Bossa, F., Giartosio, A., Riva, F., and Fasella, P. (1966), *J. Biol. Chem. 242*, 2397.
Martinez-Carrion, M., Turano, C., Riva, F., and Fasella, P. (1965), *Biochem. Biophys. Res. Commun. 20*, 206.
Riva, F., Vecchini, P., Turano, C., and Fasella, P. (1964), *6th Intern. Congr. Biochem., New York*, 329.

Reaction of Tetranitromethane with Protein Sulfhydryl Groups. Inactivation of Aldolase*

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ABSTRACT: The reactivity of protein tyrosyl and sulfhydryl groups toward tetranitromethane (TNM) was studied using aldolase of rabbit muscle. As in model compounds, oxidation of sulfhydryl groups in this protein occurs much faster than tyrosyl nitration. However, the rate of SH modification decreases with decreasing pH in contrast to previous findings with model compounds (Sokolovsky, M., Riordan, J. F., and Vallee, B. L. (1966), *Biochemistry 5*, 3582). Aldolase is rapidly and completely inactivated by a 23-fold M excess of TNM at pH 8.0. The loss in activity toward either fructose 1,6-diphosphate or fructose 1-phosphate correlates with modification of SH groups; eight to ten SH groups are oxidized in the completely inactivated enzyme while not more than 0.6 tyrosyl residue is nitrated. Other amino acid residues do not react with TNM under the mild conditions used. The same relationship between number of SH groups oxidized and degree of inactivation holds for all conditions of modification. The inactivation is only partially reversible upon incubation with mercaptoethanol suggesting only limited S–S bond formation. Other oxidized forms of cysteinyl residues occur during the reaction with TNM as evidenced by the detection of cysteic acid on amino acid analysis. Competitive inhibitors and fructose 1,6-diphosphate partially protect the enzyme against inactivation by TNM.

Nitration of tyrosine and related compounds with tetranitromethane (TNM)[1] proceeds readily under mild conditions (Riordan *et al.*, 1966). The reaction is well suited for modifying proteins (Sokolovsky *et al.*, 1966) and has proven useful for studying the tyrosyl residues of carboxypeptidase A (Riordan *et al.*, 1967). TNM has also been shown to react with sulfhydryl groups in model compounds, the product being a disulfide. Studies on sulfhydryl groups in proteins analogous to those on tyrosyl residues have not been carried out as yet and the reactivities of tyrosyl and cysteinyl residues toward TNM have not been compared in the same protein.

Aldolase seemed to be particularly suitable for such studies since it contains readily reactive tyrosyl and cysteinyl residues, both of which are thought to be involved in activity (Swenson and Boyer, 1957; Drechsler *et al.*, 1959; Kowal *et al.*, 1965; Schmid *et al.*, 1966; Kobashi and Horecker, 1967; Pugh and Horecker, 1967). However, it should be possible to examine the sulfhydryl groups independently of the tyrosyl residues. Studies with model compounds had shown that the pH dependencies of the reactions of these two residues with TNM are different (Riordan *et al.*, 1966; Sokolovsky *et al.*, 1966). Moreover, carboxypeptidase treatment removes the C-terminal tyrosyl residues of aldolase, reducing FDP activity to 5%. The residual activity of this derivative is not altered further by acetylation of tyrosine (Schmid *et al.*, 1966). Modification of sulfhydryl groups completely abolishes the activity of aldolase toward both fructose 1,6-diphosphate (FDP) and fructose 1-phosphate (F-1-P) (Kobashi and Horecker, 1967), while reactions involving the tyrosyl residues alter only activity toward FDP (Pugh and Horecker, 1967). Hence, activity can serve as a first approximation to the types of residues being modified.

