# Carboxymethylation of Yeast Enolase*

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As part of a study of the mechanism of action of yeast enolase, the enzyme was subjected to chemical modification with bromoacetic acid in an attempt to identify amino acids in the active site. When a sample of enolase reacted with sodium bromoacetate for 2 hours at *p*H 7 it lost 90% of its activity, and 2.7 histidine, 3.0 methionine, and 1.0 lysine residues per molecule of enzyme were carboxymethylated. An alteration in the tertiary structure of the protein was indicated by a lowering of its sedimentation coefficient during carboxymethylation. Another sample of enolase, which was carboxymethylated under the same conditions as the first sample except for the addition of magnesium sulfate and a substrate, D-glyceric acid 2-phosphate, to the reaction mixture, was 96% active and differed from the 10% active sample in that 1.4 fewer methionine residues were carboxymethylated. It thus appears that the inactivation of enolase by bromoacetate is due to the carboxymethylation of one or more methionine residues.

On the basis of indirect evidence, histidine residues have been implicated in both the catalytic site (Wold and Ballou, 1957) and in the metal-binding site (Malmström and Westlund, 1956) of yeast enolase (Malmström, 1961). In an attempt to get more precise information about this problem, enolase has been subjected to photo-oxidation and alkylation, and the inactive products of these reactions have been analyzed for amino acid modifications. The inactivation by photo-oxidation was associated with a rather broad destruction of histidine, methionine, and tryptophan, and gave results which were difficult to interpret (Brake and Wold, 1960). The present paper deals with the results of the modification of yeast enolase by alkylation with bromoacetate and iodoacetamide.

## RESULTS

When enolase was carboxymethylated for 2 hours at *p*H 7 and 35°, it lost 90% of its activity. However, if 0.02 M magnesium sulfate and 0.01 M D-glyceric acid 2-phosphate were included in the reaction mixture, there was only a slight loss of activity under the same conditions. At fixed magnesium concentration the extent of the protection depended on the substrate concentration (Fig. 1), and either magnesium or substrate alone gave little or no protection (Fig. 2). The competitive inhibitors, D-lactic acid phosphate and inorganic phosphate, also protected enolase from inactivation by bromoacetate in the presence of magnesium (Fig. 3). The concentrations of the three compounds required for protection correlated well with the kinetically determined affinity constants ($K_S$ [glyceric acid 2-phosphate] = $1 \times 10^{-4}$ M, $K_I$ [lactic acid phosphate] = $3.5 \times 10^{-4}$ M, and $K_I$ [inorganic phosphate] = $6.4 \times 10^{-3}$ M) (Wold and Ballou, 1957). Glycerophosphate, which does not inhibit enolase (Wold and Ballou, 1957), did not give any protection (Fig. 3).

With bromoacetate-1-$C^{14}$ used for the carboxymethylation, it was possible to determine the number of carboxymethyl groups incorporated into the protein with good precision. Under standard conditions, the unprotected (10% active) sample contained 6.2 moles carboxymethyl groups per mole of enzyme, and a protected sample under the same conditions (96% active) contained 5.3 moles carboxymethyl groups per mole of enzyme.

Table I gives the results of amino acid analyses of enolase after treatment with bromoacetate and iodoacetamide under different conditions. After reaction with bromoacetate, the only significant difference between the unprotected, 10% active sample (2.7 histidine, 3.0 methionine, and 1.0 lysine residues reacted per mole of enzyme) and the protected, 96% active sample (2.5 histidine, 1.5 methionine, and 0.6 lysine residues reacted per mole of enzyme) appears to be that 1.4 methionine residues per mole are rendered unreactive in the substrate-protected sample. The sum of reacted residues in both samples agrees well with the number of $C^{14}$-carboxymethyl groups incorporated.

As should be expected from the experiments of Stark *et al.* (1961), urea increased the number of residues reacting in a fixed period of time. Essentially all of the 8 methionine residues and half of the 13 histidine residues in enolase reacted with bromoacetate when the reaction was carried out for 2 hours in 8 M urea (Table I).

