determined in the Tri-Carb Scintillation Counter. There was no quenching by the protein under these conditions.

Carbamylmethylation.—Thirty mg of enolase was dissolved in 15 ml of 0.1 M iodoacetamide in 0.05 M potassium phosphate buffer at *p*H 7. The reaction was allowed to proceed for 2 hours at 30°, and the sample, which was partially insoluble when it was prepared in the absence of substrate, was dissolved by addition of urea. It was then passed through a Sephadex column as described above, except that 8 M urea was used for the elution. The urea was removed by dialysis, and the sample was lyophilized and hydrolyzed prior to analysis.

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Effect of Iodination on the Active Site of Several Antihapten Antibodies*

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The combining sites of four antihapten antibodies (all those studied) are affected by iodination. The order for increasing sensitivity to iodination is anti-*p*-azophenyltrimethyl ammonium $\leqslant$ anti-*p*-azobenzoate $<$ anti-3-azopyridine $<$ anti-*p*-azobenzenearsonate. That the attack is in the site is shown by the ability of hapten when present during iodination to protect the antibody against loss of activity. Decreased binding activity may be due to loss of sites or a decrease in binding constant or both. In the cases studied there was a loss of binding sites for each antibody, and this loss could be partially prevented by hapten. The stronger binding sites are preferentially lost during iodination of the antibodies, with the exception of anti-3-azopyridine, which loses weaker sites more easily. These results indicate the presence of an iodinatable group, most likely tyrosine, in the site of each antibody, but the degree of its involvement in the site is different for each.

The presence of a particular amino acid residue in the combining site of an antibody is indicated if treatment of antibody protein with reagents which react with that amino acid residue results in loss of antibody activity. Whether the amino acid is actually present in the combining site or whether the loss of activity is due to chemical modification elsewhere in the protein molecule can be tested with antihapten antibodies by performing the modification in the presence of hapten. If protection by hapten is observed, then loss of activity in the absence of hapten must be due to an attack

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on an amino acid in the site. By such means, the presence of residues reacting with iodine has been demonstrated in the combining sites of anti-*p*-azobenzoate (anti-X_p) (Pressman and Sternberger, 1951) and anti-*p*-azobenzenearsonate (anti-R_p) antibodies (Pressman and Sternberger, 1951; Koshland *et al.*, 1959). By similar protective action, Katsura (1955) has shown that the combining sites of antidiphtheria toxin antibodies contain a residue which reacts with iodine.

In the present study, we have extended the observations to effects of iodination on the antibody to a positively charged haptenic group, *p*-azophenyltrimethylammonium (anti-A_p antibody) (see Koshland *et al.*, 1961) and on the antibody to an uncharged haptenic group, 3-azopyridine (anti-P_3 antibody). We have compared these effects to those of iodination of anti-R_p and anti-X_p antibodies. Data were obtained for all four antibody systems so that it was possible to determine the fraction of the sites remaining and the changes in combining constants. From these data deductions were made regarding the relative sensitivity of the different sites toward iodination and the relative contributions of an iodinatable group to the binding activity of the various antibodies.

Experimental

Materials.—Methods of preparing and testing each of the antisera studied have been described (Pressman and Sternberger, 1951; Nisonoff and Pressman, 1957; Nisonoff and Pressman, 1958; Grossberg and Pressman, 1960). The immunizing antigens for obtaining anti-R_p and anti-P_3 antisera were prepared by coupling diazotized *p*-aminobenzenearsonate or diazotized 3-aminopyridine, respectively, to bovine γ-globulin rather than to whole beef serum as originally described. The antisera were pools of several bleedings from 5 to 12 hyperimmunized rabbits, some of which had received an initial course of injections as much as 12 months previous to the period of bleeding, with weekly injection and bleeding in the interim. A γ-globulin fraction was prepared from each pooled antiserum by precipitation with sodium sulfate (Kekwick, 1940). The γ-globulin fraction of normal rabbit serum was similarly prepared. These fractions were 95 to 98% γ-globulin as determined by free boundary electrophoresis. The contaminant was a slowly migrating β-globulin.

In a few experiments, specifically purified anti-X_p antibody was employed. This was prepared, in the manner described by Nisonoff and Pressman (1959), by dissolving a specific precipitate of the antibody and X_p-ovalbumin antigen in 0.1 M *p*-nitrobenzoate, precipitating the antigen at *p*H 5, and dialyzing the resultant antibody solution free of hapten.

The I^{131}-labeled haptens, *p*-iodophenyltrimethylammonium (Grossberg and Pressman, 1960) and -*p*iodobenzoate (Nisonoff and Pressman, 1958) were prepared by isotope exchange as previously reported. I^{131}-labeled *p*-iodobenzenearsonate was prepared from the unlabeled compound under exactly the same conditions as those employed for *p*-iodobenzoate. Radiochemical purity was 95–99%, as determined by the isotope dilution technique. Hapten concentration was determined from the optical density at 238 mμ (E^{mM}_{1cm} = 16.4).

3-Iodopyridine was prepared from 3-aminopyridine by diazotizing 10 mmoles amine in 20 ml 18 N H_2SO_4 and adding 11 mmoles KI at 0°. The precipitated iodide salt was converted to the nitrate by addition of excess $AgNO_3$, with AgI and excess silver removed as chloride. It was crystallized at below 0° from 5 ml 1 M NaCl solution, in 25% yield. A portion of this material was converted to the free base and extracted into benzene-ether (1:2) and then into aqueous HCl. The iodide salt contained the theoretical amount of iodide as determined by thiocyanate titration after addition of excess standard silver nitrate solution.

I^{131}-labeled 3-iodopyridinium nitrate was prepared by exchange with 4 mc of carrier-free I^{131} iodide at *p*H 5 by heating 3 mg in a sealed tube at 160° for 20 hours in a total volume of 0.5 to 1.0 ml. A few drops of saturated KI were then added and followed by excess $AgNO_3$. Repeated precipitations of AgI from the solution of the labeled compound were made by alternate addition of small amounts of $AgNO_3$ and KI until a radiochemical purity of 90 to 97% was achieved.

Hapten concentration of original solutions was determined from the optical density at 272 mμ (E^{mM}_{1cm} = 2.44).

