Tetranitromethane. A Reagent for the Nitration of Tyrosyl Residues in Proteins*

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ABSTRACT: Tetranitromethane (TNM) has been found to be a specific, mild reagent for the nitration of tyrosyl residues of proteins at pH 8. Oxidation of cysteine, the only other amino acid affected, can be differentiated by carrying out the reaction at pH 6 where tyrosine is not altered. By chromatography 3-nitrotyrosine has been identified as the product of the reaction of TNM with tyrosyl-containing peptides and proteins, resulting in an absorption band with a maximum at λ_{max} 428 mμ and molar absorptivity, ϵ 4100. This chromophore serves as a convenient and accurate quantitative measure of the reaction. Optimal conditions for the nitration reaction and its quantitation have been established by comparison of spectrophotometric results with those obtained by amino acid analyses.

The other products of the reaction of TNM with proteins, *i.e.*, nitroform and H, have not been found to be reliable indices for quantitative measurements of the reaction. The number of tyrosyl residues of native proteins which can be nitrated is smaller than that known to be present from total amino acid analyses, and in several instances approximates those thought to be "free" and reactive by different methods.

Procedures for the nitration of stable aromatic compounds are of limited utility for the site-specific labeling of functional groups of enzymes. Few catalytically active proteins can withstand the harsh conditions of such methods which are generally carried out in solutions of concentrated acid (Roberts and Caserio, 1964). Tetranitromethane[1] has been proposed as a nitrating reagent which can be employed under mild conditions (Herriott, 1947). It was first used for the modification of proteins by Wormall (1930) and subsequently by Ehrenberg *et al.* (1945, 1946) and Astrup (1948). In all of these instances the effect on the biological properties of the proteins under study served as the sole criterion for reaction with TNM. Although the chemistry of the reagent has been investigated thoroughly (Schmidt and Fischer, 1920) no studies have ever been reported to identify which functional groups of proteins might react and what the nature of the products might be.

Previous work from this laboratory examined the interaction of TNM with tyrosine and demonstrated the introduction of a nitro reside into the aromatic ring *ortho* to the phenolic hydroxyl group (Riordan *et al.*, 1966a). We have now examined the suitability of TNM as a reagent for nitration of amino acid residues in peptides, polymers, and proteins. The results indicate that the reagent is highly specific for tyrosyl residues, and since reaction conditions are very mild, selective modification is achieved readily. The reaction can be quantitated easily by spectrophotometry since nitrotyrosine absorbs radiation in the visible part of the spectrum and by amino acid analysis. Further, the other products of the reaction, *i.e.*, nitroform and hydrogen ions, can also serve as a means of quantitation (Riordan *et al.*, 1966a). The present study establishes optimal conditions for the nitration of tyrosyl residues in proteins by TNM and examines methods both for their qualitative and quantitative determination.

Materials

Tetranitromethane was obtained from the Aldrich Chemical Co. Tyrosine, tryptophan, tryptophan methyl ester, methionine, *N*-acetyltyrosine, carbobenzoxy-L-methionylglycine, and carbobenzoxyglycyl-L-tryptophan were obtained from Mann Research Laboratories, glutathione from Schwartz Bioresearch Laboratory, and 3-nitrotyrosine and 3,5-dinitrotyrosine from K and K Laboratories. A standard solution containing Lys, His, NH_3, Arg, Asp, Thr, Ser, Glu, Pro, Gly, Ala, Cys-, Val, Met, Ile, Leu, Tyr, and Phe (amino acid calibration mixture, Type 1, for the amino acid analyzer) each at a concentration of 2.5 μmoles/ml was obtained from Beckman Instruments, Spinco Division, Palo Alto, Calif.

