 and 600 mμ and the background protein spectra were subtracted. These spectra were determined no earlier than 1 hr or later than 2 hr after the azoprotein solution had been made alkaline. The spectra were stable during the period from 1 to 6 hr standing at room temperature in 0.1 N NaOH, as tested for several preparations and for up to 24 hr as tested for a few preparations. In view of the rather low optical densities observed, special precautions were exercised in their measurement including careful checking of the cuvet blanks. The amounts of azotyrosine and azohistidine residues present were estimated from the extinctions of an azoprotein solution at 460 and 500 mμ by use of an appropriate pair of simultaneous equations, as described by Tabachnick and Sobotka (1960). From a knowledge of the amount of protein present, values were calculated for the number of residues of azotyrosine and azohistidine coupled per mole of protein.

In order to check the accuracy of the values obtained by the method of calculation using simultaneous equations, known amounts of mono(*p*-azobenzenearsonate)-chloracetyltyrosine and mono(*p*-azobenzenearsonate)-acetylhistidine, prepared as described by Tabachnick and Sobotka (1959), were mixed. Spectra of these mixtures were determined and the calculated compositions of these mixtures were found to agree with the compositions as prepared to an accuracy of 5% or better.

Determination of Purity and Binding Constants of Antibodies. Anti-R_p antibody preparations were analyzed for their antibody content and the average value of binding constant for ^{125}I-labeled *p*-iodobenzenearsonate (K_0') by the method of equilibrium dialysis, as described previously (Grossberg and Pressman, 1960; Grossberg *et al.*, 1962) but with the modification that Tris buffer (pH 8.0) was substituted for pH 8 borate–saline buffer. Data at three to six free hapten concentrations were analyzed by use of the Sips (1948) equation. Purity is expressed as the per cent of the total protein which is antibody on the basis of two antibody sites per antibody molecule of mol wt 160,000.

Iodination of Anti-R_p Antibody. Iodination of anti-R_p antibody 3 was performed by the procedure described by Grossberg *et al.* (1962). Carrier-free [^{125}I]iodide was mixed with 8×10^{-3} μmole of ICl in 2 N HCl and 8 ×

10^{-3} μmole of KI was added. The resultant labeled I_2 was extracted into CCl_4 and then into 1.0 M glycine buffer at pH 9.0. The labeled iodinating solution was prepared by mixing 0.8 ml of the above glycine solution of ^{125}I-labeled iodine with 0.10 ml of 0.25 M ICl solution in 0.75 N HCl, and the pH was adjusted to 8.7 with 2 N NaOH. Antibody (14 mg of protein in 1.5 ml of pH 8.5 Tris buffer) was iodinated at 0° in the presence or absence of 0.1 M *p*-nitrobenzenearsonate by adding the required amounts (0.25 ml) of the iodinating solution with rapid stirring. After 90-min reaction, the protein was dialyzed exhaustively against four 4-l. portions of pH 8.0 Tris–saline buffer, to remove unreacted radiolabeled iodine and hapten. Iodine was incorporated at the level of 50 atoms of iodine/mole of protein both in the presence and absence of hapten as determined by radioactivity incorporation. Seventy per cent of the iodine was incorporated.

Estimation of Protein. For both anti-R_p antibody 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%. Nitrogen determinations of azoproteins were carried out by digestion and Nesslerization. When required, proteins were quantitatively precipitated and washed with 10% trichloroacetic acid in order to remove nitrogen-containing buffer salts.

Results

Affinity Labeling Experiments. For the experiments, four lots of specifically purified anti-R_p antibody were used. Three of them (1–3) were obtained from single rabbits and one (4) was isolated from the pooled antisera from 14 other rabbits. The antibody content and average binding constant for hapten of each of these preparations, as measured by equilibrium dialysis, are given in Table I. All of the preparations were found to contain more than 90% anti-R_p antibody. Their hapten-binding constants covered a range from 0.06 (rabbit 2) to 20 × 10^6 l./mole (rabbit 1).

