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Reaction of Tetranitromethane with Sulfhydryl Groups in Proteins*

Mordechai Sokolovsky, Daniela Harell, and James F. Riordan

ABSTRACT: The oxidation of sulfhydryl groups with tetranitromethane has been examined using a series of model compounds. The reaction stoichiometry was determined colorimetrically by measuring the formation of nitroformate (ϵ_{350} 14,400) as well as by analysis of the other reaction products. Depending on the particular thiol studied and the molar excess of tetranitromethane employed, the course of the reaction leads mainly to a disulfide or to an acidic derivative. The acidic product has been identified as a sulfinic acid by chromatographic and electrophoretic analyses and by its conversion into a sulfonic acid on oxidation with iodine. The various factors which determine the relative amounts of the two reaction products are discussed. Oxidation of protein sulfhydryl groups with tetranitromethane results primarily in disulfide formation. No indication of interprotein chain peptide-bond formation mediated by tetranitromethane could be detected. Reaction of histidine, tryptophan, and methionine peptides with tetranitromethane was observed under certain conditions demonstrating the need for caution when using this reagent to modify proteins.

Nitration with tetranitromethane has proven to be a useful procedure for the investigation of tyrosyl residues of proteins (Riordan *et al.*, 1966; Sokolovsky *et al.*, 1966). However, the reagent is not totally selective for tyrosine. Specificity studies have shown that other residues are potentially reactive toward tetranitromethane (Sokolovsky *et al.*, 1966). In particular, sulfhydryl groups of model compounds such as, *e.g.*, glutathione, are oxidized readily to yield disulfides as the major product. Recently, it was found that tetranitromethane inactivates rabbit muscle aldoase by oxidation of thiol groups but disulfide-bond formation did not appear to represent the principal reaction pathway (Riordan and Christen, 1968). A number of reactions are known to modify thiol groups of aldolase and cause inactivation (Swenson and Boyer, 1957; Kowal *et al.*, 1965; Kobashi and Horecker, 1967). In instances where disulfide-bond formation is involved, reduction by mercaptoethanol generally leads to a complete restoration of activity (Kobashi and Horecker, 1967). However, exposure of tetranitromethane-inactivated aldolase to mercaptoethanol reactivated the enzyme only partially. The degree of reactivation seemed to be related to the extent of inactivation. Slightly inactive enzyme could be restored to virtually native activity but the enzyme with little residual activity could hardly be reactivated at all. Hence, some product other than a disulfide was thought to be formed. In order to elucidate the possible oxidative pathways, studies on the reaction of tetranitromethane with sulfhydryl groups in model compounds have now been carried out.

The conditions previously adopted to examine the specificity of the reagent toward other amino acid residues had been based on results with carboxypeptidase A (Riordan *et al.*, 1967). A fourfold molar excess of tetranitromethane was sufficient to modify one tyrosyl residue in this enzyme, which does not contain free thiol groups, with concomitant alterations of esterase and peptidase activities. Further, reaction of this tyrosyl residue was most favorable at pH 8.0 but the effect of pH on the specificity of the reagent was not examined

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extensively. The present studies are intended to delineate further the specificity of the reagent over a broader range of conditions and applications.

Materials

Tetranitromethane was obtained from Aldrich Chemical Co. or from Fluka, A. G., Switzerland; reduced and oxidized glutathione from Schwartz Bioresearch Laboratory; cysteine-HCl, cystine, and 5,5′-dithiobis(2-nitrobenzoic acid), from Nutritional Biochemicals; and mercaptoethanol and thioglycolic acid from Baker Laboratory Chemicals. *N*-Carbobenzoxyglutathione (143–144°) and *N*-carbobenzoxyglutathione dibenzyl ester (158–159°) were prepared as described previously (Sokolovsky *et al.*, 1964). Ribonuclease, pepsin, lysozyme, and aldolase were obtained from Worthington Biochemical Corp.

