Effect of Cyanate on Several Anti-Hapten Antibodies: Evidence for the Presence of an Amino Group in the Site of Anti-*p*-Azobenzenearsonate Antibody*

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Treatment of anti-*p*-azobenzenearsonate antibodies (anti-Rp) with cyanate until 70–80% of free amino groups were carbamylated resulted in the loss of 20–30% of antibody sites. That this loss was due to an attack on a group in the site was shown by the fact that it could be partially prevented when hapten was present during the carbamylation reaction. Anti-*p*-azobenzoate (anti-Xp) and anti-*p*-azophenyltrimethylammonium (anti-Ap) antibody sites were not attacked by cyanate under identical conditions which resulted in attack on anti-Rp sites, as shown by experiments in which mixtures of anti-Xp and anti-Rp or anti-Ap and anti-Rp were carbamylated. Anti-Xp antibodies appear to lose activity due to an attack elsewhere than in the specific combining region; binding by anti-Ap antibody does not appear to be affected at all by carbamylation of the molecule.

There is an appreciable amount of evidence which indicates that antibodies directed against a charged group contain a charged group of opposite sign in the combining region. It has been shown definitely that antibody against the positively charged *p*-azophenyltrimethylammonium group (anti-Ap) has a carboxylate group in this region (Grossberg and Pressman, 1960). Antibodies against negatively charged groups, namely, antibody against the *p*-azobenzenearsonate group (anti-Rp) and the *p*-azobenzoate group (anti-Xp), do not contain a carboxylate group in the combining region (Grossberg and Pressman, 1960). It would appear that each has a positive charge in the combining region (Pressman, 1958); however, the amino acid residues contributing these positive charges have not been identified.

Acetylation of anti-Xp antibody reduced binding activity only after extensive acetylation, and the loss appeared largely due to acetylation of a tyrosine in the site rather than an amino group (Nisonoff and Pressman, 1959). If there is an amino group in the site it must be relatively unreactive. Anti-Rp seems somewhat more susceptible to loss of activity by acetylation, and this may implicate some contribution of an amino group (Grossberg and Pressman, 1961). Habeeb *et al.* (1959) reported that guanidination of up to 77% of amino groups in anti-Rp antibody did not reduce its specific precipitability and concluded that amino groups do not appear to be critically involved.

Cyanate, unlike acetic anhydride, reacts with amino groups and SH groups but has not been reported to react with tyrosine residues in protein. Therefore, we have studied the effects of this reagent on anti-Rp and anti-Xp antibodies in order to determine if cyanate attacks the combining site of these antibodies. We have also determined the effect of cyanate on anti-Ap antibody, which presumably does not have a positive charge in the site. We found, as reported here, that carbamylation of 70–80% of the amino groups in anti-Rp antibody leads to a 20–30% loss of binding site and this loss seems to be due to attack on an amino group in the combining region, since the loss of sites can be partially prevented by the presence of hapten during reaction. Neither anti-Xp nor anti-Ap antibody sites appear to be attacked by cyanate.

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MATERIALS AND METHODS

γ-Globulin Fractions of Antisera.—The γ-globulin fractions of pooled rabbit antisera prepared against the following haptens coupled to bovine γ-globulin were obtained by methods previously described (Grossberg *et al.*, 1962): *p*-azobenzoate (to give anti-Xp); *p*-azobenzenearsonate (to give anti-Rp); *p*-azophenyltrimethylammonium (to give anti-Ap). The γ-globulin fraction of normal rabbit sera was prepared similarly.

Reaction of γ-Globulins with Cyanate.—Protein (20–30 mg/ml) was incubated at 38° for various periods with 1 M potassium cyanate (reagent grade, Coleman and Bell Co., Norwood, Ohio), in *p*H 8 borate buffer either in the presence or absence of hapten, as noted. After incubation, reaction mixtures were dialyzed exhaustively against several changes of 1000 volumes of *p*H 8 borate buffered saline at 5° to remove cyanate and hapten.