Iodination of γ-Globulin.—Carrier-free I^{131} iodide was mixed with dilute (4×10^{-4} M) ICl, in 2 N HCl. A slight excess of KI was added and the resultant labeled iodine was extracted into CCl_4 and then into 0.2 M glycine buffer of *p*H 9. The amount of radioactivity added in an iodinating solution was sufficiently high (500–1000 cpm/10 mg protein) to allow for accurate determination of iodine incorporated into protein, but sufficiently low to be corrected for with little resultant error when the binding of I^{131}-labeled haptens to iodinated antibody was measured.

Proteins were iodinated with I^{131}-labeled ICl or KI_3. In the case of ICl, the procedure used was essentially that described by McFarlane (1958). Protein (50 mg/ml) was adjusted to *p*H 9 with the 0.2 M glycine buffer at 0° and 0.02 M IO^- (from ICl) in this buffer was added in 0.10-ml increments with rapid stirring. The reaction proceeded smoothly and rapidly, as judged by the almost instantaneous disappearance of color. After the reaction, the protein was dialyzed exhaustively against *p*H 8 buffered saline containing a small amount of KI, to remove unreacted radiolabel. In experiments in which comparison was made between effects of iodination in the presence and absence of hapten, hapten was usually added to the latter samples after iodination and then

all samples were dialyzed for 60–72 hours against three to five changes of at least 2000 volumes of *p*H 8 buffered saline to remove hapten and unreacted radiolabel. The efficiency of the iodination was 75–80% of the theoretical incorporation at levels of incorporation between 20 and 40 iodine atoms per protein molecule.

Iodination with KI_3 was carried out in similar fashion, with an efficiency of about 60% at comparable levels. Borate buffer at *p*H 9 was substituted for the glycine buffer. Iodination could not be carried out with KI_3 in the presence of 0.1 M *p*-iodophenyltrimethylammonium ion since hapten-I_3^- complex precipitated and very little iodine reacted with protein. However, hypoiodite could be used, since with that reagent no precipitation of hapten occurred and the iodination of protein proceeded to the same extent as in the absence of the hapten.

Binding of Hapten.—The binding of I^{131}-labeled haptens by the various unreacted and modified antibodies, and by unreacted and modified normal γ-globulin, was measured by the method of equilibrium dialysis, as previously described (Grossberg and Pressman, 1960). In the case of binding by a mixture of two antibodies, separate portions of the mixture were equilibrated against each hapten involved. Samples to be compared at a given hapten concentration were dialyzed against a common hapten solution so that the free hapten concentration at equilibrium was identical for all.

Analytical Methods.—Protein concentration was determined by digestion and Nesslerization, with 16.0% taken as the nitrogen content of γ-globulin. Some unmodified proteins were determined by measurement of optical density at 280 mμ, ($E^{1\%}_{1cm} = 14.6$).

Results

Effect of the Presence of Hapten on the Iodination of Antibodies.—Globulin fractions of anti-R_p, anti-A_p, and anti-P_3 sera were iodinated to the extent of approximately 40 atoms per molecule (m.w. = 160,000). Samples of these globulin preparations were iodinated to approximately the same extent in the presence of 0.1 M hapten. All samples were extensively dialyzed to remove hapten and uncoupled I^{131}. The binding of hapten to these materials and to portions of the unmodified globulins was measured, and the results are given in Table I. It is clear that for each antibody the ability to bind hapten was reduced by iodination. Further, the binding activity of each antibody was protected by the presence of its specific hapten during iodination. The binding activity of antibody iodinated in the presence of hapten was greater than that of antibody iodinated in the absence of hapten. Thus when iodinated to the extent of 36 iodines per γ-globulin molecule, anti-R_p antibody retained only 9% of the original binding activity of the unmodified antibody, while 26% of the activity was retained when iodination was carried out in the presence of 0.1 M hapten. The corresponding values for anti-P_3 antibody at 45 iodines per molecule were 12% and 32%, and for anti-A_p antibody at 40 iodines per molecule 51% and 83%.

Iodination of Mixtures of Anti-R_p and Anti-A_p Antibody.—The data in Table I show that a marked difference existed in the extent to which anti-R_p and anti-A_p antibodies were affected by a similar level of iodination. It was necessary to determine if these differences were real or merely reflected some differences in the conditions under which the separate iodinations were carried out,

TABLE I

EFFECT OF IODINATION IN THE PRESENCE AND ABSENCE OF HAPTEN ON ANTIHAPTEN ANTIBODIES

Antibody	Iodinating Method	Iodine Incorporated: Without Hapten (Atoms/Molecule)	Iodine Incorporated: With Hapten[a] (Atoms/Molecule)	Protein Concentration (mg/ml)	Free Hapten (M × 10^6)	Hapten Bound by: Unmodified Antibody	Hapten Bound by: Modified Antibody	Hapten Bound by: Antibody Modified in Presence of Hapten	Hapten Bound by: Normal γ-Globulin
						(M × 10^6)[b]			
Anti-R_p	ICl	36[c]	37	8.0	4.13[e]	3.38 ± 0.01 **100%**	0.30 ± 0.02 **9%**	0.89 ± 0.04 **26%**	0.055
Anti-A_p	ICl	40[d]	39	9.0	3.76[f]	7.31 ± 0.06 **100%**	3.68 ± 0.04 **51%**	6.03 ± 0.08 **83%**	0.17
Anti-P_3	KI_3	45	46	14.0	6.60[g]	8.47 ± 0.02 **100%**	0.96 ± 0.06 **12%**	2.71 ± 0.03 **32%**	0.38

[a] Modification of anti-R_p was in the presence of 0.1 M *p*-nitrobenzenearsonate; anti-A_p, in 0.1 M *p*-iodophenyltrimethylammonium; anti-P_3, in 0.1 M pyridine. [b] Average of duplicate determinations with the deviation from mean as indicated; values for antibody are corrected for binding by normal γ-globulin; figures in bold type are percents of the binding shown by unmodified antibody. [c] 0.1 M *p*-nitrobenzenearsonate added after iodination, before dialysis. [d] 0.1 M *p*-iodophenyltrimethylammonium added after iodination, before dialysis. [e] I^{131}-labeled *p*-iodobenzenearsonate. [f] I^{131}-labeled *p*-iodophenyltrimethylammonium. [g] I^{131}-labeled *m*-iodopyridine.