N-Acetyl-3-nitro-L-tyrosine. To an ice-cold solution of 1.13 g (5 mmoles) of 3-nitro-L-tyrosine in 30 ml of water was added 2.55 g (25 mmoles) of acetic anhydride and the pH was maintained at 7.5 for 30 min by the addition of sodium hydroxide. After stirring

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[1] Abbreviation used: TNM, tetranitromethane.

for an additional 30 min, the pH was raised to 12, and the solution was kept at this pH for 10 min. The reaction mixture was then acidified with dilute HCl and extracted twice with ethyl acetate. The extracts were combined and washed with water, dried over sodium sulfate, and evaporated to dryness *in vacuo*. The solid residue was crystallized from ethyl acetate. The yellow crystals (1.25 g, 93% yield) were collected and washed with petroleum ether (bp 30–60°), mp 180–182° (lit. (Iwasaki and Witkop (1964)) mp 182.5°). *Anal.* Calcd for $C_{11}H_{12}N_2O_6$: C, 49.25; H, 4.48; N, 10.44; neut equiv, 268. Found: C, 49.42; H, 4.50; N, 9.98; neut equiv, 271. Acid hydrolysis (5.7 N HCl, 105°, 24 hr) yielded only one ninhydrin-positive component which eluted from the amino acid analyzer at the position corresponding to 3-nitrotyrosine (*vide infra*), with a recovery of 98%.

Carboxypeptidase, ribonuclease, ovalbumin, pepsinogen, and lysozyme were all obtained from Worthington Biochemical Corp., insulin from Lilly, and bovine serum albumin from Armour Laboratories. Poly lysyl–tyrosine (10:1), poly glutamyl–lysyl–tyrosine (54:40:6), and poly glutamyl–tyrosine (9:1) were obtained from Pilot Chemical Corp.

Methods

Nitration of proteins was generally carried out in 0.05 M Tris buffer at pH 8, at room temperature (20°). Aliquots of a 0.84 M solution of TNM in 95% ethanol were added to 1 ml of solution containing approximately 10 mg of protein.

Nitration of the standard amino acid mixture was carried out at pH 8.0 on the pH-Stat. For this purpose, 5 μl (42 μM) of TNM was added to 2 ml of solution. After 1 hr at 25° the reaction mixture was acidified to pH 2 and diluted to 10 ml with pH 2.2 citrate buffer; 1 ml of this solution was placed on each column of the Spinco Model 120B amino acid analyzer, and the columns were developed according to the standard procedure of Spackman *et al.* (1958).

Hydrolyses of proteins for amino acid analysis were carried out in 6 N HCl *in vacuo* at 105° for 22 hr. Spectra were obtained with an automatic recording Cary Model 15 spectrophotometer while absorption at single wavelengths was determined with a Zeiss PMQII spectrophotometer.

Results

I. Specificity of the Reaction. To establish the specificity of the reaction of TNM with amino acids, nitration was performed on the calibration mixture (Type 1) provided for the Spinco amino acid analyzer. Chromatographic analysis of the reaction mixture revealed that 16 of the 17 component amino acids emerged in known positions relative to the control (Figure 1A) and in virtually quantitative yield (Table I). Only tyrosine, expected at 207 ml, was absent. Instead, a new peak was detected which eluted from the long column at 228 ml (relative to 213 ml for phenylalanine)

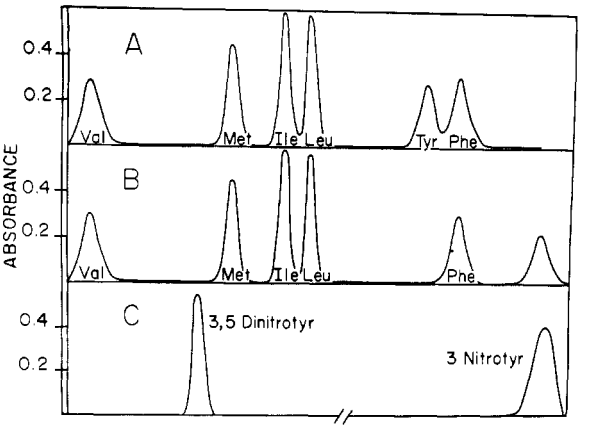


FIGURE 1: Chromatographic analysis (Spackman *et al.*, 1958) of the Spinco Type I amino acid calibration mixture after reaction with TNM. (A) Control. (B) Amino acid mixture after reaction with TNM (see text). (C) Chromatography of authentic 3,5-dinitrotyrosine and 3-nitrotyrosine. Each sample was chromatographed separately. The same portion of the chromatogram is shown in all three instances and that preceding valine, identical for A and B, has been omitted to facilitate presentation.