Determination of the Binding of Hapten by Antibody.—The binding of hapten by the various

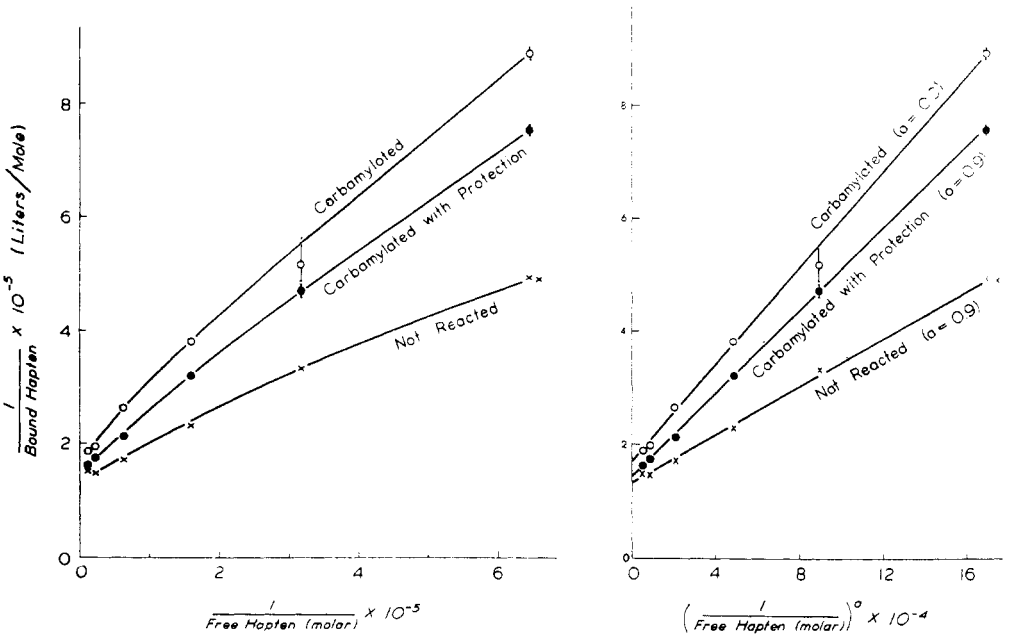

FIG. 1.—*Left*, binding of I^{131}-labeled *p*-iodobenzenearsonate: ×, to anti-Rp antibody; ○, to anti-Rp antibody treated with 1 M cyanate for 4.3 hours at *p*H 8 (77% NH_2 carbamylated); ●, to anti-Rp antibody treated with 1 M cyanate for 4.3 hours at *p*H 8 in the presence of 0.1 M *p*-nitrobenzenearsonate. All bindings were determined at a protein concentration of 12.0 mg/ml, corrected for binding to normal γ-globulin (2.7 ± 0.8% of free hapten concentration) and to carbamylated (80% NH_2 reacted) normal γ-globulin (0.0 ± 0.7% of free hapten concentration). *Right*, same data plotted with the use of a heterogeneity index, $\alpha = 0.9$.

treated and untreated proteins was determined by the method of equilibrium dialysis as previously described (Grossberg and Pressman, 1960). The preparation of I^{131}-labeled haptens for this purpose and the technique involved in assaying samples have been described (Nisonoff and Pressman, 1958; Grossberg and Pressman, 1960; Grossberg *et al.*, 1962). Binding of hapten to antibody preparations was corrected for nonspecific binding to normal (treated and untreated) γ-globulin.

Determination of Protein Amino Groups.—The extent of modification of amino groups in γ-globulin by cyanate was determined both by the method of Peters and Van Slyke (1932) and by the ninhydrin color method (Harding and MacLean, 1915). A standard curve was prepared relating ninhydrin color obtained to the per cent of amino groups remaining in samples as determined by gasometric amino nitrogen analysis. Protein concentration was determined by nitrogen analysis (digestion and Nesslerization), the value of 16.0% being used for the nitrogen content of γ-globulin.

RESULTS

Reaction of Anti-Rp Antibodies with Cyanate.—The γ-globulin fraction of anti-Rp serum was treated with 1 M cyanate at *p*H 8 and 38° in the presence or absence of hapten for 4.3 or 9 hours. The γ-globulin fraction of normal rabbit serum was also treated with 1 M cyanate. Under these conditions 76–84% of the amino groups were carbamylated (Table I). The binding of *p*-iodophenylarsonate by the preparations was measured, and the binding curves are shown in Figure 1. The concentrations of active sites and the average combining constants obtained from the curves by use of the Sips equation (Sips, 1948) are given in Table I.