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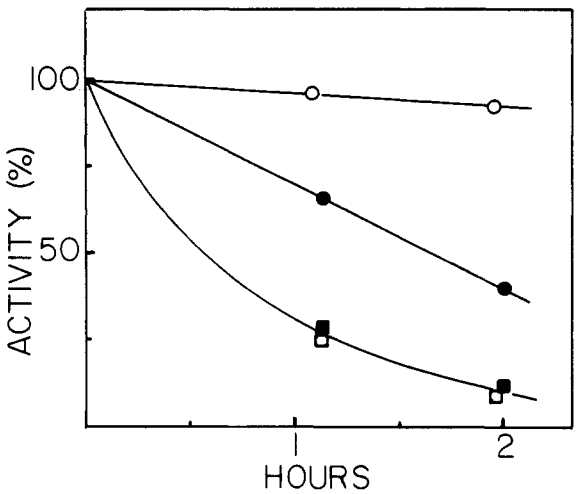



FIG. 1.—The effect of concentration of D-glyceric acid 2-phosphate on the rate of inactivation of enolase by bromoacetate. The reaction mixtures contained 0.2% protein in 0.3 M sodium bromoacetate, 0.5 M Tris buffer (*p*H 7.1), 0.02 M magnesium sulfate, and D-glyceric acid 2-phosphate at the following concentrations. O–O, 0.01 M; ●–●, 0.001 M; ■–■, 0.0001 M; □–□, none. The temperature was 35°.

When iodoacetamide was substituted for bromoacetate in an otherwise standard reaction, the enzyme was 8% active at the end of 2 hours. The product was insoluble in water but soluble in 8 M urea. A substrate-protected sample treated under identical conditions was 93% active and completely water soluble. Again the amino acid analysis indicated that 1 methionine residue was protected, although an additional difference of 0.4 histidine residue was also found (Table I).

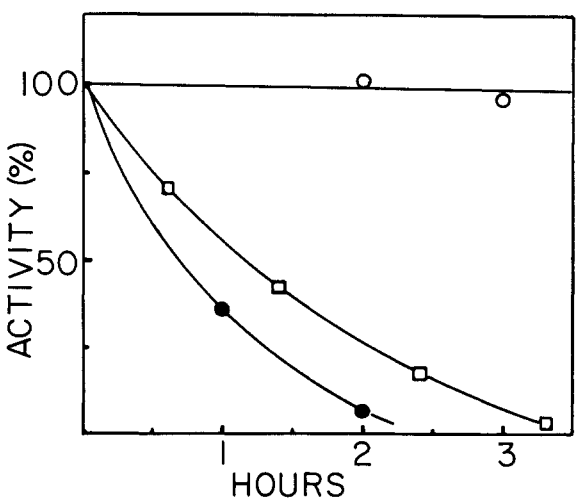



FIG. 2.—The effect of 0.02 M magnesium alone (□–□), 0.01 M substrate alone (●–●), and 0.02 M magnesium and 0.01 M substrate together (O–O) on the rate of inactivation of enolase by bromoacetate. The basic reaction mixtures and conditions as in Figure 1.

The amino acid analyses in Table I are in fairly good agreement with those obtained by Malmström *et al.* (1959). They found 13.7, 8.0, and 53.4 residues per mole, respectively, for histidine, methionine, and lysine. It is not clear why the urea-treated sample (sample number 4) should give low results, nor why lysine appears to be low in samples number 5 and 6. The results listed for samples 1, 2, and 3 are the average of several runs, and the reproducibility of the analyses is indicated.

The sedimentation coefficient of carboxymethylated enolase was determined as a measure of the extent of denaturation accompanying the carboxymethylation reaction. The results in Table II show that there was a progressive decrease in the sedimentation coefficient with increasing loss of activity in the unprotected sample, while the substrate-protected sample was found to have the same sedimentation coefficient as the native enzyme.