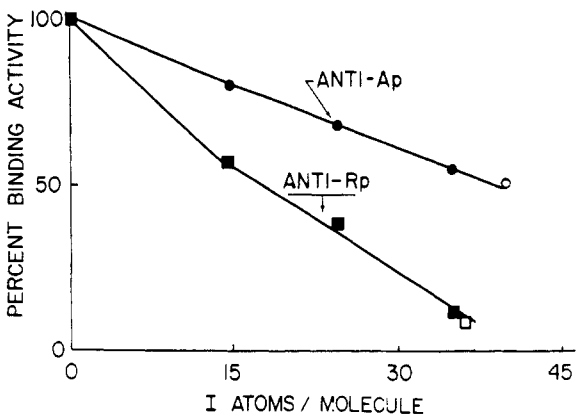

FIG. 1.—Effect of iodination on binding activity of anti-R_p and anti-A_p antibodies. A mixture of 111 mg anti-R_p γ-globulin and 105 mg anti-A_p γ-globulin was divided into 54-mg portions. Three portions (27 mg/ml) were iodinated with KI_3 to the levels indicated. Binding activity of duplicate samples (20 mg protein per ml) was determined with 7.92×10^{-6} M (free hapten) *p*-iodophenylarsonate and with 7.78×10^{-6} M (free hapten) *p*-iodophenyltrimethylammonium. Binding activity of the uniodinated mixture (100% activity) was 6.38×10^{-6} M *p*-iodophenylarsonate bound (anti-R_p) and 6.70×10^{-6} M *p*-iodophenyltrimethylammonium bound (anti-A_p). Open symbols are the determination of percentage binding activity of the preparations of anti-R_p and anti-A_p listed in Table I.

unrelated to the level of iodination. To this end anti-R_p and anti-A_p antibodies were mixed and portions of this mixture were iodinated to different levels. Under these circumstances, the two antibodies were exposed to identical reaction conditions.

The binding of haptens by the mixture of iodinated anti-R_p and anti-A_p antibodies is plotted in Figure 1, and is expressed as the percentage of binding activity present before iodination.

It is clear that anti-R_p antibody is much more sensitive to iodination than is anti-A_p antibody, since at all levels of iodination binding of hapten was decreased more for the former.

Iodination of Anti-X_p Antibodies to Different Levels in the Presence and Absence of Hapten.—In order to examine iodination of antibody in the presence and absence of hapten at varying levels of loss of activity, two preparations of specifically purified anti-X_p antibody were iodinated to increasing extents with and without hapten present. The ability of these preparations to bind hapten at a single free hapten concentration is compared in Table II. With increasing incorporation of iodine into anti-X_p molecules, hapten binding activity is progressively lost. The presence of hapten protects against this loss, the protection being more apparent at the higher levels of iodination. Thus with one antibody preparation (experiment A, Table II) binding activity decreased progressively from 75% to 30% of the activity originally present, as iodine incorporated in the absence of hapten increased from 20 to 38 iodine atoms per molecule. When the same preparation was iodinated in the presence of 0.34 M *p*-nitrobenzoate up to 56 iodine atoms per molecule were incorporated but binding activity decreased only

TABLE II
IODINATION OF SPECIFICALLY PURIFIED ANTI-X_p ANTIBODY IN THE PRESENCE AND ABSENCE OF HAPTEN

ICl Used per Mole Protein (Moles)	Iodine Incorporated (Atoms/ Molecule)		*p*-Iodobenzoate Bound (M × 10^6)	Binding Activity (%)
		Experiment A[a]		
0	0	No Hapten	2.20 ± 0.06	100
10.1	8.5	Hapten	1.99 ± 0.06	90.5
10.1	8.9	No Hapten	1.98 ± 0.01	90
25.3	19.2	Hapten	1.77 ± 0.01	78
25.3	20.0	No Hapten	1.66 ± 0.01	75
50.5	33.8	Hapten	1.48 ± 0.03	67
50.5	30.9	No Hapten	1.13 ± 0.01	51
101.0	56.2	Hapten	1.09 ± 0.03	50
101.0	38.3	No Hapten	0.65 ± 0.03	30
		Experiment B[b]		
0	0	No Hapten	4.77 ± 0.09	100
94[c]	65.5	Hapten	3.01 ± 0.02[d]	64
94[c]	65.0	No Hapten	1.13 ± 0.04[d]	24
285[c]	70.7	Hapten	0.91 ± 0.03	19
285[c]	66.9	No Hapten	0.28 ± 0.03	6

[a] Anti-X_p sample A; *p*-nitrobenzoate, when present, at 0.4 M; binding measurements at 0.50 mg/ml protein concentration in triplicate, with average deviation indicated; free hapten concentration, 2.72×10^{-6} M. [b] Anti-X_p sample B; *p*-nitrobenzoate, when present, at 0.34 M; binding at 0.84 mg/ml in triplicate except where noted, with average deviation indicated; free hapten concentration, 3.21×10^{-6} M. [c] Excess iodine was reduced with thiosulfate prior to dialysis. [d] Measurements in duplicate.

to 50%. The magnitude of the experimental error did not permit observation of the protective effect when only 10% of the binding activity was lost.

With the second anti-X_p preparation (experiment B, Table II), 0.34 M hapten prevented about half of the 75% loss of activity caused by incorporation of 65 atoms of iodine. When samples of this preparation were exposed to three times the amount of ICl, little additional incorporation was achieved although considerably more activity was lost. Hapten was much less effective in preventing this additional loss.