Reduced ribonuclease and lysozyme were prepared according to the procedures of Haber and Anfinsen (1962). The sulfonic acid derivative of reduced glutathione was obtained by performic acid oxidation.

Methods

The reactions of tetranitromethane can be followed conveniently by virtue of the high absorptivity of the product, nitroformate (Christen and Riordan, 1968). Stock solutions of tetranitromethane in 95% ethanol were used within 30 min after preparation. Reactions were carried out at 20° and were followed by measuring the change in absorption at 350 mμ using 1-cm cuvets. Spectra were obtained with an automatic recording Cary Model 15 spectrophotometer while absorption at single wavelengths was determined with a Beckman DB spectrophotometer.

Electrophoresis on Whatman No. 3MM paper was run at 50 V/cm in pyridine–acetate buffer (pH 3.6) using a Savant HVE flat-plate Model FP-22A apparatus. Amino acid analyses were carried out on a Beckman Unichrome amino acid analyzer according to the procedures of Spackman *et al.* (1958). Sulfhydryl groups were determined by reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman, 1959).

Results

The nitroformate ion, a product of the reaction of tetranitromethane with thiols, absorbs intensely at 350 nm (ε 14,400) (Glover and Landsman, 1964) and, hence, its formation can be employed both to follow the time course and to estimate the stoichiometry of the reaction. By this criterion oxidized glutathione does not react with tenfold molar excess of tetranitromethane during the first 20 min of incubation in 0.1 M acetate buffer, at pH 5.5. However, similar treatment of reduced glutathione results in maximum nitroformate production within 4 min (Figure 1). The increase in absorbance at 350 mμ indicates that more than 1 mole of nitroformate is produced per 2 moles of reduced glutathione. The increase in absorbance over and above that expected for the formation of oxidized glutathione could be due to either a secondary or an alternate reaction pathway(s) or both. These possibilities were examined by ion-exchange chromatography of the reaction mixture in order to identify the nature and number of products formed.

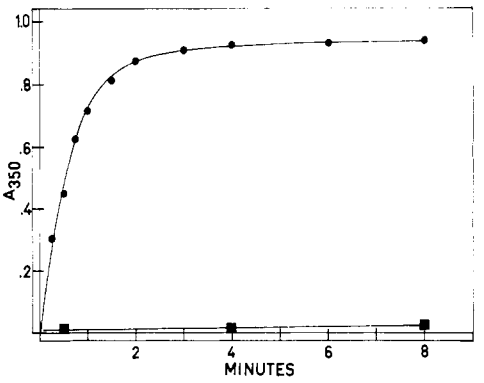

FIGURE 1: Increase in A_{350} on addition of a tenfold molar excess of tetranitromethane to 10^{-4} M reduced glutathione (●) and oxidized glutathione (■) in 0.1 M acetate, pH 5.5, 20°.

Reduced glutathione emerges from the long column of the amino acid analyzer as a single peak at 85 ml, separate and distinct from oxidized glutathione which emerges at 105 ml (Figure 2). The major product of the tetranitromethane–glutathione reaction elutes in the position of oxidized glutathione (Figure 2C) establishing a reduced glutathione → oxidized glutathione pathway. Another component emerges at 55 ml. Its acidic behavior suggests that it might be analogous to cysteic acid. The relative proportions of oxidized glutathione and of the acidic components change as a function of the molar excess of tetranitromethane employed (Table I). As the tetranitromethane concentration is increased, there is a corresponding increase in the amount of acidic component and a decrease in the amount of disulfide indicative of a second oxidative pathway.