Materials

Fructose 1,6-diphosphate aldolase from rabbit muscle was obtained from Worthington Biochemical Corp. as a crystal suspension in ammonium sulfate. Before use, the enzyme was dialyzed against the appropriate buffer. The specific activity at 25° was 11.3 IUB units/mg with fructose 1,6-diphosphate as substrate. Other materials and suppliers were: carboxypeptidase A, Worthington Biochemical Corp.; glycerol 1-phosphate dehydrogenase, triose phosphate isomerase, fructose 1,6-diphos-

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[1] Abbreviation used that is not listed in *Biochemistry 5*, 1445 (1966), is: TNM, tetranitromethane.

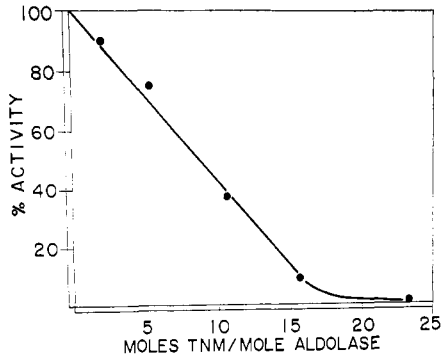

FIGURE 1: Inactivation of aldolase with increasing molar excesses of TNM. The reaction mixtures contained 2.5 mg of aldolase in 0.5 ml of 0.05 M Tris-Cl–0.3 M NaCl (pH 8.0). Aliquots of a 0.0084 M solution of TNM in 95% ethanol were added and after 30 min at 20° aliquots were assayed for FDP activity.

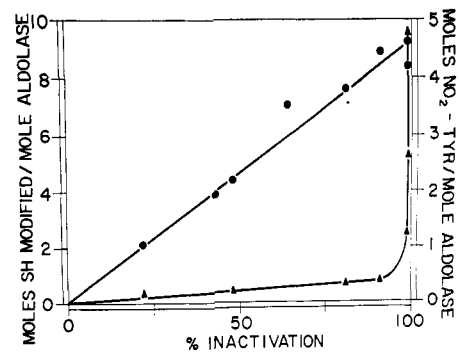

FIGURE 2: Inactivation of aldolase by TNM. Degree of inactivation *vs.* number of modified cysteinyl and tyrosyl residues. The reaction mixture contained 5 mg of aldolase in 1 ml of 0.05 M Tris-Cl–0.3 M NaCl (pH 8.0), 25°. The different degrees of inactivation were attained by varying either the amount of 0.034 M ethanolic TNM which was added (molar excess 4–63-fold), or the reaction time (5, 15, or 30 min). After reaction the protein so'ution was passed through a Bio-Gel P-4 column in 0.05 M Tris-Cl–0.3 M NaCl (pH 8.0) to remove both excess reagent and the nitroform produced during the reaction. Activity toward FDP and sulfhydryl (●) and nitrotyrosyl (▲) content were then determined as described under Methods. The difference in sulfhydryl content between native and TNM-inactivated aldolase was taken as the number of thiol groups modified.

phate tetracyclohexylammonium salt, and fructose 1-phosphate dicyclohexylammonium salt, Boehringer Mannheim Corp.; NADH, Sigma Chemical Co.; tetranitromethane, Aldrich Chemical Co.; 2-mercaptoethanol, Eastman Organic Chemicals; and 5,5-dithiobis(2-nitrobenzoic acid), Pierce Chemicals. Bio Gel P-4 was a product of Bio-Rad Laboratories.

Methods

Aldolase was assayed spectrophotometrically (Racker, 1947) in a Unicam SP 800 spectrophotometer at 25°. The assay mixture consisted of 0.1 M Tris-Cl (pH 7.6), 2.5×10^{-4} M NADH, 4×10^{-4} M fructose 1,6-diphosphate or 0.012 M fructose 1-phosphate, 10 μg of glycerol 1-phosphate dehydrogenase, and 1 μg of triosephosphate isomerase in a total volume of 2.5 ml.

Protein concentrations were calculated from the absorption at 280 mμ, $A^{1\%}_{1\,cm}$ 9.1 (Baranowski and Niederland, 1949). Absorption at a single wavelength was determined with a Zeiss PMQ II spectrophotometer. The molecular weight of aldolase was taken to be 158,000 (Kawahara and Tanford, 1966).