(Figure 1B). Authentic 3-nitrotyrosine, Figure 1C, emerged in precisely the position found for the unknown product of nitration in the amino acid mixture, thereby provisionally establishing its identity. 3,5-Dinitrotyrosine, when chromatographed either alone, Figure 1C, or together with a standard mixture of amino acids, eluted as a discrete peak between valine and methionine with an elution volume of 156 ml (relative to 213 ml for phenylalanine), distinctly different from 3-nitrotyrosine. No peak corresponding to 3,5-dinitrotyrosine could be detected in the nitrated amino acid mixture.[2]

This calibration mixture (Type 1) contains neither cysteine nor tryptophan and, hence, the reaction of TNM with indole and thiol residues was investigated separately. Qualitatively, a reaction between TNM and glutathione in 0.05 M Tris, pH 8, can be detected by the appearance of a yellow color immediately after mixing. One-dimensional descending paper chromatography with 1-butanol–acetic acid–water (3:1:1) as the solvent system identified the product of the reaction as oxidized glutathione.

Addition of TNM to either 10^{-4} M tryptophan, tryptophan methyl ester, or carbobenzoxyglycyl-L-tryptophan at pH 8 in 0.05 M Tris neither increased the absorbance at 350 mμ (*vide infra*) nor caused the release of protons when followed on the pH-Stat. Aliquots of the carbobenzoxy-L-tryptophan mixture

[2] If the reaction of TNM with tyrosine is allowed to continue for much longer times, 3,5-dinitrotyrosine may be formed, of course.

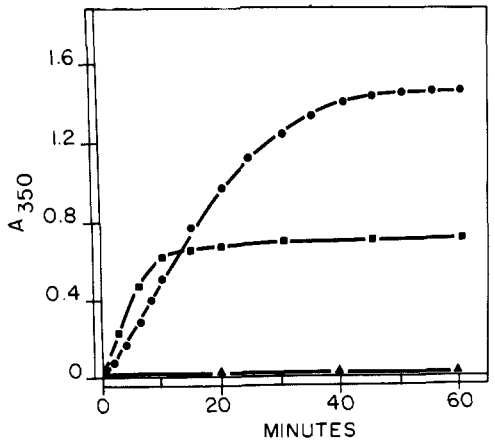


FIGURE 2: Increase in absorbance at 350 mμ on nitration of *N*-acetyltyrosine (●), glutathione (■), and *N*-carbobenzoxyglycyl-L-tryptophan, *N*-acetylhistidine, or *N*-carbobenzoxy-L-methionylglycine (▲), all 10^{-4} M; TNM, 5 μl (42 μmoles)/3 ml; 0.05 M Tris, pH 8.0, 20°. The data for *N*-acetyltyrosine are corrected for the absorbance due to *N*-acetyl-3-nitrotyrosine.

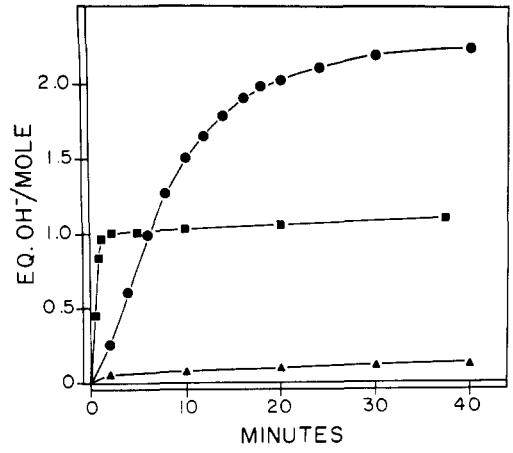


FIGURE 3: Nitration of *N*-acetyltyrosine (●), GSH (■), and *N*-carbobenzoxyglycyltryptophan, *N*-acetylhistidine, or *N*-carbobenzoxyglycyl-L-methionine (▲) with TNM. The reaction was performed with 10 mmoles of each peptide on the pH-Stat at pH 8.0, 20°, using a fivefold molar excess of TNM; the protons released were titrated with 0.10 N NaOH.