Treatment with cyanate in the absence of hapten to such an extent that 77% of the amino groups were carbamylated (4.3 hours) resulted in a loss of only 22% of the binding sites. Longer exposure (9 hours) to the reagent (84% loss of amino groups) did not result in any greater loss of sites.

The apparent binding constant of the remaining antibody is lower (55% of the original), and becomes still lower (20% of the original) with longer exposure.

When treatment was in the presence of *p*-nitrobenzenearsonate the total loss of binding site (11%) was less than in the absence of hapten (22%). The binding constant of the remaining antibody is not significantly greater than observed in the absence of hapten. Since the hapten protects the antibody site against action of cyanate it appears that the site does contain a residue attacked by cyanate.

Reaction of Anti-Xp Antibodies with Cyanate.—The γ-globulin fraction of anti-Xp serum was treated with 1 M cyanate at *p*H 8 at 38° for 4.5 hours. This resulted in 75% loss of amino groups.

TABLE I

EFFECT OF CYANATE ON ANTI-RP ANTIBODIES IN THE PRESENCE AND ABSENCE OF HAPTEN

Time of Treatment (hr)	Anti-Rp Untreated	Anti-Rp Treated with 1 M KOCN		
		No Hapten Present		Hapten Present[a]
		4.3	9.0[b]	4.3
Free NH_2 (%)	100	23	16	24
Active site concentration[c] (M × 10^5)	0.78 ± 0.01	0.61 ± 0.02	0.61 ± 0.03	0.69 ± 0.01
Average combining constant[c] (× 10^{-5})	2.3 ± 0.1	1.2 ± 0.05	0.45 ± 0.02	1.4 ± 0.05

[a] 0.1 M *p*-nitrobenzenearsonate. [b] The binding curve for this sample is not reproduced in Figure 1. The same procedure was used for obtaining the extrapolated values from the data; the heterogeneity index, *a*, was 0.6. [c] Values were obtained from the extrapolated linear plots of binding data (Fig. 1b). The uncertainty indicated was estimated visually.

TABLE II

EFFECT OF CYANATE ON THE BINDING OF HAPTEN BY ANTI-XP ANTIBODIES[a]

	Free NH_2 (%)	Free Hapten (M × 10^5) 0.39	0.80	1.66	4.10	8.48	Active Site Concentration[c] (M × 10^5)	Average Combining Constant[c] (× 10^{-5})
		Concentration of *p*-Iodobenzoate Bound (M × 10^5)[b]						
Untreated anti-Xp	100	0.99	1.24	1.54	1.86	2.19	3.00 ± 0.05	0.68
Carbamylated anti-Xp	25	0.77	0.96	1.20	1.54	1.80	3.00 ± 0.05	0.31

[a] Binding determinations in duplicate at a protein concentration of 14.4 mg/ml; values are averages with average deviation = ±1.4%. [b] Values for hapten bound to antibody are corrected for hapten bound to normal γ-globulin, 5.0 ± 1.1% of free hapten concentration; with carbamylated (20% free NH_2) normal γ-globulin, 1.4 ± 0.5% of free hapten concentration. [c] These values were obtained by extrapolation of binding curves to infinite free hapten concentration, the Sips equation with heterogeneity constant $a = 0.5$ being used in each case.

TABLE III

EFFECT OF CYANATE ON ANTI-XP ANTIBODIES IN THE PRESENCE AND ABSENCE OF HAPTEN[a]

Treatment	Hapten Present During Treatment	Free NH_2 (%)	Free Hapten (M × 10^5) 0.66	3.91	7.38	Active Site Concentration[c] (M × 10^5)	Average Combining Constant[c] (× 10^{-5})
			Concentration of *p*-Iodobenzoate Bound (M × 10^5)[b]				
None	None	100	0.43	0.78 ± 0.01	0.89 ± 0.03	1.00 ± 0.04	1.1 ± 0.1
1 M KOCN	None	23	0.33	0.59 ± 0.02	0.63 ± 0.02	0.73 ± 0.04	1.2 ± 0.1
1 M KOCN	0.1 M *p*-nitrobenzoate	22	0.33	0.61	0.69	0.80 ± 0.05	1.0 ± 0.1

[a] Binding determinations in duplicate at a protein concentration of 18 mg/ml; values are averages, with average deviation of less than 1%, except where noted. [b] See footnote *b*, Table II. [c] These values were obtained by extrapolation of the binding curves, using the Sips equation and a heterogeneity index, $a = 0.9$. The estimated error of the extrapolation is relatively large owing to the limited number of points.