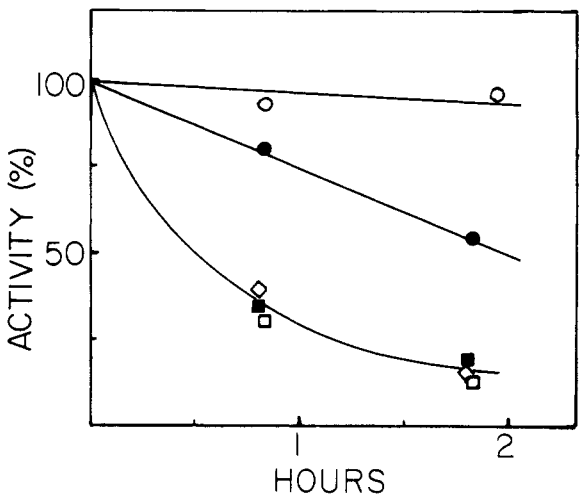



FIG. 3.—The effect of substrate analogues on the rate of inactivation of yeast enolase by bromoacetate. The reaction mixtures and conditions were the same as in Figure 1. All samples contained 0.02 M magnesium sulfate and in addition the following compounds. O–O, 0.01 M D-lactic acid phosphate; ●–●, 0.1 M potassium phosphate; ■–■, 0.01 M potassium phosphate; ◇–◇, 0.1 M sodium glycerophosphate (38% α- and 62% β-); □–□, no additions.

## DISCUSSION

There are several points of uncertainty in interpreting the above results. Malmström (1955) has shown that the substrate can be bound to sites other than the active site, and it is thus important to establish if the substrate protection exerts itself specifically at the active site. In an electron spin resonance study of substrate and metal binding to yeast enolase, Malmström *et al.* (1958) showed that nonspecific binding is greatly diminished in solutions containing low concen-

TABLE I

AMINO ACID ANALYSIS OF CARBOXYMETHYLATED SAMPLES

| Sample[a] No. | cm-his[b] | his[b] | cm-his + his[b] | cm-met[b] | met[b] | cm-met + met[b] | cm-lys[b] | lys[b] | cm-lys + lys[b] |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 0.00 | 13.7 ± 0.1 | 13.7 ± 0.1 | 0.00 | 8.98 ± 0.10 | 8.98 ± 0.10 | 0.00 | 47.3 ± 2.2 | 47.3 ± 2.2 |
| 2 | 2.73 ± 0.09[c] | 10.7 ± 0.6 | 13.4 ± 0.7 | 2.95 ± 0.05 | 6.07 ± 0.38 | 9.12 ± 0.43 | 0.99 ± 0.42 | 46.7 ± 0.6 | 47.3 ± 1.0 |
| 3 | 2.50 ± 0.12 | 11.1 ± 0.2 | 13.6 ± 0.3 | 1.53 ± 0.41 | 7.98 ± 0.60 | 9.51 ± 1.01 | 0.58 ± 0.14 | 47.3 ± 1.3 | 47.9 ± 1.4 |
| 4 | 5.49 | 7.2 | 12.7 | 7.62 | 0.00 | 7.62 | 3.82 | 46.0 | 49.8 |
| 5 | 1.35 | 12.8 | 14.2 | 2.59 | 5.90 | 8.49 | 0.34 | 44.0 | 44.3 |
| 6 | 0.94 | 13.2 | 14.1 | 1.66 | 8.02 | 9.66 | 0.33 | 44.5 | 44.8 |

[a] The samples were prepared as follows. 1. A control to test the effectiveness of the Sephadex column for the removal of bromoacetate. Prepared in 0.3 M bromoacetate for zero time. 2. Carboxymethylated for 2 hours, 10% active. 3. Carboxymethylated for 2 hours in the presence of the substrate, 96% active. 4. Carboxymethylated for 2 hours in 8 M urea. 5. Carbamylmethylated for 2 hours, 8% active. 6. Carbamylmethylated for 2 hours in the presence of the substrate, 93% active. (Reaction conditions are described in the Experimental Procedure section.) [b] The following abbreviations are used. cm-his, carboxymethylated histidine; cm-met, carboxymethylated methionine; and cm-lys, carboxymethylated lysine. The amino acid analyses are expressed in moles of amino acid per mole of enolase. [c] The ± indicates the range of experimental results.

TABLE II

SEDIMENTATION COEFFICIENTS OF CARBOXYMETHYLATED ENOLASE

| Reaction Time (hr.) | Addition | Activity (%) | $s_{20}$ (sec. × $10^{13}$) |
|---|---|---|---|
| 0.0 | None | 100 | 5.73 |
| 0.5 | None | 73 | 5.75 |
| 1.5 | None | 23 | 5.12 |
| 3.0 | None | 10 | 4.57 |
| 3.0 | MgGA2P[a] | 96 | 5.72 |

All samples were prepared in 0.3 M sodium bromoacetate at *p*H 7 and 32°. The sedimentation experiments were performed in 0.01 M potassium phosphate buffer at *p*H 6.9, with a protein concentration of 0.4%.