TABLE I: Recoveries of Amino Acids after Incubation with TNM as Determined by Amino Acid Analysis.[a]

	μmoles
Lys	1.00
His	0.99
Arg	0.98
Asp	1.01
Thr	0.99
Ser	1.03
Glu	1.02
Pro	0.99
Gly	1.00
Ala	1.02
Cys	0.93
Val	1.04
Met	0.91
Ile	1.02
Leu	1.02
Tyr	...
Phe	0.98
NO_2-Tyr	0.98

[a] Nitration of the Type 1 amino acid calibration mixture was carried out at pH 8.0 on the pH-Stat for 1 hr at 25°. The reaction mixture was then acidified, diluted to 10 ml with pH 2.2 citrate buffer, and chromatographed according to Spackman *et al.* (1958).

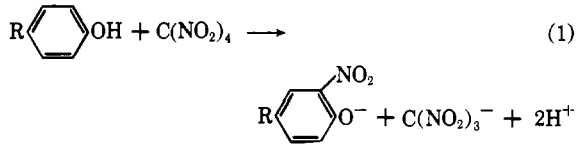
were removed at various times up to 60 min and their analysis with *p*-dimethylaminobenzaldehyde (Spies and Chambers, 1949) failed to reveal any loss of tryptophan (Table II).

TABLE II: Determination of Tryptophan after Reaction of Cbz-Gly-Try with TNM.[a]

Time of Reaction (min)	% Recov
0	100
0	100
15	96
15	102
30	92
30	95
60	95
60	97

[a] Cbz-Gly-Try (10 μmoles) was incubated with 5 μl (42 μmoles) of TNM in 0.05 M Tris, pH 8.0, 20°. Aliquots were removed at various times and tryptophan was analyzed colorimetrically (Spies and Chambers, 1949).

II. Quantitation of the Reaction. The most probable scheme for the reaction of TNM with tyrosine or cysteine is indicated in eq 1 and 2.



$$R'SH + C(NO_2)_4 \longrightarrow R'SNO_2 + C(NO_2)_3^- + H^+$$
$$R'SNO_2 + R'SH \longrightarrow R'SSR' + H^+ + NO_2^- \quad (2)$$

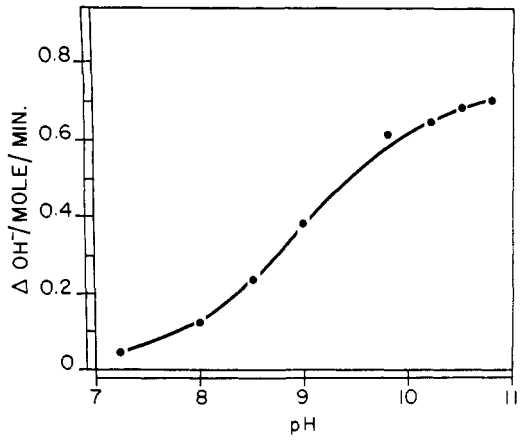


FIGURE 4: Dependence of the rate of nitration of *N*-acetyltyrosine. Rates were calculated from the initial linear slope of the titration curves obtained when nitrations were performed on the pH-Stat.