The specifically purified anti-R_p antibody preparations were affinity labeled by treating them with 1.0 mole of *p*-(arsonic acid)benzenediazonium fluoroborate/mole of protein (mol wt 160,000) at pH 8.5. After 30 min the reaction was stopped and the absorption spectra of the resultant azoproteins were determined. In addition, spectra were determined for anti-R_p antibody which had 0.01 M *p*-nitrobenzenearsonate present during the coupling reaction. The latter are referred to as protected samples and those reacted without hapten present, as unprotected samples. For comparison, the spectra of azo derivatives of rabbit normal γ-globulin obtained after reaction for 60 min were also determined. The results which follow are representative of those obtained in several experiments run at different times with the antibodies from these particular rabbits.

The spectra of the various azoprotein preparations are shown in Figure 1. As can be seen, the absorption spectra of the derivatives obtained by affinity labeling of the individual antibody preparations exhibit distinct differences. The spectrum of azoprotein from anti-R_p antibody 1 was closely similar to that of the corresponding monoazo derivative of *N*-chloroacetyltyrosine, showing a maximum at about 480 mμ and a minimum near 380 mμ (Tabachnick and Sobotka, 1959). The absorption spectrum of azo derivatives from anti-R_p antibody preparation 4 was similar to that of antibody preparation 1. The spectrum of azoprotein from anti-R_p antibody 2 was more complex. It showed a spectrum that could not be attributed solely to azotyrosine. It corresponded to the spectrum that would be expected for a mixture of azohistidine and azotyrosine, *i.e.*, a shift of the maximum toward lower wavelength and a much higher absorption at the minimum (380 mμ). The spectrum of azoprotein from anti-R_p antibody 3 was very different and was indistinguishable from that of the reaction product of normal γ-globulin, indicating that essentially no affinity labeling reaction had taken place. Both of these latter spectra indicated that only minute amounts of azotyrosine and azohistidine were formed during the time of the coupling reaction.

The presence of hapten during the coupling reaction altered the spectra of azo derivatives obtained from anti-R_p preparations 1 and 2 so that they were similar to the spectrum of azo derivative from normal γ-globulin. The presence of hapten during coupling did not alter appreciably the spectrum of the azo derivative obtained with anti-R_p preparation 3 in accord with the observation that there was no affinity labeling effect with this preparation.

On the basis of extinctions at 460 and 500 mμ, amounts of azotyrosine and azohistidine formed were calculated for each preparation using the method of Tabachnick and Sobotka (1960). This calculation does not need correction for the possible presence of azolysine derivatives, since their absorption is negligible at these wavelengths. The amounts of azo derivatives found are shown in Table II. The most significant observation was that essentially no specific azotyrosine or azohistidine formation was found for anti-R_p antibody from rabbit 3. In anti-R_p antibody preparations 1, 2, and 4, the labeling reagent reacted with the specific antibody to form larger amounts of azo derivatives compared with those formed with nonspecific γ-globulin. These larger amounts were not formed when hapten was

TABLE I: Hapten-Binding Activity of Anti-R_p Antibody Preparations.[a]

Lot of Anti-R_p Antibody	Purity[a] (%)	Binding Constant, K_0 (l./mole × 10^{-6})
Rabbit 1 (2453)	100	20
Rabbit 2 (3763)	96	0.06
Rabbit 3 (3527)	90	0.14
Pooled antisera 4	100	0.16

[a] Determined by equilibrium dialysis.

TABLE II: Affinity Labeling of Anti-R_p Antibodies.[a]

Lot of Anti-R_p	Azotyrosine Formed		Azohistidine Formed	
	Unprotected (moles/mole of Ab)	Protected (moles/mole of Ab)	Unprotected (moles/mole of Ab)	Protected (moles/mole of Ab)
Rabbit (1)	0.52	0.02	0.03	0.03
Rabbit (2)	0.17	0.03	0.09	0.01
Rabbit (3)	0.02	0.02	0.04	0.03
Pooled antisera (4)	0.15	0.04	0.07	0.05
Normal γ-globulin[b]	0.05	0.05	0.07	0.06
Rabbit (2) 2 moles of reagent/mole of protein[b]	0.31	0.13	0.31	0.18

[a] Diazonium reagent (1 mole) was used per mole of protein and values were obtained after 30-min reaction time, except as noted. [b] Values were obtained after 60-min reaction time.

present. The specific azo derivatives formed, defined as the difference between those observed in the unprotected and protected samples, varied not only quantitatively but also qualitatively, depending on the individual anti-R_p preparation. Anti-R_p antibody from rabbit 1 and from pooled sera preparation 4 showed specific azotyrosine formation, whereas specific azohistidine formation of 0.08 mole/protein in addition to 0.14 mole of azotyrosine was estimated with anti-R_p antibody from rabbit 2.