The results of measurement of *p*-iodobenzoate bound by the carbamylated and the original unreacted anti-Xp antibodies are summarized in Table II. Cyanate did not reduce the total number of active sites of anti-Xp antibody, in spite of the large reduction of amino groups. However, the average combining constant decreased to one half of the original—from 6.8 × 10^4 for the nontreated anti-Xp to 3.1 × 10^4 for treated anti-Xp. The reaction of a different pool of anti-Xp with cyanate was also carried out both in the presence and absence of hapten. The data in Table III show that there is about 25% loss of sites after carbamylation of this preparation, but that there appears to be no significant protection by hapten, indicating that any loss of antibody activity does not appear to be due to attack in the antibody combining region. The combining constants (Table III, footnote *c*) are essentially the same for all preparations.

Reaction of a Mixture of Anti-Xp and Anti-Rp Antibodies with Cyanate.—In order to compare

TABLE IV

EFFECT OF CYANATE ON A MIXTURE OF ANTI-RP AND ANTI-XP ANTIBODIES IN THE PRESENCE AND ABSENCE OF HAPTENS

Treatment	Free NH_2 (%)	Hapten Present During Treatment	Hapten Concentration Bound[a]: I^{131}-Labeled *p*-Iodobenzenearsonate[b] (M × 10^5)	Hapten Concentration Bound[a]: I^{131}-Labeled *p*-Iodobenzoate[c] (M × 10^5)
Untreated	100	None	0.96 ± 0.01	0.45 ± 0.033
Incubated only	100	None	0 93 ± 0.01	0.40 ± 0.007
Incubated with 1 M KOCN	15	None	0.48 ± 0.003	0.31 ± 0.01
Incubated with 1 M KOCN	15	0.1 M	0.64 ± 0.001	0.32 ± 0.002
Incubated with 1 M KOCN	15	0.1 M	0.47 ± 0.011	0.31 ± 0.005

[a] Values are average of duplicates, with deviation from average, determined at 26 mg/ml protein concentration and corrected for binding by normal γ-globulin and treated normal γ-globulin (15% free NH_2); see footnote *b*, Table II. [b] Free hapten concentration; $0.63_0 \pm 0.002 \times 10^{-5}$ M. [c] Free hapten concentration; $0.68_5 \pm 0.001 \times 10^{-5}$ M.

TABLE V

EFFECT OF CYANATE ON A MIXTURE OF ANTI-RP AND ANTI-AP ANTIBODIES

	Anti-Rp		Anti-Ap	
	Untreated	Carbamylated[a]	Untreated	Carbamylated[a]
Active site concentration[b] (M × 10^5)	1.35 ± 0.05	1.00 ± 0.05	1.23 ± 0.02	1.25 ± 0.02
Average combining constant[b] (× 10^{-5})	1.15 ± 0.05	0.62 ± 0.02	14 ± 0.5	14 ± 0.5

[a] Free NH_2 = 25% of that of the untreated mixture, after 20 hours' incubation. [b] These values were obtained by extrapolation of the binding curves (Fig. 2), converted to linear plots through use of a heterogeneity index, a = 0.7 in each case, as in Figure 1. The uncertainty indicated was estimated visually.

more precisely the effects of cyanate on anti-Rp and anti-Xp antibodies, a mixture of the globulin fractions of anti-Rp and anti-Xp sera was incubated with 1 M cyanate at *p*H 8 at 38° for 20 hours. Similar reaction mixtures were incubated in the presence of *p*-nitrobenzoate (0.1 M) or benzene phosphonate (0.1 M). The extent of binding of *p*-iodobenzoate and of *p*-iodobenzenearsonate to all these samples was determined at a single concentration of each hapten. The results are collected in Table IV. It may be seen that there is loss of binding activity due to cyanate treatment, both for anti-Rp and anti-Xp, but hapten protects against loss of activity only in the case of anti-Rp antibody. The protection is due to specific binding of hapten to the anti-Rp site, since benzene phosphonate, which is known to bind to this site (Erlenmyer and Berger, 1932; Pressman *et al.*, 1945), does not protect the anti-Xp site, and *p*-nitrobenzoate does not protect the anti-Rp site (Table IV).