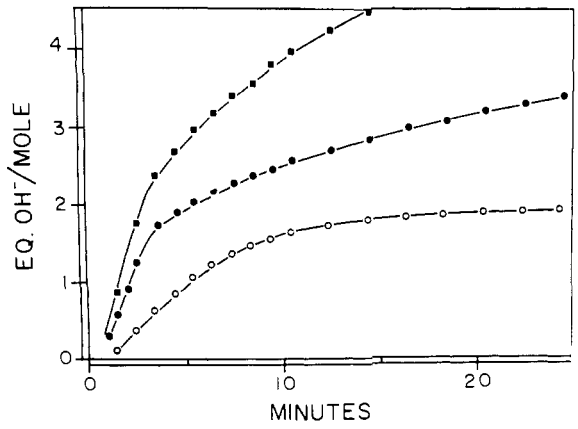


FIGURE 5: Nitration of tyrosine (■) and *N*-acetyltyrosine with a fivefold molar excess of TNM as followed on the pH-Stat. The nitration of tyrosine (10 mmoles) was carried out at pH 10.5 while that of *N*-acetyltyrosine (10 mmoles) was at pH 8.5 (○) and 10.5 (●).

TNM is thought to exist in a partially ionic form which can dissociate to form nitronium ions and nitroformate ions. In eq 1, the phenolic group of tyrosine is considered to react with nitronium ions leading to the formation of mononitrotyrosine and liberating protons. In eq 2, the thiol residue of cysteine is thought to react with the nitronium ion leading to the formation of an intermediate, indicated here as a sulfenyl nitrate, and also liberating a proton. The intermediate then might react with a second mole of thiol leading to the formation of a disulfide bond, releasing a nitrite ion. On the basis of these schemes, three alternatives should potentially be suitable to quantitate the reaction of TNM with proteins. The first is based on the formation of nitroformate anions which are colored bright yellow due to an absorption band at 350 mμ. The reaction can also be followed by titration of the protons released or by determination of the amount of product formed. The high molar absorptivity of the nitroformate anion, ϵ 14,400, λ_{max} 350 mμ (Glover and Landsman, 1964), would seem to provide the most sensitive and convenient characteristic of this product for quantitation.

The nitration of *N*-acetyltyrosine with a 140-fold molar excess of TNM at pH 8.0 was followed by measuring the increase in absorbance at 350 mμ as a function of time (Figure 2). Under these conditions, the reaction is complete within 30 min. Allowing for the absorption due to the formation of *N*-acetyl-3-nitrotyrosine, the increase in absorbance corresponds to the formation of 1 mole of nitroformate/mole of tyrosine, consistent with the scheme indicated in eq 1. The reaction between TNM and glutathione was also carried out using a 140-fold molar excess of reagent at pH 8.0 (Figure 2). The reaction proceeds quite rapidly and is complete within 5 min. The increase in absorbance at 350 mμ corresponds to the release of 1 mole of nitroformate/2 moles of glutathione, consistent with the mechanism shown in eq 2. This method was also employed in an attempt to detect a reaction between TNM and *N*-acetylhistidine, carbobenzoxyglycyl-L-tryptophan, and carbobenzoxymethionylglycine. With each of these compounds the increase in absorbance at 350 mμ was negligible over a period of 1 hr (Figure 2).

The reaction of TNM can also be quantitated by titrating the release of protons. Nitration of *N*-acetyltyrosine with a fivefold molar excess of TNM at pH 8.0 releases 2 moles of protons/mole of tyrosine, one due to displacement by the nitro group and the second due to ionization of the phenolic hydroxyl group (Figure 3). Under the same conditions, 1 proton/mole of glutathione is released. Further, this method also demonstrates the lack of reaction between TNM and histidyl, tryptophanyl, or methionyl residues (Figure 3).

The rate of nitration of *N*-acetyltyrosine is markedly dependent on pH, as determined on the pH-Stat. It proceeds most rapidly at pH 10 or above but hardly at all at pH values below 7 (Figure 4). It would appear that the optimal conditions for the nitration of tyrosyl residues in proteins would be at about pH 10. However, two factors suggest that a lower pH might be preferable operationally. First, many proteins are unstable at such high pH values, thus unnecessarily introducing potential complications into the effect of nitration on biological function. Second, at high pH values TNM is also unstable (Schmidt, 1919). This is evident from titration studies carried out at different pH values (Figure 5). The titration curve for the nitration of *N*-acetyltyrosine at pH 8 is compared with a similar one at pH 10.5. At the higher pH two different rate processes are apparent. The initial portion of the curve corresponds to the release of protons due to nitration of the tyrosyl residue while the second part