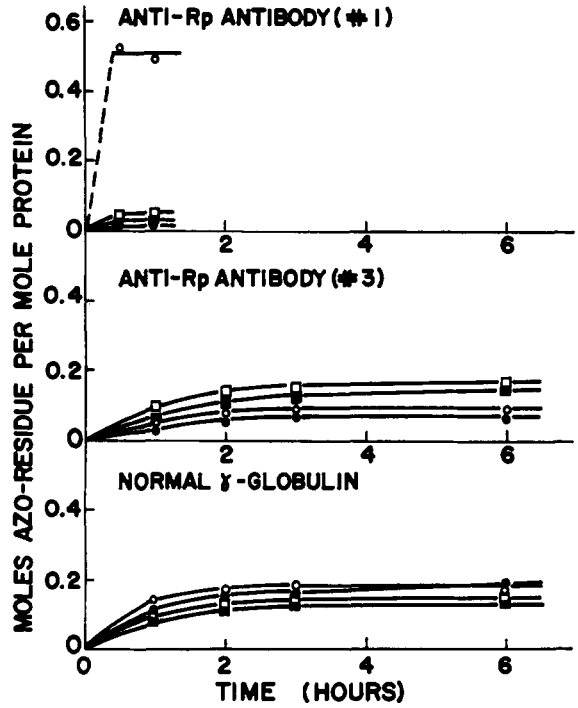

FIGURE 2: The extent of formation with time of azo derivatives of normal rabbit γ-globulin and of specifically purified anti-R_p antibody preparations. Affinity labeling reagent (*p*-(arsonate)benzenediazonium fluoroborate) (1 mole) per mole of protein was reacted at pH 8.5 in the absence of hapten (open symbols) or with 0.1 M *p*-nitrobenzenearsonate present (closed symbols); (○—○, ●—●) tyrosylmono-(*p*-azobenzenearsonate); (□—□, ■—■) histidylmono(*p*-azobenzenearsonate).

In another experiment testing the effect of a larger amount of labeling reagent, antibody from rabbit 2 was treated with 2 moles of reagent/mole of protein for 60 min instead of with 1 mole for 30 min (Table II, bottom line). Specific azohistidine formation of 0.13 mole per mole of protein and of 0.18 mole of azotyrosine was observed although the amounts of azo derivatives formed in the protected samples increased.

It is interesting that labeling of the protected antibody varies from labeling of the normal γ-globulin control. This is in accord with the fact that the antibody represents a certain restricted population of γ-globulins, while the normal control represents a different population and this reflected in a difference in reaction with the labeling reagent.

It is not valid to use the difference spectrum between the unprotected and protected samples to calculate the number of azo groups coupled to histidine and tyrosine by the affinity labeling reaction since more of the reagent is available for coupling elsewhere in the protected sample where affinity labeling is prevented.

In order to study further the variability in affinity labeling observed, the time course of reaction was determined (Figure 2) for two anti-R_p antibodies (1 and 3) and also for rabbit normal γ-globulin. While the affinity labeling reaction for antibody 1 was completed after as short a period as 30 min and showed predominantly azotyrosine formation, affinity labeling could not be observed with antibody 3 even after 6 hr. For the latter antibody, the rate curves indicated slow formation of azo derivatives. The same slow formation was also observed with the protected sample and even with normal γ-globulin. A doubling in the amount of diazonium salt added (to 2 moles/mole of protein, Figure 3) did not cause any significant increase in the amount of azotyrosine formed since it was almost the same as that observed with the 1 mole of reagent (Figure 2). However, more than twice as much azohistidine formation was observed with the 2 moles of reagent/mole of protein. Al-