Sulfhydryl groups were determined by reaction with 5,5-dithiobis(2-nitrobenzoic acid) according to the procedure of Ellman (1959). The change in absorbance at 412 mμ of both the native and modified proteins were followed at 25° in 1-cm cuvets. To 2 ml of an aldolase solution (0.5 mg/ml) in 0.05 M Tris-Cl–0.3 M NaCl (pH 8.0) was added 50 μl of a 0.01 M solution of the Ellman reagent in 0.1 M phosphate (pH 7.0). In the native protein, about 10.5 sulfhydryl groups were found to react during the first 15 min; beyond this time the reaction proceeded very slowly and at identical rates for the native and the modified proteins. Calculations of the number of thiol groups modified were based on the measurements at 15 min.

Nitrotyrosine was determined in alkaline solution by virtue of its absorption at 428 mμ using a molar absorptivity, ϵ 4200 M^{-1} cm^{-1} (Sokolovsky *et al.*, 1966). Spectra were recorded with a Cary Model 15 spectrophotometer.

Amino acid analyses were performed on proteins hydrolyzed in 6 N HCl *in vacuo* at 105° for 22 hr using a Spinco Model 120 B amino acid analyzer.

Results

Increasing molar excesses of TNM progressively abolish the activity of aldolase toward FDP (Figure 1). The protein precipitates when more than a 40-fold M excess of TNM is employed at low ionic strength (0.05 M Tris). However, in the presence of 0.3 M NaCl nitration can be carried out with up to a 60-fold M excess of reagent without precipitation. At pH 8.0 and at 20°, a 23-fold M excess of TNM almost completely inactivates aldolase in 20 min.

The loss of activity is the same whether FDP or F-1-P

TABLE I: Inactivation of Aldolase by TNM. Activities toward FDP and F-1-P.[a]

Min after first Addn of TNM	% Act.	
	FDP	F-1-P
5	70	70
10	64	62
20	36	40
25	32	30

[a] To 2.0 mg of aldolase in 4 ml of 0.05 M Tris-Cl–0.3 M NaCl (pH 8.0), 25°, was added 25 μl (6.3-fold M excess) of a 0.0034 M solution of TNM in 95% ethanol. The same amount of TNM was added again after 15 min. At the indicated times aliquots were assayed for activity toward FDP and F-1-P.

TABLE II: Amino Acid Analysis of TNM-Inactivated Aldolase.[a]

	Native Aldolase	+8-fold M Excess TNM, 61% FDP Act.	+25-fold M Excess TNM, 1% FDP Act.
Lys	105.0	106.0	105.4
His	42.9	43.6	42.2
Arg	60.5	58.8	60.9
Cysteic acid	0	1.5	4.9
Asp	114.7	114.8	115.3
Thr	82.2	81.3	83.6
Ser	78.6	79.8	78.0
Glu	152.2	155.0	151.2
Pro[b]	88.0	84.5	69.7
Gly	123.0	117.2	122.2
Ala	165.2	163.9	168.3
Cys/2	2.8	14.5	19.0
Val	61.0	62.4	59.8
Met	12.7	12.4	12.1
Ile	57.0	58.6	56.3
Leu	131.4	129.7	128.0
Tyr	41.2	41.9	41.3
Phe	27.4	27.4	27.8

[a] Aldolase (5.47 mg) in 1 ml of 0.05 M Tris-Cl–0.3 M NaCl, pH 8.0, was allowed to react with an 8- or 25-fold M excess of TNM at 25° for 15 min. Aliquots were then assayed for FDP activity. The samples were dialyzed against water, quantitatively transferred to hydrolysis tubes, and evaporated to dryness in a rotatory evaporator prior to hydrolysis. The values are given as moles per mole of aldolase. [b] Cysteine also emerges in the position of proline.

is employed as substrate (Table I). Tyrosyl modification does not affect F-1-P activity (Pugh and Horecker, 1966) but sulfhydryl modification does (Kobashi *et al.*, 1967). Hence, these data indicate that the inactivation may be due to sulfhydryl modification rather than to nitration of tyrosyl residues.