The products of the reaction were also examined by high-voltage paper electrophoresis at pH 3.6 where the reduced and oxidized forms of glutathione are separated readily (Figure 3). Treatment of reduced glutathione with a tenfold molar excess of tetranitromethane leads to the formation of a

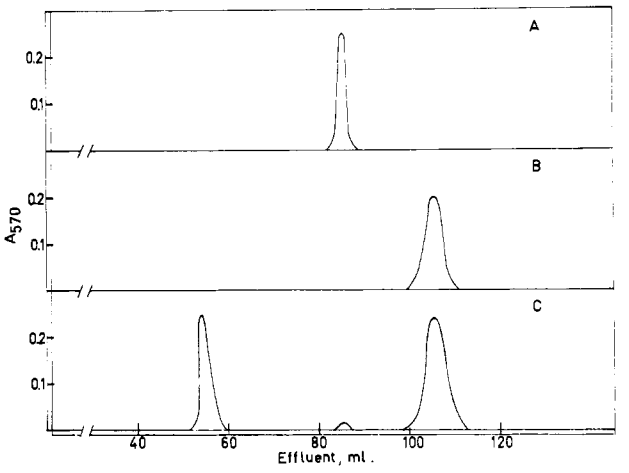

FIGURE 2: Chromatographic analysis (Spackman *et al.*, 1958) of the reaction between reduced glutathione and tetranitromethane on the long column of Beckman Unichrome amino acid analyzer. (A) Unreacted reduced glutathione, (B) unreacted oxidized glutathione, and (C) the reaction mixture of reduced glutathione and a tenfold molar excess of tetranitromethane.

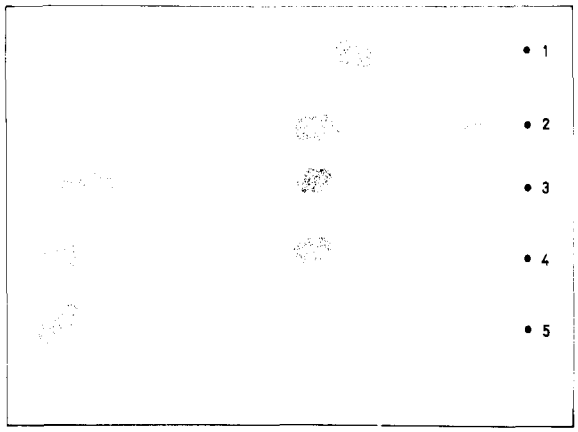

FIGURE 3: High-voltage paper electrophoresis in pyridine-acetate buffer (pH 3.6), 50 V/cm, 105 min, 50–80 mA. Mobility is from right to left, the points of application being indicated by the numbered spots: (1) reduced glutathione, (2) oxidized glutathione, (3) reduced glutathione reacted with tetranitromethane, (4) sample 3 oxidized with iodine, and (5) sample 2 oxidized with performic acid.

considerable amount of material in the position of oxidized glutathione as well as a small amount of a more acidic component. The mobility of this latter product is less than that of the sulfonic acid derivative of reduced glutathione. However, oxidation with iodine converts this product into one having the mobility of glutathione sulfonic acid.

A number of other sulfhydryl model compounds were reacted with tetranitromethane. Based on the production of nitroformate, the reaction of thioglycolic acid, cystamine, mercaptoethanol, and *N*-carbobenzoxyglutathione dibenzyl ester all yield close to 1 mole of nitroformate/2 moles of thiol. On the other hand, excess nitroformate production, as observed with glutathione, is also found with cysteine and *N*-carbobenzoxyglutathione (Table II).

The reaction of cysteamine and cysteine with tetranitromethane was compared by high-voltage paper electrophoresis (Figure 4). Virtually all of the reaction product derived from cysteamine has a mobility similar to that of the disulfide.