Complete Binding Curves.—Complete binding curves were obtained for various preparations in order to determine the effect of iodination. From such data it is possible to determine what part of the effect is due to loss of binding sites by destruction or complete blockage and what part is due to alteration of the binding constant, K_0. Globulin fractions of anti-R_p, -P_3, -A_p, and -X_p sera were iodinated in the absence of hapten. The level of iodination was different for each antibody preparation, since their apparent extent of loss of binding activity with degree of iodination is in the order anti-R_p > -P_3 >-X_p ≥ -A_p. Iodination levels were chosen from previous experience to give a similar loss of binding activity for all the preparations. Thus the anti-R_p, -P_3, -X_p, and -A_p preparations were iodinated to the extents of 20, 33, 33, and 50 iodine atoms per molecule, respectively, as shown in Table III, at which levels they retained comparable amounts of binding activity. Other portions of the same preparations were iodinated to these levels in the presence of hapten in order to determine what effect hapten has on the alteration of binding activity. In addition, portions of normal rabbit γ-globulin were iodinated to the various extents to serve—along with uniodinated normal γ-globulin—as controls for nonspecific binding.

The binding data, corrected for nonspecific binding, were plotted as the reciprocal of bound hapten concentration against the reciprocal of free hapten concentration (Fig. 2, 3, 4, and 5). Extrapolation of such curves to infinite free hapten concentration, *i.e.*, the ordinate intercept, provides a measure of the total concentration of binding sites. From the slope and the concentration of sites the binding constant, K_0, can be calculated. If such a plot is linear, the data can be interpreted as representing binding by a population of sites with identical binding constants. Curvature of the plot indicates heterogeneity of the binding constants for the population of sites. As has been previously discussed (Nisonoff and Pressman, 1958), the heterogeneity can be analyzed by use of equation (1), derived from the Sips equation (Sips, 1948),

$$1/b = (1/A_0) + (1/K^a{}_0 A_0)(1/c^a) \qquad (1)$$

in which b and c are bound and free hapten con-

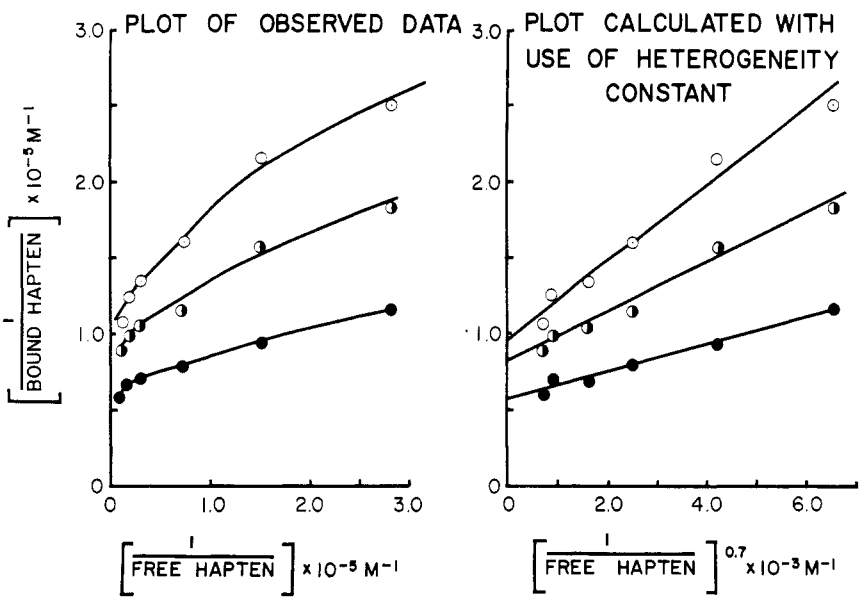

FIG. 2.—Binding of I^{131}-labeled *p*-iodophenylarsonate by anti-R_p preparations (15.0 mg protein per ml): ●, not iodinated; $A_0 = 18.2 \times 10^{-6}$ M; ◑, iodinated with ICl (19 atoms I incorporated/mole) in the presence of 0.1 M *p*-nitrophenylarsonate; $A_0 = 13.0 \times 10^{-6}$ M; ○, iodinated with ICl (20 atoms I incorporated/mole) in the absence of hapten; $A_0 = 10.2 \times 10^{-6}$ M. Each point is the average of duplicate determinations, with average deviation ± 2.7%. Values are corrected for nonspecific binding by normal γ-globulin, 1.7 ± 0.5% of free hapten concentration bound; by iodinated normal γ-globulin (22 I atoms/mole), 0.4 ± 0.5% of free hapten concentration bound.

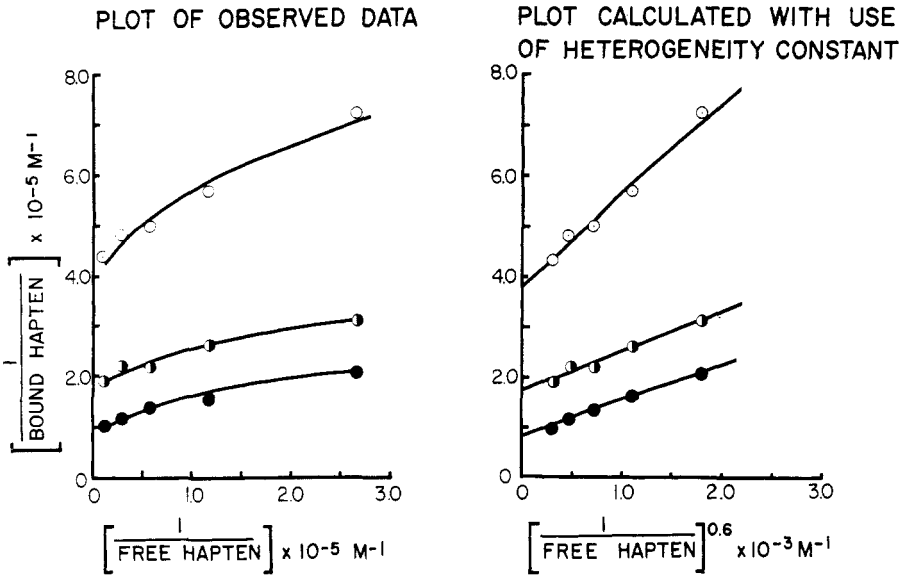

FIG. 3.—Binding of I^{131}-labeled 3-iodopyridine by anti-P_8 preparations (10.0 mg protein per ml): ●, not iodinated; $A_0 = 11.5 \times 10^{-6}$ M; ◑, iodinated with ICl (34 atoms I incorporated/mole) in the presence of 0.1 M pyridine; $A_0 = 5.8 \times 10^{-6}$ M; ○, iodinated with ICl (33 atoms I incorporated/mole) in the absence of hapten; $A_0 = 2.6 \times 10^{-6}$ M. Each point is the average of duplicate determinations with average deviation ± 2.3%. Values are corrected for nonspecific binding by normal γ-globulin, 3.5 ± 0.5% of free hapten concentration bound; by iodinated normal γ-globulin (32 atoms I incorporated/mole), 4.9 ± 0.6% of free hapten concentration bound.