The change in aldolase activity produced by varying both the time of reaction and molar excess of TNM employed was examined, therefore, as a function of the free sulfhydryl group content of the enzyme and of the degree of tyrosyl nitration (Figure 2). The number of sulfhydryl groups oxidized correlates well with the degree of inactivation. Oxidation of thiol groups proceeds much faster than nitration of tyrosyl residues. When 8 to 10 thiol groups have been lost, almost all of the aldolase activity is destroyed while only 0.6 tyrosyl residue has been nitrated. On incubation with higher molar excesses of TNM up to five tyrosyl residues can be modified but no further loss of thiol groups occurs under these conditions (Figure 3). Attempts to nitrate all of the "free" tyrosyl residues of aldolase by repeated additions of TNM (Sokolovsky *et al.*, 1966) precipitated the protein. With a 23-fold M excess of TNM, no residues other than cysteinyl or tyrosyl were modified, as determined by amino acid analysis (Table II). The apparent change in "proline" content is a reflection of cysteine oxidation (*vide infra*). The absorption spectrum of TNM-inactivated aldolase was identical with that of the native enzyme from 240 to 600 mμ except for small peaks at 428 and 290 mμ due to the 0.6 residue of nitrotyrosine/molecule. Further evidence that TNM inactivates aldolase through oxidation of sulfhydryl groups was obtained using carboxypeptidase-treated aldolase. This enzyme was inactivated at the same rate as the native enzyme (Table III). Moreover, the rate of reaction with TNM, as measured by the increase in absorbance at 350 mμ due to the formation of nitroform, was identical for the two enzymes. The production of nitroform observed with both native and carboxypeptidase-treated aldolase is greater than would be expected from the degree of sulfhydryl plus tyrosine modification (*vide infra*).

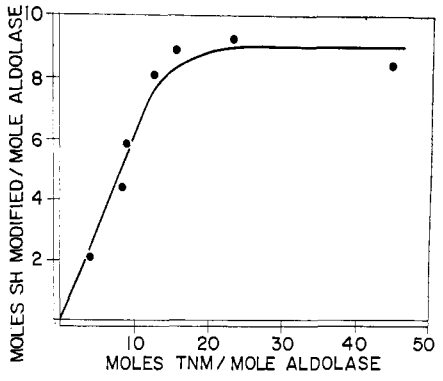

FIGURE 3: Inactivation of aldolase by TNM. Number of sulfhydryl groups modified *vs.* molar excess of TNM. Reaction conditions as in legend of Figure 2. Appropriate aliquots of a 0.034 M solution of TNM in 95% ethanol were added. After 15 min the protein solution was passed through a Bio-Gel P-4 column and sulfhydryl groups were determined (*cf.* legend of Figure 2).

The rate of inactivation of aldolase is substantially reduced by the presence of inhibitors or substrates. Phosphate and sulfate, both of which are competitive inhibitors of aldolase (Kowal *et al.*, 1965), protect against inactivation, as does FDP (Table IV). In the presence of FDP and aldolase, the breakdown of tetranitromethane is enhanced significantly, the rate of nitroform production being greater than the sum of the rates found in the presence of either one of these components (*vide infra*). This phenomenon is accompanied by the appearance of a new protein absorption maximum at 370 mμ.

With model sulfhydryl compounds the reaction of TNM proceeds almost exclusively to the formation of disulfides. With aldolase this does not appear to be the case. Inactive aldolase is only partially reactivated by incubation with mercaptoethanol (Table V), and the degree of reactivation is inversely proportional to the number of sulfhydryl groups which have been oxidized. Increasing the mercaptoethanol concentration or time of incubation did not produce further reactivation.