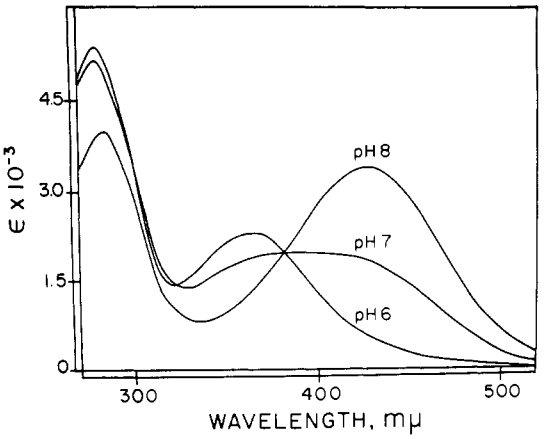


FIGURE 6: Effect of pH on the absorption spectra of *N*-acetyl-3-nitrotyrosine in 0.05 M Tris.

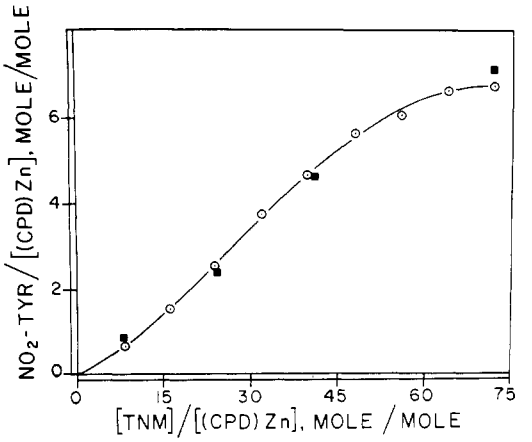


FIGURE 7: Nitration of carboxypeptidase A: formation of 3-nitrotyrosyl residues with increasing amounts of TNM. The reaction was performed at pH 8.0 (20°) and the degree of nitration was determined either spectrophotometrically at 428 mμ (○) or by amino acid analysis (■) as described in the text.

is due to the spontaneous breakdown of TNM, probably due to hydroxide ion catalysis. Other general bases can also catalyze the decomposition of TNM, and this becomes a significant problem when trying to quantitate the reaction of TNM with proteins. Comparison of the titration curves at pH 10.5, for the nitration of *N*-acetyltyrosine and tyrosine, suggests that the free amino group of tyrosine may also participate in the catalytic breakdown of TNM, perhaps even at lower pH values. As a result, the increase in absorbance at 350 mμ or the release of protons as measured on the pH-Stat while suitable for model compounds cannot be considered reliable gauges of the degree of nitration of the tyrosyl residues of proteins. Therefore, the number of moles of nitrotyrosine per mole of protein formed must be determined directly.

Two procedures can be employed for this purpose. The first is based on the spectral characteristics of 3-nitrotyrosine (Figure 6). At pH 8.0, the tyrosine absorption maximum at 275 mμ has a molar absorptivity, ε 1360. Nitration increases this molar absorptivity to 4000 and, in addition, generates a new absorption band with maximal absorption, λ_{max}, at 428 mμ and ε 4100. The spectrum of 3-nitrotyrosine, like that of tyrosine, is pH dependent. The absorption maximum at 428 mμ, observed at alkaline pH, is shifted to 360 mμ at acid pH with an isosbestic point at 381 mμ and ε 2200. This can be employed for direct quantitation of the degree of nitration, independent of the pH at which the measurement is made or of the p*K*(s) of the nitrotyrosyl residues being measured. In general, this method is only suitable for high molecular weight substances which can be easily separated from nitroform by gel filtration. The high molar absorptivity of nitroformate at 350 mμ interferes significantly with the measurement of low molecular weight substances at 381 mμ.

Amino acid analysis can be employed as another method to quantitate the nitration of tyrosyl residues in proteins. No loss of nitrotyrosine could be detected after heating at 105° *in vacuo*, in 6 N HCl for 24 hr, and 3-nitrotyrosine appears as a discrete peak in the elution profile of the amino acid analyzer (Figure 1).