Reaction of a Mixture of Anti-Rp and Anti-Ap Antibodies with Cyanate.—A direct comparison of the effect of cyanate on anti-Ap antibody with the effect on anti-Rp antibody was made by treating a mixture of the γ-globulin fractions of anti-Rp and anti-Ap sera with 1 M cyanate at *p*H 8 and 38° for 20 hours. The binding curves are shown in Figure 2 and the results are given in Table V. It can be seen that cyanate had no effect at all on the binding of *p*-iodophenyltrimethylammonium ion by anti-Ap antibodies There was no decrease in sites and no alteration of binding constant. However, under the identical conditions prevailing in this experiment cyanate attacks the anti-Rp antibodies with a consequent loss of sites and a decrease in the average combining constant of the remaining anti-Rp antibodies.

Amino Acid Residues Modified by Cyanate.—In order to determine if reactive groups in γ-globulin other than α-amino and ε-amino could have reacted with cyanate, attempts were made to detect modification of the hydroxyl group of tyrosine and the guanidinium group of arginine.

No detectable change occurred in the absorption at 280 mμ of γ-globulin treated with cyanate even when 80–85% of amino groups were carbamylated. This indicates that there was no major alteration of the tyrosines. Lack of reactivity of tyrosine hydroxyl under these conditions was shown by the observation that there was no change in absorption when free tyrosine was treated at *p*H 8 with 1 M cyanate for 17 hours at 38° (all amino carbamylated).

The effect of cyanate on the guanidinium group was determined by reacting free arginine (5×10^{-3} M) with 1 M cyanate at *p*H 8 for 4.5 hours

at 38°. The reaction mixture was subjected to two-dimensional separation on paper, with high-voltage electrophoresis in 0.9 M formic acid in one direction, and chromatography with a butanol–acetic acid–water system (40:10:50) in the other direction. Chromatograms were developed with Sakaguchi reagent and pentacyanoaquoferriate reagent (Smith, 1958). Complete carbamylation of α-amino occurred, but there was no indication of reaction at the guanidinium group.

The presence of a sulfhydryl group was investigated by treating anti-Rp (and also anti-Xp and anti-Ap) with iodoacetamide under conditions known to alkylate one SH group (the other appears very unreactive to this reagent). This does not result in appreciable loss of binding activity and indicates that if any SH is involved in the binding sites of any of these antibodies and is responsible for the inactivation of anti-Rp by cyanate, it must be unreactive toward iodoacetamide.

Discussion

The loss of anti-Rp activity owing to chemical alteration of this antibody by cyanate seems to be due to an attack in the combining region of the antibody, since the loss (though small) can be partially prevented by hapten (Tables I and IV). These observations have been controlled by observing the effect of cyanate on mixtures of anti-Rp antibody with either anti-Xp or anti-Ap antibodies. Most significantly, in each case the cyanate treatment of the mixtures in which both antibodies in the pairs studied were exposed to identical conditions of reaction resulted in loss of anti-Rp sites. There was no loss of anti-Ap sites in the mixture with anti-Rp. There was apparently some effect on the binding activity of anti-Xp, as well as anti-Rp, due to attack elsewhere than in the combining region. This effect can be distinguished from that due to attack in the anti-Rp site by the fact that hapten protects against the loss of anti-Rp activity whereas hapten does not protect against loss of anti-Xp activity.

The group in the anti-Rp site which is attacked by cyanate seems most likely to be the ϵ-amino group of lysine or α-amino group, on the basis of the observations that amino groups are carbamylated in γ-globulin whereas neither the guanidinium group of free arginine nor the hydroxyl group of tyrosine seem to be attacked by the reagent. Although the presence of a sulfhydryl has not been ruled out, it would appear that if one were present it would have to be one unreactive toward iodoacetamide.

The results of Habeeb *et al.* (1959) concerning the absence of any effect of guanidination on anti-Rp antibody (which they report as casting doubt on the participitation of an amino group in the site) were based on the precipitation reaction, which is quite sensitive to chemical alteration of the antibody elsewhere than in the site. For example, acetylation is known to greatly decrease precipitability of antibody without affecting the site (Marrack and Orlans, 1954; Nisonoff and Pressman, 1959). On the other hand, Pressman and Radzimski (1962) have shown that iodination of antibody elsewhere than in the combining region caused increased precipitability of antibody.