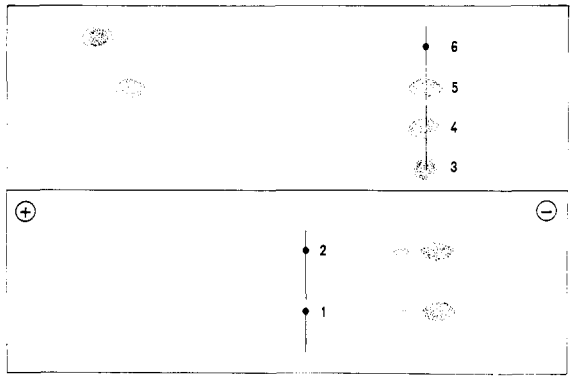

FIGURE 4: High-voltage paper electrophoresis in pyridine-acetate buffer (pH 3.6), 50 V/cm, 50–80 mA. The points of application are indicated by the numbered spots: (1) cystamine, (2) cysteamine reacted with tetranitromethane, (3) cystine, (4) cysteine, (5) cysteine reacted with tetranitromethane, and (6) cysteic acid. Samples 1 and 2 were run for 20 min while samples 3–6 were run for 105 min.

TABLE I: Formation of Oxidized Glutathione and "Acidic Product" on Oxidation of Reduced Glutathione with Tetranitromethane.

Moles of Tetranitromethane/Mole of Reduced Glutathione	% Oxidized Glutathione	% "Acidic Product"
5	80	20
10	75	25
30	60	40
70	55	45

cystamine. On the other hand, the product of the reaction with cysteine has the mobility of an acidic component, similar to but not identical with cysteic acid.

The reaction of tetranitromethane with sulfhydryl groups was examined with a series of proteins. The disulfide bonds of ribonuclease, pepsin, and lysozyme were reduced by treatment with mercaptoethanol and the products were isolated by gel filtration. Prior to treatment with tetranitromethane their sulfhydryl contents were determined by reaction with the Ellman reagent. Reaction was carried out at pH 5.5 in 0.1 M acetate buffer using a tenfold molar excess of tetranitromethane per SH group at 20° for 8–10 min. Under these conditions no reoxidation was found to occur unless tetranitromethane was present in the reaction mixture. Aldolase, denatured in 8 M urea but without exposure to mercaptoethanol, was examined also. In each case an increase in absorbance at 350 mμ was observed due to the production of nitroformate

TABLE II: Oxidation of Thiols with Tetranitromethane[a]–Nitroformate Production.

Thiol[b]	ΔA_{350}	$\epsilon_{350} \times 10^3$	Moles of $C(NO_2)_3^-$/Mole of SH
Thioglycolic acid	0.69	6.9	0.48
Cystamine	0.73	7.3	0.50
Mercaptoethanol	0.76	7.6	0.53
N-Z-Reduced glutathione dibenzyl ester	0.80	8.0	0.55
Reduced glutathione	0.96	9.6	0.67
Cysteine	1.12	11.2	0.78
N-Z-Reduced glutathione	1.12	11.2	0.78

[a] The reaction was carried out at pH 5.5 in 0.1 M acetate buffer using a tenfold molar excess of tetranitromethane, 20°, 10 min. [b] Prior to reaction the concentration of free thiol (10^{-4} M) was determined with 5,5′-dithiobis(2-nitrobenzoic acid).

TABLE III: Reaction of Proteins with Tetranitromethane[a]–Nitroformate Production.

Protein	SH Content[b]	$\epsilon_{350} \times 10^4$	ϵ_{350}/SH $\times 10^3$	Moles of $C(NO_2)_3^-$/ Moles of SH
Reduced RNase	7.6	6.04	7.9	0.55
Reduced lysozyme	10.1	8.19	8.1	0.56
Reduced pepsin	6.1	4.91	8.0	0.55
Aldolase[c]	29	24.1	8.3	0.57
Bovine serum albumin	1.0	10.1	10.1	0.70

[a] The reaction was carried out at pH 5.5 in 0.1 M acetate buffer with a tenfold molar excess of tetranitromethane per mole of thiol, 20°, 8–10 min. [b] As determined with 5,5′-dithiobis(2-nitrobenzoic acid). [c] Reaction with tetranitromethane carried out in 8 M urea.