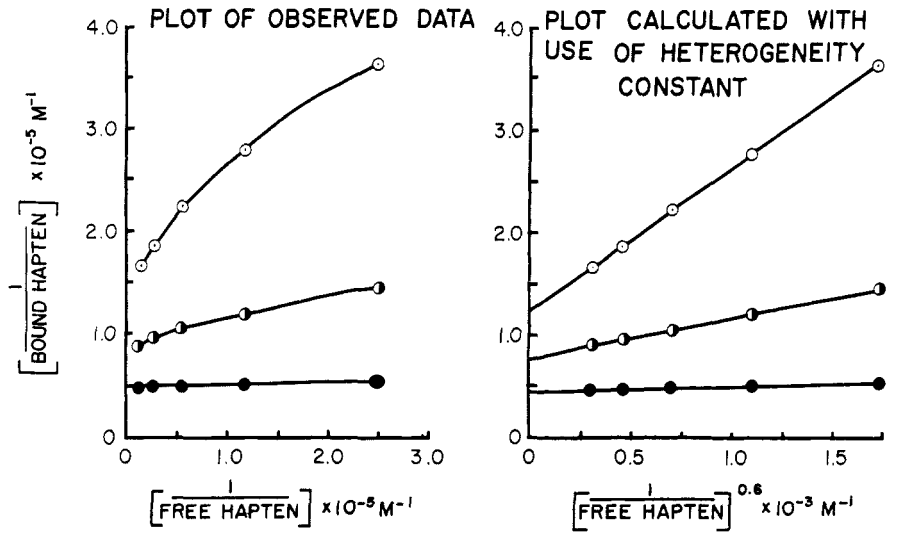

FIG. 4.—Binding of I^{131}-labeled *p*-iodophenyltrimethylammonium by anti-A_p preparations (10.0 mg protein per ml): ●, not iodinated; $A_0 = 22.0 \times 10^{-6}$ M; ◑, iodinated with ICl (52 atoms I incorporated/mole) in the presence of 0.1 M *p*-iodophenyltrimethylammonium; $A_0 = 13.0 \times 10^{-6}$ M; ○, iodinated (48 atoms I incorporated/mole) in the absence of hapten; $A_0 = 8.0 \times 10^{-6}$ M. Each point is the average of duplicate determinations with average deviation ± 3.0%. Values are corrected for nonspecific binding by normal γ-globulin, 3.2 ± 0.8% of free hapten concentration bound; by iodinated normal γ-globulin (54 atoms I incorporated/mole), 11.0 ± 3.0% of free hapten concentration bound.

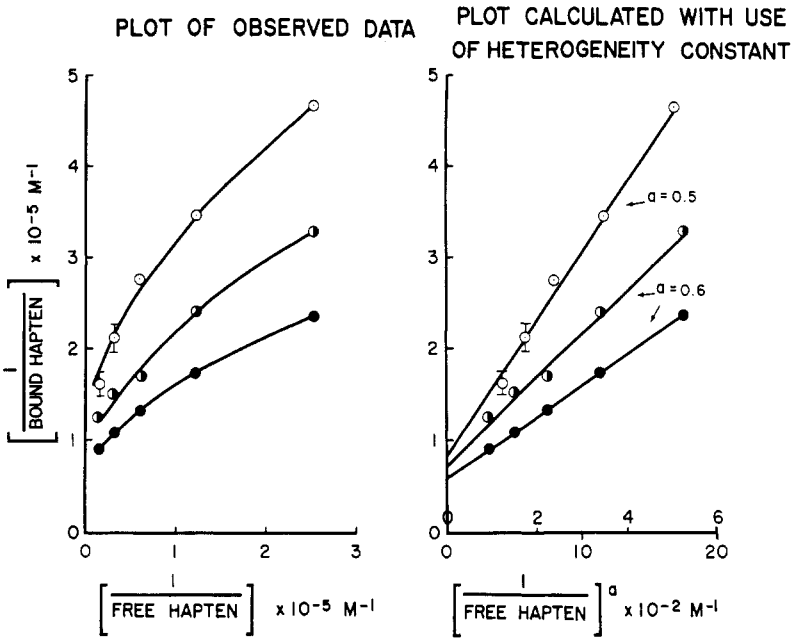

FIG. 5.—Binding of I^{131}-labeled *p*-iodobenzoate by anti-X_p preparations (protein concentration, 15.0 mg/ml): ●, not iodinated; $A_0 = 16.7 \times 10^{-6}$ M; ◑, iodinated with ICl (33 atoms I incorporated/mole) in the presence of 0.1 M *p*-nitrobenzoate; $A_0 = 13.3 \times 10^{-6}$ M; O, iodinated (33 atoms I incorporated/mole) in the absence of hapten; $A_0 = 12.5 \times 10^{-6}$ M. Upper abscissa scale is for $a = 0.5$; lower scale is for $a = 0.6$. Each point is the average of duplicate determinations, with deviation as shown; average deviation is ±3.0%. Values are corrected for nonspecific binding by normal γ-globulin, 3.4 ± 0.3% of free hapten concentration bound; by iodinated normal γ-globulin (35 atoms I incorporated/mole), 4.5 ± 0.7% of free hapten concentration bound.

centrations, respectively, A_0 is the total concentration of sites, and a is a heterogeneity constant expressing the distribution of binding constants. Thus by choosing the proper value of a so that the data best fit a straight line, the value of the average binding constant, K_0, can be calculated. The values of K_0 and a thus obtained are in Table III. Extrapolation of the data yields a value for A_0. In Table III there are given values for the percentage of the sites retained during iodination and the amount of active antibody present in the original uniodinated γ-globulins obtained from the extrapolation data.