[a] Magnesium sulfate (0.02 M) and D-glyceric acid 2-phosphate (0.01 M).

trations of manganese ion and substrate and high concentrations (0.5 M) of potassium chloride. When enolase was carboxymethylated in this reaction mixture giving minimal "nonspecific" binding, complete protection occurred, strongly indicating that the protective effect was exerted at the active site.

The protection afforded by a competitive inhibitor (lactic acid phosphate), but not by a similar compound which is not an inhibitor (glycerophosphate), and the absolute requirement for divalent metal are also consistent with the concept of active site protection.

One also needs to validate the assumption that the same histidine and lysine residues and the same two methionine residues react in the presence and absence of substrate, in order to justify the simple subtraction of residues which gave the difference only in methionine reactivity in the substrate-protected enzyme. Unfortunately, direct evidence in favor of this assumption is not easy to obtain. It appears, however, that it would be a rather unlikely coincidence that the addition of substrate to the reaction mixture should shift the carboxymethylation to a new set of residues of composition so similar to the original one. It may be of interest in this connection to mention that we have recently found a clear substrate protection in the inactivation of enolase by photo-oxidation. Again, the substrate protects a methionine residue, but further experiments are required to establish whether other residues are protected as well. The common evidence for methionine involvement obtained in the two completely different types of protein modifications appears to favor the thesis that a specific methionine residue is essential for enolase action.

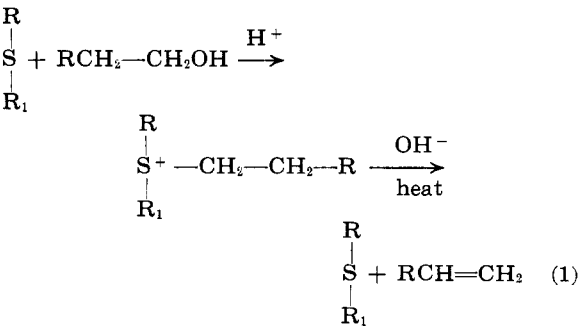
Substrate protection similar to that observed here in the carboxymethylation of enolase has also been reported for the carboxymethylation of ribonuclease (Barnard and Stein, 1959) and the iodination of an antibody (Koshland *et al.*, 1959).

The simplest explanation for substrate protection is that the substrate shields residues in or near the active site, making them unreactive toward the modifying reagent. Another possibility is that the substrate may stabilize the tertiary structure of the enzyme, an explanation often proposed to explain substrate protection from denaturation by heat (see for example London *et al.*, 1958), so that modification of a residue which is essential for maintaining the tertiary structure no longer causes denaturation. It is unlikely that this latter case is the sole cause of substrate protection of enolase, since removal of the protective agent from the carboxymethylated enzyme by dialysis did not lead to inactivation. On the other hand, the carboxymethylation in urea shows that drastic changes in the tertiary structure will expose several new residues to reaction with bromoacetate, and one cannot disregard the possibility that the unfolding accompanying the reaction in the absence of substrate could be partially responsible for the exposure of an additional 1 or 2 methionine residues.

Accepting, however, the arguments for specific active site protection by the substrate, one is left with the conclusion that one or two methionine residues are involved in the active site of yeast enolase. Such a methionine involvement is not without precedent. Kinetic analysis of the photooxidation of phosphoglucomutase has given good evidence for methionine involvement in that enzyme (Ray and Koshland, 1960). Ribonuclease is also inactivated by alkylation of methionine residues, and when several residues of methionine have been alkylated, there is evidence for an alteration in the tertiary structure of the enzyme (Stark *et al.*, 1961). In a study of the modification of ribonuclease-*S* with iodoacetate, Vithayathil and Richards (1961) obtained evidence that carboxymethylation of methionine greatly reduced the binding of *S*-peptide to *S*-protein. In the latter case the role of the methionine appears to be a structural one, but the possibility of methionine involvement in the catalytic site of phosphoglucomutase and enolase cannot be excluded on the basis of the available evidence.