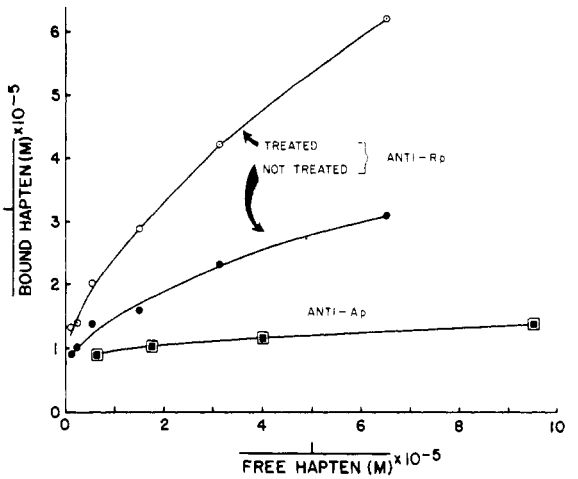

FIG. 2.—Binding of haptens to untreated and to carbamylated (75% NH_2 reacted) mixtures of anti-Rp and anti-Ap antibodies at a total protein concentration of 26 mg/ml. Anti-Rp binding was determined with I^{131}-labeled *p*-iodobenzenearsonate (PIR), anti-Ap binding was determined with I^{131}-labeled *p*-iodophenyltrimethylammonium (PIQ) on separate portions of the same mixtures. All determinations were corrected for binding to normal γ-globulin and carbamylated normal γ-globulin. (See legend of Figure 1 for these values for PIR; for PIQ the values are 9.0 ± 1.0% and 21 ± 2% of free hapten concentration respectively.) ●, PIR binding to untreated mixtures; ○, PIR binding to carbamylated mixture; ■, PIQ binding to untreated mixture; □, PIQ binding to carbamylated mixture.

The carbamylation of anti-Rp antibodies resulted in only 30% loss of sites after 20 hours of incubation with 75% loss of amino groups (Table V). There is some indication from the data (Tables I and V) that the loss was progressive during this period whereas the bulk of amino groups were lost early in the alteration. Since more than 15% of amino groups were unreacted after even the most extensive alteration observed, it is probable that they represent groups buried in the molecule or unavailable to the reagent for other reasons. It is possible that the group in the anti-Rp site attacked by cyanate is one of these relatively unreactive amino groups. It is also possible that more than one kind of anti-Rp molecule was present in the preparations studied, and that only a portion of the anti-Rp sites present contained a group able to react with cyanate.

Analysis of the alteration in average binding constants of antibody remaining after cyanate alteration confirms the idea that carbamylation

of residues elsewhere than in the combining region of anti-Rp and anti-Xp molecules results in molecules which bind hapten less firmly than unaltered antibody. This may be due to conformational changes in the molecule due to the large change in net charge of the molecule, and in part to nonspecific electro-repulsions of the anionic haptens by the more negatively charged antibody molecules, as previously discussed (Grossberg *et al.*, 1962). It is noteworthy in this respect that the binding constant of anti-Ap antibody is not altered at all by carbamylation. This may be due either to the fact that any conformational changes which may have been produced in this antibody do not affect this particular site or that a change is not detected because it is just balanced by an increased strength of binding due to the nonspecific electrostatic attraction of the cationic hapten to the more negative antibody. (For the magnitude of the nonspecific binding of this hapten to carbamylated γ-globulin, see the legend to Figure 2.)

An alternative interpretation of the observations concerning cyanate effects on anti-Xp is that loss of activity is due in part to an attack on a group in or near the combining region, but that 0.1 M *p*-nitrobenzoate even though combined with the site is not able to protect against the loss. It has been noted previously that *p*-nitrobenzoate is less effective in protecting the anti-Xp site against alteration by iodination than is *p*-nitrobenzenearsonate in protecting the anti-Rp site against iodination effects, and incomplete blocking of tyrosine toward iodination has been implicated. It may be that there is an amino group at one side of the site which can still be carbamylated by attacks on the side away from the hapten.

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