TABLE III: Reaction of Native and Carboxypeptidase-Treated Aldolase with TNM. Inactivation and Production of Nitroform.[a]

	% FDP Act.		ΔA_{350}	
Min	Native	Carboxypeptidase Treated	Native	Carboxypeptidase Treated
0	100	100		
4	15	9	0.485	0.462
11	4	7	0.669	0.636
22	1	2	0.870	0.837

[a] Aldolase (0.5 mg/ml) was incubated with carboxypeptidase A (120:1, w/w) in 0.05 M Tris-Cl–0.3 M NaCl (pH 8.0) at room temperature for 20 min and then dialyzed overnight against the same buffer. After dialysis the activity toward FDP was 4% of the native enzyme. To 2 ml of native and of carboxypeptidase-treated aldolase in spectrophotometer cuvets was added an aliquot of a 0.034 M solution of TNM in 95% ethanol to give a 100-fold M excess. At the indicated times an aliquot was withdrawn and assayed for FDP activity. The zero-time activity of each enzyme is taken as 100%.

In model compounds the oxidation of thiol groups by TNM is independent of pH between 6.0 and 9.0. With aldolase, however, the rate of inactivation is strongly dependent on pH (Figure 4A). Only 10% of the activity is lost after 10-min exposure to a 25-fold M excess of TNM at pH 6.0, while over the same time interval activity has virtually disappeared at pH 9.0. A plot of activity remaining after 5-min reaction *vs.* pH gives a sigmoid curve with an inflection at pH 7.4 (Figure 4B).

The relationship between the degree of inactivation

TABLE IV: Inactivation of Aldolase by TNM. Protection by Substrate and Inhibitors.[a]

Addition	12-fold M Excess of TNM, % Act.	40-fold M Excess of TNM, % Act.
None	42	0
Sulfate	58	
Phosphate	69	15
FDP	87	48

[a] Aldolase (0.5 mg/ml) was incubated at 25° in 0.05 M Tris-Cl–0.3 M NaCl (pH 8.0) containing 0.01 M FDP, 0.1 M phosphate, or 0.09 M sulfate with a 12- and 40-fold M excess of TNM. After 30 min, aliquots were assayed for activity toward FDP.

TABLE V: Reactivation of Tetranitromethane–Inactivated Aldolase by 2-Mercaptoethanol.[a]

Residual Act. after Reaction with TNM (%)	Act. after Incubn with ME (%)	% Reactiv $\left(\frac{\text{Act. Recov}}{\text{Act. Lost}} \times 100\right)$
77	91	61
57	70	30
48	60	23
36	50	22
1	7	6

[a] Aldolase was incubated with tetranitromethane as described in the legend of Figure 2. After gel filtration on a Bio-Gel P-4 column one aliquot was taken for activity assay, and another was adjusted to a final concentration of 0.1 M 2-mercaptoethanol. Activity of the second aliquot was determined after 30-min incubation at 0°.

of aldolase and loss of free sulfhydryl groups is the same under all conditions of modification with TNM. If the reaction is carried out at pH 6.0 or 8.0, in the presence or absence of FDP, or with varying molar excesses of TNM over different periods of time, loss of activity is always proportional to the extent of oxidation of free sulfhydryl groups (Figure 5). Complete inactivation occurs when eight to ten sulfhydryl groups per mole have been modified.

Discussion

Reaction of aldolase with low molar excesses of TNM results in a rapid loss of free sulfhydryl groups and a concomitant abolition of both FDP and F-1-P activities. This inactivating modification appears to be limited to sulfhydryl groups; less than one tyrosyl residue is nitrated under conditions which oxidize all the sulfhydryl groups available for modification. This is consistent with previous findings demonstrating that the oxidation of glutathione by TNM proceeds approximately ten times faster than nitration of *N*-acetyltyrosine (Sokolovsky *et al.*, 1966). It probably does not reflect any difference in the availability of sulfhydryl *vs.* tyrosyl residues in aldolase, since both types of residues are readily acetylated with acetylimidazole (Pugh and Horecker, 1967).