It is interesting to speculate about the possible role of methionine in a dehydrating enzyme. Yeast enolase forms a stable complex with mercuric ion, and is, as a matter of fact, crystallized as the mercuri-complex (Warburg and Christian, 1941). The complex contains one mole of mercury per mole of enzyme (Bücher, 1947), is completely inactive, and is also very susceptible to denaturation in solutions of low ionic strength. Since there is no cystine or cysteine in yeast enolase, some other residue or residues must be responsible for the high affinity of the enzyme for mercury. Methionine may be involved here, since it is known that dialkyl sulfides will form stable coordinate complexes with mercuric salts. (For an extensive review of the properties of sulfides see Reid, 1960.)

Another interesting reaction of sulfides is the formation of sulfonium salts in the reaction with alcohols (Reid, 1960). The sulfonium salt may subsequently be decomposed with alkali to give an olefin, and the simplified reaction sequence indicated in equation (1) represents the dehydration of a primary alcohol.




(1)

Although we have some misgivings about a mechanism based on a similar sequence of steps for the dehydration reaction catalyzed by enolase, such a mechanism warrants consideration.

## Experimental

*Materials.*—D-Glyceric acid 2-phosphate was prepared by the method of Ballou and Fischer (1954). Bromoacetic acid (Eastman Chemicals, reagent grade) was recrystallized from benzene-pentane. Iodoacetamide (Sigma Chemical Co.) was used without further purification. All other chemicals were C.P. reagent grade.

Enolase was prepared from brewers' bottom yeast, generously supplied by the Anheuser-Busch Brewery in St. Louis. The yeast, which was obtained as a slurry, was washed three times with cold water, then filtered with suction on a 32-cm stainless steel Büchner funnel. The isolation procedure was essentially that of Warburg and Christian (1941), except for the following modifications: (1) Autolysis with toluene was substituted for autolysis in water, since, in addition to eliminating the time-consuming drying process, it was found to increase the yield of enzyme in the extract 1.5- to 2-fold. Five kg of filtered, but not dried, yeast were stirred with 2.7 liters of toluene and brought to 37°. After standing for 3 hours at room temperature the mixture was stirred well with 5.4 liters of water and left overnight at 4°. The lower, aqueous layer was siphoned off and filtered with suction after addition of Celite 535 (Johns-Manville) as a filter aid. (2) In the acetone and ethanol fractionations suction filtration was used in place of centrifugation, facilitating the handling of larger quantities. In order to achieve satisfactory filtration it was necessary to use a filter aid, Celite 535. (3) It was necessary to modify the first ethanol fractionation slightly in order to obtain the best results. The fraction which precipitated between 38% and 53% ethanol was found to contain most of the enolase. (4) The second ethanol fractionation was omitted, since

it gave little further purification and resulted in a loss of activity. (5) Yeast ribonucleic acid (Nutritional Biochemicals Corp.) was used for the nucleic acid fractionation. (6) Streptomycin sulfate (Eli Lilly and Co.) was used in place of protamine for the removal of nucleic acid after the nucleic acid precipitation. Thirty-five ml of a 5% solution of streptomycin sulfate was added per 100 ml of the solution of nucleic acid–protein complex at *p*H 6.0–6.5, and the resulting precipitate was removed by centrifugation. The subsequent steps were carried out according to the original procedure. The final crystalline mercuri-enzyme was freed from mercury and its purity was tested. It was found to be homogeneous in the ultracentrifuge, and its ultraviolet absorption spectrum and turnover number agreed with the reported values for the pure enzyme (Malmström, 1961).