Discussion

The effect of iodination on antibody activity may be considered as a sum of two effects—first, an effect on the number of sites, A_0, and, second, an effect on the binding constant, K_0.

Effect of Iodination on Number of Sites.—Examination of the data (Table III) for the effect of iodination in the presence and absence of hapten on A_0 reveals that iodination in the absence of hapten causes the loss of binding sites in each antibody system. This loss is partially prevented when hapten is present during iodination, as represented graphically in Figure 6. This shows that each preparation contains antibodies with an iodinatable group in the site itself. Iodination of this group completely blocks the site.

The prevention by hapten of loss of sites due to iodination is more easily observed, in the experiments reported, the larger the proportion of sites affected. Thus this protection effect was greatest in the experiments reported (Table III) for anti-A_p and anti-P_3 antibodies, which lost 64% and 78% of sites, respectively. The protection effect was of lesser magnitude in the case of the anti-R_p preparation, in which only 44% of sites were blocked, and was least for the anti-X_p preparation, in which only 25% of sites were blocked. In our experience protection of anti-X_p binding activity by 0.1 M *p*-nitrobenzoate is less than the protection of anti-R_p activity by 0.1 M *p*-nitrobenzenearsonate, but always becomes very appreciable when iodination is at higher levels.

Effect of Iodination on Binding Constants.—Iodination in the absence of hapten results in preparations which have decreased binding constants (binding energy) for the remaining sites of anti-R_p, anti-X_p, and anti-A_p antibodies and increased binding constants for the remaining anti-P_3 sites. The presence of hapten during iodination tends to keep the value of K_0 observed from lowering in the anti-R_p, anti-X_p, and anti-A_p system. The observations are summarized by the bar graphs in Figure 7.

TABLE III

EFFECT OF IODINATION OF ANTI-HAPTEN ANTIBODIES IN THE PRESENCE AND ABSENCE OF HAPTEN ON TOTAL BINDING SITES (A_0) AND BINDING CONSTANTS (K_0)

	Anti-R_p	Anti-P_3	Anti-A_p	Anti-X_p
Antibody content of γ-globulin (mg/mg total protein)[a]	0.097	0.092	0.176	0.089
Iodine incorporated (atoms/molecule)				
In absence of hapten	20	33	48	33
In the presence of hapten[b]	19	34	52	33
Binding sites remaining after iodination				
In absence of hapten (% of original)	56	23	36	75
In presence of hapten (% of original)	72	50	59	80
Binding constants, K_0 (liters/M $\times 10^{-5}$)[c]				
Before treatment	2.5	1.6	11.0	0.44
After iodination in absence of hapten	1.5	3.5	1.0	0.11
After iodination in presence of hapten	2.1	3.6	3.5	0.36
Heterogeneity constant, a (same for iodinated and uniodinated antibody)	0.7	0.6	0.6	0.5–0.6

[a] Obtained by extrapolation of binding curves to infinite hapten concentration (Fig. 2, 3, 4, 5). [b] Haptens employed (all at 0.1 M) were: for anti-R_p, *p*-nitrobenzenearsonate; for anti-A_p, *p*-iodophenyltrimethylammonium; for anti-P_3, pyridine; for anti-X_p, *p*-nitrobenzoate. [c] Haptens employed for binding were the homologous iodo derivatives labeled with I^{131}.

In interpreting the observations shown in Figure 7 it is noteworthy that all the binding curves indicate that the preparations were heterogeneous with respect to binding constants. It is probable that, for each system, the various antibodies comprising such a heterogeneous population would have somewhat different binding sites as reflected by the different binding constants. The different sites in each system may also have different susceptibilities to alteration. If iodination results in a preferential loss of binding sites of high K values, there results a population with lower average binding constant after iodination. On the other hand, if iodination causes a preferential loss of binding sites with a low K, then K_0 (the average) observed after iodination would be larger.

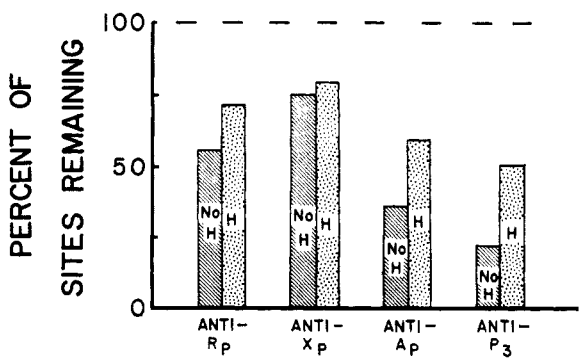

FIG. 6.—The percentage of antibody sites remaining after iodinations of different antibody preparations in the presence (H) or absence (NoH) of hapten; data taken from Table III.

A hapten protects only those sites that bind it. The degree of protection depends on the strength of binding and on the importance of the iodinatable group in the site. From these considerations, it follows that with the preparations of anti-R_p, anti-X_p, and anti-A_p antibodies studied, iodination in the presence of hapten results in preparations that bind more strongly than those obtained after iodination in the absence of hapten because the stronger binding sites are better protected and are more sensitive to iodination than the weaker binding sites.

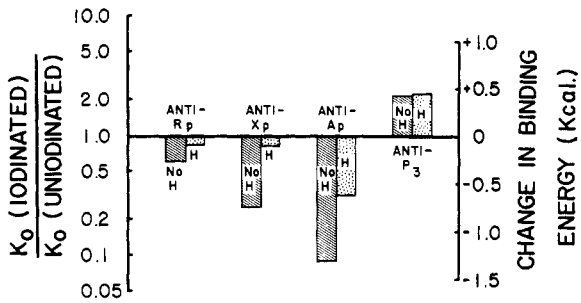

FIG. 7.—The change in average combining constantr (combining energy) of antibody sites remaining aftes iodination of different antibody preparations in the presence (H) or absence (NoH) of hapten; data taken from Table III.