Inactivation of aldolase is essentially complete when eight to ten thiol groups have been modified (Figure 2). With silver, 28 to 29 sulfhydryl groups are titratable (Benesch *et al.*, 1955) while only 10 react readily with *p*-mercuribenzoate (Swenson and Boyer, 1957). These 10 residues may be considered to be "free" or exposed while the remaining 18–19 seem to be "buried." None of the buried sulfhydryl groups appear to react with TNM, since there is no change in the number of sulfhydryl groups oxidized as the molar ratio of TNM to aldolase is increased from 16 to 45 (Figure 3).

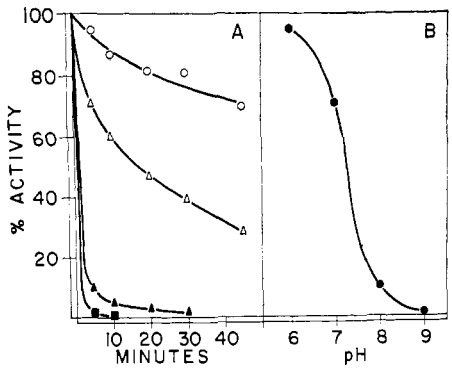

FIGURE 4: Inactivation of aldolase by TNM. Dependence on pH. The reaction mixture contained 0.5 mg/ml of aldolase in 0.3 M NaCl, 0.05 M sodium citrate (pH 6, ○), or 0.05 M Tris-Cl (pH 7, △; 8, ▲; and 9, ■) 25°. A 25-fold M excess of TNM was added and at the indicated times aliquots were assayed for activity toward FDP (A). In part B the activity remaining after 5-min reaction is plotted *vs.* pH.

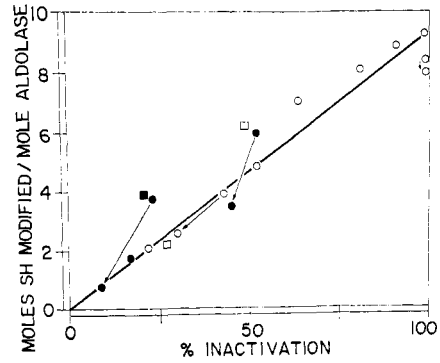

FIGURE 5: Inactivation of aldolase by TNM at pH 6 and 8, in the presence and absence of substrate, and reactivation with mercaptoethanol; degree of inactivation *vs.* number of modified cysteinyl and tyrosyl residues. The inactivation reactions at pH 8 (○) were carried out as described in the legend of Figure 2; the reactions at pH 6 (□) were in 0.05 M sodium citrate–0.3 M NaCl. FDP (0.01 M) was present as indicated (● at pH 8, ■ at pH 6). Activity and sulfhydryl content were determined as in Figure 2. The arrows indicate changes in activity on treatment of inactivated aldolase with 0.1 M 2-mercaptoethanol (*cf.* Table V). After incubation and prior to the determination of sulfhydryl groups and activity the samples were dialyzed exhaustively against 0.05 M Tris-Cl–0.3 M NaCl (pH 8.0).

All of the sulfhydryl groups appear to react with TNM at the same rate and inactivation is directly proportional to their degree of oxidation. Moreover, the same relationship holds for all conditions of modification. Thus, if the reaction with TNM is carried out either at pH 6.0 or 8.0, in the presence or absence of a substrate, loss of activity correlates with oxidation of sulfhydryl groups. Extrapolation to zero activity always indicates the involvement of eight to ten residues (Figure 2). While this could mean that all eight to ten of these groups are involved in catalytic activity, only some of them may be essential but exhibit the same reactivity toward TNM as the others.