TABLE III: Loss of Sites by Iodination of Anti-R_p Antibody (3) and Its Prevention by Hapten.[a]

	Binding Sites Remaining (%)	Binding Constant, K_0 (l./mole $\times 10^{-5}$)
Before treatment	100	1.4
After iodination		
In absence of hapten	33	0.4
In presence of hapten	91	0.4

[a] Both iodinated preparations contained 50 I atoms/mole of protein.

though differences in azotyrosine formation between the unprotected and protected samples of antibody preparation were almost negligible, azohistidine formation was slightly less in the protected sample at the early stage of the reaction (Figure 3). The presence of hapten did not affect azohistidine formation in normal γ-globulin. The results in Figures 2 and 3 thus reveal remarkable differences between the two anti-R_p antibody preparations 1 and 3 in their behavior with the coupling reagent.

Iodination of Anti-R_p Preparation 3. The absence of tyrosine available for affinity labeling in anti-R_p 3 was surprising in view of the fact that the presence of tyrosine residues in the active sites of many anti-R_p antibody preparations has been shown previously (Pressman and Sternberger, 1951; Koshland *et al.*, 1959; Pressman and Roholt, 1961; Grossberg *et al.*, 1962) by loss of active sites due to iodination and protection against this loss by hapten. Therefore, we examined the effect of iodination in the presence of hapten and in the absence of hapten on the anti-R_p antibody preparation 3. As can be seen from Table III, this anti-R_p antibody lost 67% of its original binding sites by iodination to the level of 50 atoms/mole of protein. This loss was essentially prevented by the presence of 0.1 M *p*-nitrobenzenearsonate during the iodination. Only 9% of the sites was lost. This result indicates that anti-R_p antibody 3 has iodinatable residues, presumably tyrosine, in its active sites and affinity labeling does not detect these tyrosine residues.

It would be fortuitous if the loss of activity on iodination were caused by attack of a key residue outside the antibody site, resulting in a conformational change affecting the site, while the presence of hapten in the site would act by reducing the reactivity of this key tyrosine, thus protecting the site (Grossberg *et al.*, 1962). The steric protection of a tyrosine in the site is a much more direct interpretation of this observation.

Discussion

Wofsy *et al.* applied the method of affinity labeling to three antihapten–antibody systems: anti-*p*-azobenzenearsonate antibody labeled with *p*-nitrobenzenediazonium fluoroborate (1962), anti-2,4-dinitrophenyl antibody labeled with *m*- and *p*-nitrobenzenediazonium fluoroborate (Metzger *et al.*, 1963; Lenard and Singer, 1966; Good *et al.*, 1967), and anti-*p*-azophenyltrimethylammonium antibody labeled with *p*-(trimethyl)benzenediazonium fluoroborate (Fenton and Singer, 1965). They reported that in each case affinity labeling took place and the label was attached to tyrosine residues. Wofsy *et al.* (1967a,b) reported that affinity labeling took place with antibodies specific for *p*-azophenyl β-lactoside and *p*-azophenyl β-galactoside but that for these anticarbohydrate antibodies some of the labeling was on residues other than tyrosine (or histidine). Differences in reactivity of the antibodies composing the population in a given system, particularly an absence of reactivity by some antibody, can be overlooked especially when pooled sera are used.