In the case of the anti-P_3 preparation, the weaker binding sites are more sensitive to iodination. The value of K_0 for anti-P_3 antibodies which remain after iodination in the presence of hapten is the same high value (compared to the uniodinated preparation) as observed for the preparation obtained by iodination in the absence of hapten. If all sites were equally sensitive to iodination, K_0 would rise somewhat owing to greater protection of those with high K. If the sites with a high value of K were less sensitive the

rise would be smaller with protection. Since the value of K_0 is about the same whether or not hapten was present during iodination, it appears that some of the sites with high values of K as well as those with low values are affected by iodination.

Loss of activity even in the presence of hapten occurs during iodination, and there are several possible explanations. The most apparent possibility is that iodination takes place in the sites of antibodies uncombined with hapten, due to the equilibrium state. Other possibilities exist for change of activity by iodination of the antibody, or antibody-hapten complex, elsewhere than in the site. Some sites may be lost by complete unfolding of the protein in the region composing the site owing to iodination or oxidation elsewhere. This destruction would not be protected against by hapten.

effects such as contraction or expansion of the whole molecule, as described by Habeeb *et al.* (1958). This could be in part a cause of observed lowering of K_0 in the iodinated anti-R_p, anti-X_p, and anti-A_p preparations or the increase of K_0 for anti-P_3 (Fig. 7). Another possibility is that, in a site which is combined with hapten, a tyrosine contributing to the site may still be iodinated even though blocked on one side by hapten. This could result in a change in the binding energy of hapten-site interaction by increased ionization of the tyrosine hydroxyl to yield a negative charge in the site. Such ionization would decrease binding of an anionic hapten. Any reduction of K_0 would increase the lability of a susceptible site by permitting subsequent iodination within the site itself due to increased dissociation of the hapten.

Apparent Sensitivity to Iodination.—The apparent effect of iodination, as stated above, is a com-

TABLE IV

APPARENT SENSITIVITY OF ANTIHAPTEN ANTIBODIES TO IODINATION

Antibody	Hapten Bound[a]		Iodine Incorporated (Atoms/Molecule) (1)	% Loss of Binding Activity[b] (2)	% Loss of Sites[c] (3)	Loss of Activity (% per Atom Iodine Incorporated) (2)/(1)	Loss of Sites (% per Atom Iodine Incorporated) (3)/(1)
	Unmodified Antibody (M × 10^6)	Iodinated Antibody (M × 10^6)					
Anti-R_p	9.80	4.35	20	56	44	2.8[d]	2.2
Anti-P_3	5.12	1.52	33	70	78	2.1	2.4
Anti-X_p	4.65	2.04	33	56	25	1.7[e]	0.76
Anti-A_p	18.5	3.00	48	84	64	1.7[d]	1.3

[a] Values obtained by interpolation from the binding curves (Fig. 2, 3, 4, and 5) at a free hapten concentration of 5.0×10^{-6} M. [b] Calculated as $100\left(1 - \frac{\text{hapten bound to iodinated antibody}}{\text{hapten bound to unmodified antibody}}\right)$. [c] Taken from the data in Table III. [d] The corresponding values for different preparations of anti-R_p and anti-A_p in the experiment of Figure 1 are: for anti-R_p, 2.5; for anti-A_p, 1.2. [e] The corresponding value for the preparation of specifically purified anti-X_p in experiment A, Table II, is 1.3.

An example of data which may be considered to demonstrate this type of effect is provided in experiment B, Table II. The samples of specifically purified anti-X_p which were exposed to 285 moles of ICl per mole of protein incorporated little more iodine atoms than other samples exposed to only 94 moles of ICl, but lost much more activity —a loss which could not be protected by hapten. It seems a reasonable presumption that the lack of increased incorporation in the more drastically treated samples can be correlated with increased oxidative reactions occurring in these samples, which could well lead to destruction of the site in the manner suggested. However it is possible that the loss of activity may be due to increased incorporation of iodine in the site while oxidation effects occurring elsewhere result in a lower total iodine content for the whole molecule.

The possibility also exists that iodination (or oxidation) in other parts of the molecule lowers (or raises) the binding constant of uniodinated sites by electrostatic effect due to change of charge, or

bination of loss of sites and modification of binding constants. However, sensitivity to iodination can be approximately determined by the loss of binding activity (as measured at a suitable single hapten concentration) with increasing extent of iodination. Table IV shows the percentage loss in binding activities as calculated for the four iodinated antihapten antibodies from the curves used to obtain the data of Table III. Also included are the percentage loss in sites (from the data in Table III) and the calculated losses of activity and sites per iodine atom. Anti-R_p is clearly more sensitive to iodination than anti-A_p. This is the same relationship that obtains in Figure 1, where mixtures were iodinated to different extents. Anti-P_3 is of intermediate sensitivity (Table IV). This intermediate value is of particular interest since anti-P_3 loses sites more rapidly than anti-R_p or anti-X_p or anti-A_p (Table IV), but this is compensated by the fact that those lost comprise the weaker binding antibodies.

The differences in sensitivity of the various

antibodies toward iodination may be accounted for in part by the considerations mentioned above regarding the importance of the iodinatable group in the sites for the specific bindings involved. In addition, differences in sensitivity can be caused by differences in the reactivity of the iodinatable group toward the reagent.

Groups Within the Site Affected by Iodination.—That iodination affects a group within the site in each of the four antibodies is clearly shown by the experiments given.[1] Although loss of activity can be due to nonspecific alteration such as denaturation or charge alteration which result in molecular expansion, increased hydrophobic character, etc., thereby destroying antibody sites because of alteration of the molecule elsewhere, specific protection of a site by hapten during iodination is interpreted as showing that the attack must be in the site itself (or on a group important for the integrity of the site).[1] Protected and unprotected molecules are altered nonspecifically (in regions other than the site) to the same extent.

Residues which may be affected by iodination are tyrosine and histidine, which can incorporate iodine atoms, and tryptophane, cysteine, methionine, and cystine, which can undergo oxidation. Sulfhydryl and amino, which may possibly react with iodine to give sulfenyl iodide or iodoamino groups, would hydrolyze to the sulfenic acid or revert to the amine under the conditions of the treatment subsequent to iodination (addition of KI, dialysis, and, in those cases where excess iodine was used, addition of thiosulfate).