In previous studies not all of the free sulfhydryl groups were found to be important to catalytic function. Reaction of ten sulfhydryl groups with *p*-mercuribenzoate did not affect activity (Swenson and Boyer, 1957). Similarly, the changes in activity accompanying reaction of aldolase with *N*-acetylimidazole were not brought about by acetylation of ten sulfhydryl groups but rather by tyrosyl modification (Pugh and Horecker, 1967). Arylation of three to four thiols with chlorodinitrobenzene did not alter activity, but modification of an additional eight residues led to complete inactivation (Kowal *et al.*, 1965). Complete inactivation was also observed on reaction of 14 sulfhydryl groups with either carboxyethyl disulfide or *N*-ethylmaleimide (Kowal *et al.*, 1965). The same results were encountered when four to five intrachain disulfide bonds were formed by autoxidation catalyzed by a cupric-*o*-phenanthroline complex (Kobashi and Horecker, 1967). Apparently the nature of the chemical modification of sulfhydryl groups of aldolase is a significant variable in conditioning loss of activity since some modification will affect activity while others do not. The interesting questions raised by these circumstances cannot be answered at present. The causes may be related to changes in structure accompanying modification, to the nature of the product formed in each instance, or to the particular sulfhydryl groups being modified.

A 23-fold M excess of TNM, the amount required for virtually complete inactivation, does not modify other residues of aldolase to any significant extent. Only small differences in amino acid composition are noted between native and TNM-modified aldolases (Table II). The apparent change in "proline" content is really due to a loss of cysteine, which elutes in the position of "proline" under the usual conditions of amino acid analysis (Moore and Stein, 1963) and as evidenced by a change in A_{440}/A_{670} on the recorder tracing. Loss of "proline" is accompanied by a corresponding increase in both cystine and cysteic acid. Less than one tyrosyl residue is nitrated with a 23-fold M excess of TNM and only five tyrosyl residues are nitrated when a 45-fold M excess is employed, less than either the total number of tyrosines in aldolase, *i.e.*, 41 to 42 (Table II) or even the number of those that are thought to be "free" (Donovan, 1964). This is not meant to imply, of course, that five is the maximal number of tyrosyl residues in aldolase which can be nitrated. However, attempts to achieve a greater degree of nitration thus far have always resulted in precipitation; hence, a maximal value could not be determined.

The oxidation of glutathione by TNM was shown to proceed almost exclusively to the formation of the disulfide. Such does not seem to be the case with the sulfhydryl groups of aldolase. Only a small fraction of the loss in activity can be reversed on treatment with mercaptoethanol (Table V) whereas total reactivation would be expected if all of the sulfhydryl groups had been oxidized to disulfides (Kobashi and Horecker, 1967). Hence, some other oxidized species would seem to be formed. If the first step in the oxidation of sulfhydryl groups involves the formation of a sulfenyl nitrate, then various products could arise by further interactions of this intermediate. Reaction with a second sulfhydryl group would lead to a disulfide bond, while reaction with hydroxide ions could result in other oxidized prod-

ucts. Amino acid analysis indicates increasing amounts of cysteic acid as a function of the molar excess of TNM employed. There is a reasonable correlation between cysteic acid content and thiol groups oxidized.

The extent to which TNM-inactivated aldolase can be reactivated with mercaptoethanol seems to be inversely related to the degree of inactivation. Thus, if only 23% of the native activity is lost, treatment with mercaptoethanol restores all but 9%. Hence, 61% of the lost activity is recovered. However, only 6% can be recovered after virtually complete inactivation (Table V). This suggests that low molar excesses of TNM result in greater disulfide-bond formation. This is to be expected, of course, since under such conditions the number of free sulfhydryl groups available to react with the proposed sulfenyl nitrate intermediate to give a disulfide would be much greater than when high molar excesses of TNM are employed.

Tritium-exchange studies have demonstrated that carboxypeptidase digestion of aldolase affects the rate of proton addition to the carbanionic species of the enzyme–substrate complex (Rose *et al.*, 1965). The activity toward FDP of the modified enzyme is about 5% that of the native enzyme while its activity toward F-1-P is unchanged (Drechsler *et al.*, 1959). The residual activity of carboxypeptidase-treated aldolase to both of these substrates is abolished on addition of TNM. Moreover, the per cent inactivation obtained with a given excess of TNM is identical with that observed for native enzyme (Table II). Hence, some other step in the enzymatic mechanism appears to be altered by TNM oxidation.