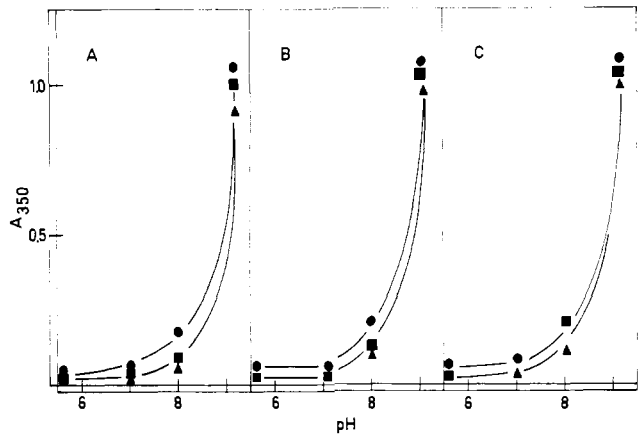

FIGURE 5: Changes in A_{350} on reaction of 10^{-4} M Z-Try-NH_2 (A), Z-Met-Ala-OBz (B), and Z-His-Leu (C) with a (▲) 50-, (■) 100-, and (●) 200-fold molar excess of tetranitromethane in 0.1 M acetate (pH 5.5), 0.05 M Tris (pH 7.0 and 8.0), and in 0.05 M Veronal (pH 9.2), all for 2 hr, 20°.

(Table III, column 2). The molar absorptivity increase per SH group was determined based on the measured sulfhydryl content of the proteins prior to oxidation with tetranitromethane (Table III, column 3). The value exceeds that expected if disulfide-bond formation were to take place exclusively. Treatment of native ribonuclease, lysozyme, or pepsin with tetranitromethane did not result in changes in absorbance at 350 mμ.

Oxidation of reduced ribonuclease by tetranitromethane gave a product which was inactive against RNA, and was heterogeneous with regard to molecular weight when examined by gel filtration on G-75 Sephadex in 0.1 M acetic acid. However, after exposure to mercaptoethanol in 8 M urea, the protein eluted in the position of the initial reduced ribonuclease and heavier material could no longer be detected.

The effects of large molar excesses of tetranitromethane on histidine, methionine, and tryptophan derivatives were examined. At pH 7.0 or below virtually no reaction of these three amino acids can be detected with a 200-fold molar excess of tetranitromethane (Figure 5). However, above pH 7.0 there is rapid production of nitroformate which is somewhat slower when 50- and 100-fold molar excesses are employed.

Discussion

The stoichiometry of the reaction of tetranitromethane with thiols deduced from nitroformate production and proton release had initially suggested Scheme I (Sokolovsky *et al.*, 1966). An intermediate sulfenyl nitrate was postulated to react with a second mole of thiol to form a disulfide releasing nitrate ions. According to this scheme, 1 mole of tetranitromethane reacts to produce 1 mole of nitroformate/2 moles of thiol. If this scheme were valid, nitroformate could be employed to gauge the stoichiometry of the reaction.

In fact, the reaction of tetranitromethane with glutathione produces more than 1 mole of nitroformate/mole of thiol group. The excess production of nitroformate must arise from the oxidation of thiol groups to derivatives other than disulfides. While oxidized glutathione is the major reaction product, ion-exchange chromatography reveals some material having the elution properties of a strongly acidic amino acid. High-voltage paper electrophoresis indicates that this oxidized species is not identical with that obtained by performic acid oxidation of glutathione. But since the derivative can be converted into a sulfonic acid by iodine oxidation (Simonsen, 1933), it would appear that it is the sulfinic acid derivative of glutathione. The sulfinic acid could arise from the intermediate sulfenyl nitrate *via* displacement of nitrate by a general base catalyzed hydrolysis. Under the reaction conditions employed oxidation of the sulfenic to the sulfinic acid derivative would occur spontaneously (Scheme II). The reaction of tetranitromethane might be considered analogous to the reaction of iodine with the sulfhydryl group of TMV protein (Fraenkel-Conrat, 1955) or glyceraldehyde 3-phosphate dehydrogenase (Parker and Allison, 1969) in which stable sulfenyl iodine intermediates have been found.