Tyrosine is known to incorporate iodine when proteins are iodinated. Hughes and Straessle (1950) found iodine in iodinated human serum albumin to be primarily in combination with tyrosine. More iodine than could be accounted for by the iodine in tyrosine could be incorporated, probably in the form of iodohistidine, although none of the iodohistidine was isolated. Iodohistidine has been shown to be formed during iodination of certain proteins, *e.g.*, lysozyme and lima bean trypsin inhibitor (Fraenkel-Conrat, 1950).

Any of these alterations, involving either incorporation of iodine or oxidation, can conceivably affect the activity of an antibody. The groups within an antibody site would probably be neither iodinated nor oxidized when the site is combined with hapten, and thus the site itself would be protected against the effects of iodination.

Evidence that iodine is incorporated in a group in the site of anti-X_p antibody was obtained in this laboratory by the demonstration that peptides derived from the site[1] contained iodine (Pressman and Roholt, 1961). Moreover, in the case of anti-X_p antibody, the presence of tyrosine in the site is further implicated by the following facts: (*a*) There is a group in the site which has a dissociation constant equivalent to that of tyrosine (Pressman *et al.*, 1960); (*b*) there is a group which can be acetylated (Nisonoff and Pressman, 1960); (*c*) this acetylation is easily reversed by hydrolysis (Pressman *et al.*, 1960). Koshland *et al.* (1960) have presented evidence that histidine and tyrosine residues are iodinated in antibody and that tryptophane and methionine residues are oxidized. It would appear that tyrosine is definitely involved in the sites of anti-X_p antibodies and is probably involved in those of the other three antibodies studied, although the evidence is not as complete.

Nonspecific Effect of Iodination of γ-Globulin on Binding of Haptens.—There is an effect of charge on the nonspecific binding of hapten to normal γ-globulin, as shown by the fact that iodination, which increases negative charge of the protein (by increasing the dissociation constant of the tyrosine hydroxyl), alters the nonspecific binding of hapten. In accord with simple electrostatic theory, nonspecific binding of negatively charged haptens is decreased and binding of positively charged haptens is increased, as shown by the data in legends of Figures 2 through 5. The opposite effect was observed with esterified globulins, in which the negative charge was decreased (Grossberg and Pressman, 1960). The magnitude of this nonspecific charge effect on hapten binding by modified antibody is not clear. However the differences observed in binding by antibodies iodinated to the same extent in the presence and absence of hapten *must be due to differences other than those associated with any such nonspecific charge effect*, since this latter effect must be the same for both preparations.

[1] The single reservation exists that it is possible that an antibody when combined with hapten changes configuration in some way, and thus a group elsewhere (which is important for the integrity of the site) is prevented from being iodinated. In this hypothesis, the iodination of such a group would destroy antibody activity but such a situation would protect antibody activity by hapten because the group is exposed for iodination only when hapten is absent.

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The Reversible Masking of Amino Groups in Ribonuclease and Its Possible Usefulness in the Synthesis of the Protein

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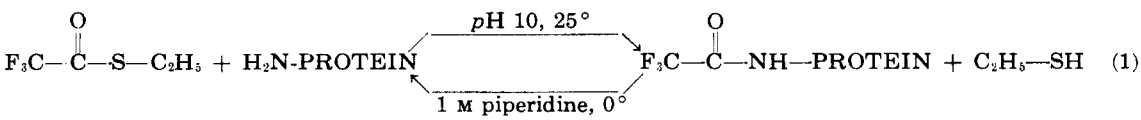
In the present report a method is described by which the amino groups of ribonuclease can be trifluoroacetylated by reaction with ethyl thioltrifluoroacetate. By this method the lysyl bonds in the protein molecule are rendered resistant to hydrolysis by trypsin, while the arginyl bonds are left susceptible. The amino acid compositions of the five peptides isolated from a tryptic digest of trifluoroacetylated ribonuclease (in its oxidized form) were shown to correspond with the known compositions of the segments of ribonuclease expected upon cleavage of arginyl bonds. The trifluoroacetyl groups can be removed from the trifluoroacetylated enzyme by exposure to 1.0 M piperidine at 0°. The material thus obtained lacks enzymic activity, presumably owing to the presence of incorrectly paired half-cystine residues. Rearrangement of disulfide bonds, under conditions known to favor the assumption of "native" configuration, leads to the regeneration of full enzymic activity. The method for the reversible masking of amino groups is discussed in relation to its possible usefulness in the organic synthesis of ribonuclease.

The availability of the complete formula for the covalent structure of bovine pancreatic ribonuclease (RNase),[1] together with an extensive body of knowledge concerning its chemical and enzymic properties, make it reasonable to undertake the "organic" synthesis of this enzyme. Such a project is feasible only because the last step in the over-all synthesis, the correct formation of four disulfide bonds and the assumption of native tertiary structure, takes place spontaneously during air oxidation of the reduced polypeptide chain (Anfinsen and Haber, 1961; White, 1961; Anfinsen *et al.*, 1961).

The stepwise synthesis of RNase by methods such as those employed by Hofmann and his colleagues (Hofmann, 1960) for adrenocorticotropic hormone appears to be beyond the possibilities afforded by presently available techniques. However, these methods might ultimately be applicable to segments of the polypeptide chain. A prerequisite for total synthesis is the demonstration that such segments can be isolated from partial enzymic digests and subsequently rejoined in proper sequence with restoration of enzymic activity. The present paper reports on the masking of the amino groups of the protein by reaction with ethyl thioltrifluoroacetate (Schallenberg and Calvin, 1955), as shown in equation (1). The resulting trifluoroacetyl-enzyme (TFA-RNase), both in its intact form and after conversion to the extended polypeptide chain by oxidation or reduction of disulfide bonds, is cleaved by trypsin specifically at the four arginyl bonds in the molecule. The specificity of this cleavage is the result of the modification of ε-amino groups



(1)

[1] Abbreviations: Ribonuclease (RNase); oxidized ribonuclease (Ribox); trifluoroacetyl (TFA); trifluoroacetylated ribonuclease (TFA-RNase); trifluoroacetylated oxidized ribonuclease (TFA-Ribox). Abbreviations for individual amino acids are as recommended by Brand and Edsall (1947).