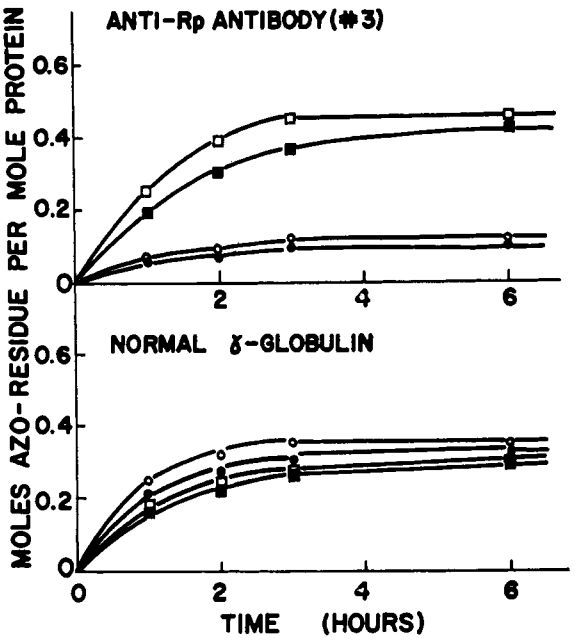

FIGURE 3: The extent of formation of azo derivatives with time in the reaction of specifically purified anti-R_p antibody preparation 3 and of normal rabbit γ-globulin with 2 moles of *p*-(arsonate)benzenediazonium fluoroborate/mole of protein, in the absence or in the presence of 0.1 M *p*-nitrobenzenearsonate. Symbols are as in Figure 2.

Our results show that the affinity labeling effect varies significantly and depends on the individual antibody preparation even with antibodies formed in response to the same antigen. Anti-R_p antibody from rabbit 1 and pooled antisera preparation 4 from 14 rabbits formed azotyrosine exclusively, in accord with the observations of Wofsy *et al.* (1962). However, anti-R_p antibody from rabbit 2 appeared to produce azohistidine besides azotyrosine.

Our results show that affinity labeling can distinguish between different antibody populations. While in many anti-R_p antibody populations azotyrosine is the principle product of affinity labeling, there seems to be an antibody population which has other reactive residues, perhaps histidine, in or near the active sites, which are capable of coupling with the reagent as exemplified by some of the antibodies of rabbit 2.

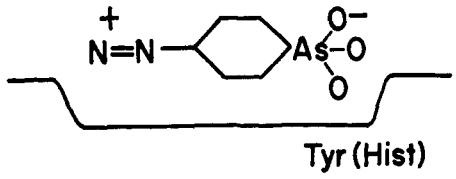

FIGURE 4: Diagrammatic illustration of the inability of an affinity labeling reagent to couple with a tyrosyl (or histidyl) residue in the site because the reactive group of the reagent is in an unfavorable orientation.

Moreover, there is a third population of anti-R_p antibodies which are not capable of being affinity labeled with the reagent used, as exemplified by anti-R_p antibody from rabbit 3. In the anti-R_p antibodies from this rabbit, neither tyrosine nor histidine was available for the affinity labeling reaction. The presence of all three types of antibodies may be the usual case with the histidine reactive one occurring in low amount. The completely unreactive one may often be present in a large amount but has remained undetected. It was fortuitous that we obtained a preparation which was almost exclusively of the unreactive type. Thus antibody 3 probably represents a relatively restricted population in that it contains none of the reactive types.

The three rabbits whose anti-R_p antibodies were affinity labeled were selected at random with no previous basis for expecting that their affinity labeling might differ. In subsequent examination of additional anti-R_p preparations from four other individual rabbits, we have observed affinity labeling spectra which implicate azotyrosine formation only, as exemplified by rabbit 1. Thus in a total of seven anti-R_p preparations from individual rabbits, only rabbit 3 was sufficiently devoid of antibodies reactive toward affinity labeling to allow demonstration of unreactive ones. The spectrum of affinity labeled anti-R_p 2 is also the only one of its kind among the seven individual preparations examined. Since the conditions of affinity labeling and examination of the spectra were identical for all preparations, the existence of such a difference in spectra certainly suggests differences in the residues being labeled. However, since the spectra may very well be sensitive to solvation effects and to the nature of neighboring amino acid residues in the different proteins, the identification and quantitation of the residue other than tyrosine which appears to be labeled in anti-R_p 2 cannot be considered established. This limitation may also apply to the absolute values given for the numbers of azotyrosine residues found in the different preparations examined. Such a limitation in quantitation does not detract from the demonstration of the marked absence of affinity labeling for anti-R_p 3.

There does not appear to be a strict relationship between the rates of affinity labeling and binding constant for hapten. The fact that anti-R_p antibody 1, which showed the highest rate of affinity labeling, had the highest binding constant (20×10^6 l./mole) seems to support the possibility which has been suggested by Metzger *et al.* (1963) and Good *et al.* (1967) that the antibody sites which are most rapidly labeled with a coupling reagent are those which bind the reagent most strongly. However, this cannot be the whole story since antibody 3 is not affinity labeled, although its binding constant (0.14×10^6 l./mole) is higher than that of anti-R_p antibody 2 (0.066×10^6 l./mole) which is affinity labeled.