If products other than disulfide or the proposed sulfinic acid intermediate are formed, they occur in very small quantities and, hence, do not constitute a major reaction pathway. This is also indicated by the fact that the nitro-

SCHEME I

$$RSH + C(NO_2)_4 \longrightarrow RSNO_2 + C(NO_2)_3^- + H^+$$

$$RSNO_2 + RSH \longrightarrow RSSR + NO_2^- + H^+$$

SCHEME II

$$RSNO_2 + H_2O \longrightarrow RSOH + NO_2^- + H^+$$

$$RSOH + 0.5O_2 \longrightarrow RSO_2H$$

formate production correlates quite well with the scheme proposed when 1 mole of nitroformate is produced per mole of disulfide, and another mole is produced per mole of sulfinic acid.

The relationship between the amount of disulfide produced, on the one hand, and of sulfinic acid, on the other, is dependent both on the molar excess of reagent employed and on the nature of the thiol compound studied. This is to be expected based on the mechanism of Scheme II. The ratio of disulfide to sulfinic acid depends on the relative rates of reaction of the sulfenyl nitrate with thiol or water. Increasing concentrations of tetranitromethane would lead to an increase in the amount of $RSNO_2$ but also decrease the free thiol, RSH. This would slow down the first reaction and hence lower the ratio of disulfide to sulfenic acid. The second reaction, the general base catalyzed hydrolysis of the intermediate sulfenyl nitrate, will depend not only on the concentration of intermediate but also on the concentration and nature of the general base. This may explain the differences observed between thiol models and proteins as well as between the models themselves. Thus, for example, the thiol itself can act either as a nucleophile or as a general base to give a disulfide or sulfenic acid, respectively.

The reaction of tetranitromethane with sulfhydryl groups in proteins leads largely to the formation of disulfides and is thus much more analogous to the reaction of cysteamine than of glutathione. For each of the proteins studied, more than 90% of the reaction proceeds in the direction of disulfide-bond formation, based on the extent to which nitroform exceeds the expected 2:1 stoichiometry. In fact, the apparent absorptivity of 8000 (Table III) might even be useful to quantitate the sulfhydryl content of proteins and perhaps even to distinguish between free and buried sulfhydryl groups, though obviously this would not be totally reliable. The results with fructose 1,6-diphosphate aldolase were quite different (Riordan and Christen, 1968). With this enzyme, virtually all of the 8–10 active-center sulfhydryl groups were oxidized to the sulfinic acid as evidenced by the lack of reactivation of the modified enzyme on treatment with mercaptoethanol. Similar results have recently been reported for 2-keto-4-hydroxyglutarate aldolase (Lane, 1969). It might be significant that for both of these enzymes the active center contains a reactive lysyl residue.

These studies on the specificities of the reaction of tetranitromethane with thiols were carried out at pH 5.5. Preliminary investigation had shown that this reaction is independent of pH between 5 and 9 (Riordan *et al.*, 1966). Below pH 5 the rate of reaction falls off with pH. Moreover, at pH 4 and below, nitrite, a by-product of the reaction, becomes a potential problem since nitrous acid can react with thiol, amino, and phenolic groups. On the other hand, the pH must be kept below neutrality to avoid reaction of ionized tyrosyl residues. Consequently, pH 5.5 was selected as most suitable for the reaction. At this pH a tenfold molar excess of sodium nitrite was found to have no effect on the tetranitromethane–thiol reaction.

Since the tetranitromethane reaction is quite specific at pH 5.5, all of the nitroformate produced must be derived from thiol reactions. Certain sulfhydryl groups depending on their environment may be particularly sensitive to the formation of the sulfinic acid, however. In addition, it is possible that in some proteins unusually reactive tyrosyl or other residues might react with tetranitromethane even at pH 5.5. In other instances, steric factors may prevent disulfide formation thus interfering with the detection of sulfhydryl groups. In such instances, production of nitroformate would be misleading.