Studies on the pH dependence of the nitration of tyrosine either in proteins or in model compounds have demonstrated that only the phenolate species reacts with TNM. Nitration does not occur below pH 7.0. On the other hand, oxidation of glutathione was found to proceed equally well between pH 6.0 and 9.0 (Riordan *et al.*, 1966; Sokolvsky *et al.*, 1966). However, the inactivation of aldolase by TNM, due to sulfhydryl oxidation, is markedly dependent on pH (Figure 4). A plot of activity remaining after 5 min *vs.* pH is sigmoidal with an inflection at pH 7.4. The significance of this pH dependence is not immediately apparent. The studies with model compounds demonstrate that it is not due to any change in the intrinsic reactivity of TNM. It could reflect the pK' of the eight to ten reactive sulfhydryl groups, though reaction of glutathione with TNM proceeds quite readily with the undissociated species. Alternatively, the pH dependence may indicate the participation of some other ionizable group in the reaction of TNM with the sulfhydryl groups. Further, there may be a change in the availability of the sulfhydryl groups as the pH is lowered due to conformational alterations. Thus, while pH dependence can differentiate the reaction of TNM with tyrosyl *vs.* sulfhydryl groups of *model* compounds, this criterion will not in all instances pertain to *proteins*.

The protective effect of substrates and inhibitors on the inactivation of aldolase by TNM (Table IV) suggests that the sulfhydryl groups may be components of the active center. On the other hand substrates and inhibitors may induce structural changes in the enzyme which render the sulfhydryl groups unavailable for reaction. These two possibilities have not been resolved.

One of the by-products of the reaction of proteins with TNM is nitroform. It has been observed that the amount of nitroform produced is greater than can be accounted for by tyrosyl nitration and sulfhydryl oxidation. This has been attributed to a catalytic breakdown by TNM by side-chain groups of the protein (Sokolovsky *et al.*, 1966). Excessive production of nitroform was also found with native and carboxypeptidase-treated aldolase (Table III) and was especially marked when the TNM reaction was carried out in the presence of substrate. In the latter instance, however, the effect has been shown to be due to an interaction of TNM with an aldolase–substrate intermediate. This will be the subject of a separate communication.

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References

Baranowski, T., and Niederland, T. R. (1949), *J. Biol. Chem. 180*, 543.

Benesch, R. E., Lardy, H. A., and Benesch, R. (1955), *J. Biol. Chem. 216*, 663.

Donovan, J. W. (1964), *Biochemistry 3*, 67.

Drechsler, E. R., Boyer, P. D., and Kowalsky, A. G. (1959), *J. Biol. Chem. 234*, 2627.

Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82*, 70.

Kawahara, K., and Tanford, C. (1966), *Biochemistry 5*, 1578.

Kobashi, K., and Horecker, B. L. (1967), *Arch. Biochem. Biophys. 121*, 178.

Kowal, J., Cremona, T., and Horecker, B. L. (1965), *J. Biol. Chem. 240*, 2485.

Moore, S., and Stein, W. H. (1963), *Methods Enzymol. 6*, 827.

Pugh, E. L., and Horecker, B. L. (1967), *Arch. Biochem. Biophys. 122*, 196.

Racker, E. (1947), *J. Biol. Chem. 167*, 843.

Riordan, J. F., Sokolovsky, M., and Vallee, B. L. (1966), *J. Am. Chem. Soc. 88*, 4104.

Riordan, J. F., Sokolovsky, M., and Vallee, B. L. (1967), *Biochemistry 6*, 358.

Rose, I. A., O'Connell, E. L., and Mehler, A. H. (1965), *J. Biol. Chem. 240*, 1758.

Schmid, A., Christen, P., and Leuthardt, F. (1966), *Helv. Chim. Acta 49*, 281.

Sokolovsky, M., Riordan, J. F., and Vallee, B. L. (1966), *Biochemistry 5*, 3582.

Swenson, A. D., and Boyer, P. D. (1957), *J. Am. Chem. Soc. 79*, 2174.