*Instruments.*—Optical densities were determined with a Beckman Model DU Spectrophotometer equipped with thermospacers. Sedimentation velocities were determined with a Beckman/Spinco Model E Ultracentrifuge. Reactions at constant *p*H were conducted in an automatic titrator (International Instrument Corp.). Radioactivity was determined with a Packard Instruments Tri-Carb Scintillation Counter. Amino acids and carboxymethylamino acids were determined in a Beckman/Spinco Model 120 Amino Acid Analyzer after hydrolysis in constant boiling HCl for 21 hours in evacuated, sealed tubes. Carboxymethylamino acids were identified by their elution volumes, as determined by Gundlach *et al.* (1959). The carboxymethyl histidine and lysine values reported in Table I are the sum of the monocarboxymethyl derivatives (2 for histidine and 1 for lysine) and the dicarboxymethyl derivatives. Because *S*-carboxymethyl methionine is unstable to acid hydrolysis, the amount of this amino acid is determined by the sum of the amount of its breakdown products, namely, homoserine, homoserine lactone, *S*-carboxymethylhomocysteine, and methionine. The first three quantities can be determined directly, but the last must be determined indirectly, since the methionine resulting from decomposition of *S*-carboxymethylmethionine cannot be differentiated from the methionine which was not attacked by bromoacetate. However, a correction factor can be calculated as suggested by Stein (1960). Oxidation of carboxymethylenolase with performic acid converts the methionine (but not the *S*-carboxymethylmethionine) to methionine sulfone. The actual amount of S-carboxymethylmethionine in the sample is then found by subtracting the amount of methionine sulfone from the amount of methionine which is present in enolase. It was found that 26% of the *S*-carboxymethylmethionine reverted to methionine during hydrolysis, so equation (2) could be used for calculating the amount of *S*-carboxymethylmethionine in a carboxymethylenolase sample

$$[\text{S-cm-met}] = \frac{[\text{homoser}] + [\text{homoser lac}] + [\text{S-cm-homocys}]}{0.74} \quad (2)$$

prior to hydrolysis, where the brackets stand for the number of micromoles of the compound indicated. The methionine values were corrected by the expression:

$$[\text{met}] = [\text{met (found)}] - 0.26\,[\text{S-cm-met}].$$

*Enolase Assays.*—Enzymatic activity was determined by the spectrophotometric procedure of Warburg and Christian (1941), with the assay mixture consisting of 0.001 M D-glyceric acid 2-phosphate, 0.008 M magnesium sulfate, 0.1 M potassium chloride, and 0.05 M imidazole, adjusted to *p*H 7.4 with hydrochloric acid. Protein concentration was determined from the optical density at 280 mμ (Warburg and Christian, 1941). All assays were run at 25°.

*Carboxymethylation.*—The reaction was carried out in a *p*H-stat or in a buffered solution (0.5 M Tris) at *p*H 7.0. The temperature was kept constant at 35°. The reaction mixture (15 ml) consisted of 0.3 M sodium bromoacetate, 15–40 mg of enolase, and other additions as required for a particular experiment. Aliquots for measurement of activity were removed at intervals. To stop the reaction, the sample was freed of bromoacetate by passing at 4° through a 4 × 40 cm column of Sephadex G-50 (Pharmacia, Uppsala) mixed with about 0.1 volume of acid-washed Celite to improve its flow rate, with 0.01 M potassium phosphate, *p*H 7, as eluent. The enzyme was located by its absorption at 280 mμ, lyophilized, and hydrolyzed in 6 N hydrochloric acid at 110° for 21 hours in a sealed tube which had been evacuated with an efficient water aspirator. After removal of hydrochloric acid with a flash evaporator, the hydrolysate was analyzed for amino and carboxymethylamino acids. Attempts were also made to carboxymethylate enolase at *p*H 5.5. At this *p*H enolase is unstable, however, and rapid denaturation and precipitation of the enzyme accompanied the reaction.

*Carboxymethylation with Radioactive Bromoacetate.*—Bromoacetate-1-$C^{14}$ (New England Nuclear Corporation) was mixed with unlabeled bromoacetic acid to give a specific activity of 0.02 μc per mg. Ten mg of enolase was carboxymethylated in 5 ml of 0.3 M bromoacetate-$C^{14}$ in the manner described above and then freed of bromoacetate by dialysis for 48 hours against running deionized water. After lyophilization the sample was placed in a tared 5-dram screw-cap vial and dried to constant weight at 100° in an Abderhalden vacuum drying apparatus. The weight of the sample was determined by difference, and the sample was then dissolved in 1 ml of Hyamine hydroxide (1 M solution in methanol) (Packard Instruments) by heating to 50° for 1 or 2 hours. Ten ml of 0.1% diphenyloxazole in toluene was added, and the radioactivity of the sample was

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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