It is interesting to note that the active sites of antibody 3 apparently contain tyrosine residues as revealed by protection with hapten against inactivation by iodination. These tyrosine residues are not reactive with the affinity labeling reagent. A plausable reason for this apparent difference is that the tyrosine residues in the active site of this antibody are not accessible to the affinity labeling. It may be that the reagent actually is protecting such a residue from reaction by sterically interferring with the attack by the reactive diazonium group, as diagrammed in Figure 4. This possibility is quite likely, since the affinity labeling reaction apparently depends on combination of the reagent to the active sites region in a fixed orientation which determines the locus of the residues with which its reactive group can couple.

Acknowledgments

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The Effects of Complete Modification of Amino Groups on the Antibody Activity of Antihapten Antibodies. Reversible Inactivation with Maleic Anhydride*

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ABSTRACT: The effect of reversible modification of free amino groups in the combining sites of antibodies directed against negatively charged haptens was studied. Two different pools of rabbit antisera directed against *p*-azobenzenearsonate (anti-R_p) were immunospecifically purified, and essentially 100% of their amino groups was selectively modified with maleic anhydride under conditions for which tyrosines are not modified. Both preparations lost a significant proportion, but not all, of their combining sites following complete maleylation of their amino groups. This loss of sites could be prevented by the presence of specific hapten during maleylation and could be reversed by hydrolytic removal of maleyl groups. A similar reversible loss of a proportion of the combining sites, following complete maleylation, was obtained with antibodies prepared against two other negatively charged haptens (*p*-azobenzoate and *p*-azobenzenephosphonate). The loss of antibody combining sites in anti-R_p antibodies and antibodies directed against *p*-azobenzenephosphonate was shown not to be due to any possible conformational changes caused by modification of amino groups outside of the antibody combining sites since loss of sites could be prevented completely by the presence of hapten during maleylation, and since the modification of all the amino groups in antibodies prepared against a neutral hapten (3-azopyridine) and against a positively charged hapten (*p*-azophenyltrimethylammonium) resulted in no change in either their number of antibody combining sites or their average binding constants. These results establish the presence of two kinds of chemically different combining sites in antibodies against negatively charged haptens, those containing an amino group and those that do not. In addition they establish the absence of amino groups in the combining sites of antibodies prepared against a positively charged and a neutral hapten.

Antibodies directed against several negatively charged haptens have a positive charge in their combining regions as demonstrated by the specific binding activities of various inorganic anions (Pressman *et al.*, 1961). The presence of free amino groups in the combining sites of some antibodies prepared against two negatively charged haptens (*p*-azobenzenearsonate and *p*-azobenzenephosphonate) was recently reported in studies utilizing the *N*-carboxyanhydride of DL-alanine (Freedman *et al.*, 1968). The failure to modify 100% of the amino groups in these antibody molecules with the alanine anhydride initiated the work reported in this communication.

Recently, Butler *et al.* (1967) reported on the reversible blocking of free amino groups in protein molecules using maleic anhydride. The authors suggested that the maleylation procedure was completely specific for amino groups. Maleic anhydride modifies essentially all of the amino groups present in protein molecules, and can be easily removed by acid hydrolysis.

This report describes the effect on antibody combining sites following chemical modification of all of the free amino groups in antibody molecules by maleic anhydride under mild conditions. The results demonstrate the direct involvement, in varying amounts, of amino groups in the combining sites of antibodies prepared against certain negatively charged haptens (*p*-azobenzenearsonate, *p*-azobenzenephosphonate, and *p*-azobenzoate), and the absence of amino groups in the combining sites of antibodies prepared against a positively charged (*p*-azophenyltrimethylammonium) and a neutral (3-azopyridine) hapten.

Materials and Methods

Buffers. The buffers used in this study included: Tris buffer (pH 8.0) (0.1 M Tris-HCl, 0.002 M EDTA), Tris-NaCl buffer (pH 8.0) (0.02 M Tris-HCl, 0.15 M NaCl, 0.002 M EDTA), and borate buffer (pH 9.0)

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