The influence of environmental factors on the nature of the product obtained on reaction of protein sulfhydryl groups with certain reagents has been observed in other instances. Under conditions where a series of reagents oxidized the free sulfhydryl group of Baker's yeast cytochrome *c* without forming disulfides, they converted glutathione into the disulfide dimer (Little and O'Brien, 1966). The sulfhydryl groups of rabbit muscle glyceraldehyde 3-phosphate dehydrogenase have been oxidized with linoleic acid hydroperoxide. Enzyme which had been inactivated by short-term incubation could be fully reactivated by treatment with cysteine, while enzyme inactivated to the same extent by long-term treatment with lower molar excesses of reagent could not be reactivated (Little and O'Brien, 1966).

The formation of disulfide bonds in proteins can be either inter- or intrachain. The results with ribonuclease may be characteristic in this regard. Tetranitromethane oxidation of the reduced protein leads to high molecular weight products as evidenced by gel filtration. Only a small fraction of the product elutes in the position corresponding to the native protein. This material is totally inactive toward RNA. Thus, it would appear that a considerable number of interchain disulfide bonds were formed.

On reduction of tetranitromethane-oxidized ribonuclease with mercaptoethanol all of the heavy material disappeared and was converted into a product having the elution characteristics of the original reduced ribonuclease. It is possible that during the tetranitromethane–thiol reaction nitrate esters might be formed. Such active esters could react with, *e.g.*, amino groups leading into inter- and intramolecular peptide bonds. However, these gel filtration experiments indicate the absence of high molecular weight components after reduction, hence polymerization due to intramolecular peptide-bond formation can be ruled out. However, the possibility that such peptide bonds could form in other proteins remains a possibility. The polymerization reported to occur on nitration of collagen and γ-globulin (Doyle *et al.*, 1968) might be due to such a process although others have not been ruled out.

The reaction of tetranitromethane with tyrosyl residues in proteins carried out at pH 8 with low molar excesses of reagent seems to differ from that observed with model compounds. It has been reported (Bruice *et al.*, 1968) that the yield of nitrophenols observed on reaction with several phenols is less than quantitative and that a number of other by-products are formed *via* a free-radical mechanism. However, in most of the proteins examined so far (Vallee and Riordan, 1969) the major product appears to be 3-nitrotyrosine and the sum of nitrotyrosine plus unreacted tyrosine found by amino acid analysis, equals the total tyrosine content of the proteins. A similar influence of protein environment may lead to the predominance of disulfide-bond formation found in the case of protein sulfhydryl groups.

The effect of high molar excesses of tetranitromethane on the ratio of the disulfide to sulfinic acid production with thiol compounds prompted an analysis of the effect of such high molar excesses of reagent on the reactivity of other

amino acid residues in proteins. Earlier specificity studies were carried out under conditions which had been found optimal for specificity modification of carboxypeptidase A. Such studies failed to reveal that tetranitromethane alters residues such as tryptophan, histidine, and methionine. The reactivity residues, however, have a marked pH dependence. Below pH 8 little or no nitroformate production is observed but above pH 8 it is enhanced considerably. In addition to pH other reaction conditions could also influence the reactivity of these residues particularly in the case of tryptophan (M. Sokolovsky, M. Fuchs, and J. F. Riordan, in preparation). As yet the products of these reactions have not been identified and it is not known if nitration or oxidation constitutes the major pathway. However, preliminary evidence indicates that multiple reaction products are obtained with histidine and tryptophan. Work is in progress to further characterize the products. It should be noted that in most instances studied, thus far, modification of residues other than tyrosine and cystine has not been observed. However, as with virtually all chemical modification procedures, nitration of proteins with tetranitromethane may not always be specific for these residues. The possibility that others, because of their protein environment, might be especially sensitive to tetranitromethane should not be discounted.

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