The nitration reaction was carried out as a function of TNM concentration to determine the optimal molar excess to be employed. Carboxypeptidase A of bovine pancreas was the protein selected for study since it has been examined previously from a similar point of view with other reagents. This protein contains a total of 19 tyrosyl residues, six to seven of which are "free" as judged by pH titration and by reaction with acetylimidazole (Simpson *et al.*, 1963). TNM was diluted tenfold with 95% ethyl alcohol and aliquots corresponding to an eightfold molar excess were added to a 3×10^{-4} M solution of the protein in 0.05 M Tris buffer at pH 8. The reaction was allowed to proceed for 30 min, an aliquot was removed and passed through a Biogel P4 column, and nitrotyrosine was determined both spectrally and by amino acid analysis. Another eightfold molar excess of TNM was added to the remainder of the carboxypeptidase solution and the reaction was allowed to proceed for another 30 min. Again, the nitrotyrosyl content was determined and the process was repeated. Maximal nitration corresponding to 6.7 nitrotyrosyl residues/mole was achieved after eight cycles, *i.e.*, a 64-fold molar excess of TNM (Figure 7). In a different experiment, 5 μl of TNM was added to a 3×10^{-4} M solution of carboxypeptidase in 0.05 M Tris–1 M NaCl at pH 8 and the reaction mixture was allowed to stand at room temperature overnight. Separate aliquots were then assayed for nitrotyrosine both by spectra and by amino acid analysis. The results are identical with those obtained using a 64-fold molar excess over a 4-hr period (Figure 7).

Both methods of addition and modes of analysis were employed to study the nitration of a number of proteins

TABLE III: Nitration of Proteins with Tetranitromethane.[a]

Protein	Nitrotyrosine (mole/mole) At $\lambda_{428\ m\mu}$	Nitrotyrosine (mole/mole) Amino Acid Anal.
Insulin	2.0	1.8
Lysozyme	2.8	2.5
Ribonuclease	3.1	2.6
Bovine serum albumin	4.4	4.8
Ovalbumin	5.6	5.8
Carboxypeptidase A	7.1	6.9
Pepsinogen	10.4	9.5

[a] The nitration reaction was carried out by both methods described in the text; samples were then measured both spectrophotometrically at 428 mμ and by amino acid analysis after acid hydrolysis (Spackman *et al.*, 1958). Except for lysozyme, the numbers of tyrosyl residues nitrated were less than are known to be present by amino acid analysis.

(Table III). In all instances, good agreement both between the two methods of addition of TNM and of determination of nitrotyrosine content was obtained. In most instances, the number of nitrotyrosyl residues found is less than the total number of tyrosyl residues known to be present by amino acid analysis.

In addition to proteins, a number of tyrosyl-containing copolymers were nitrated with TNM (Table IV).

TABLE IV: Nitration of Polymers with TNM.[a]

Polymer	Nitration (%)
Lys-Tyr (10:1)	74
Glu-Tyr (9:1)	100
Glu-Lys-Tyr (54:40:6)	97

[a] The degree of nitration was estimated from the recoveries of tyrosine and 3-nitrotyrosine after acid hydrolysis.

In each instance, essentially 100% nitration of the tyrosyl residues was achieved, as judged by amino acid analysis.

Discussion

The increasing awareness of the importance of tyrosyl residues both in stabilizing the structure of proteins and in participating in their function focuses attention on reagents suitable for the specific, chemical modification of these residues. Both pH titration (Crammer and Neuberger, 1943) and the effects of agents, such as iodine (Cha and Scheraga, 1963; Donovan, 1963) and acetylimidazole (Riordan *et al.*, 1965a,b) have served to delineate this structural role. The effects of TNM are somewhat similar to those of acetylimidazole, an agent which has proven relatively specific and convenient for the differentiation of "free" and "buried" tyrosyl residues. The difference in the products of the reactions of acetylimidazole and TNM with tyrosyl residues should prove valuable in extending this knowledge and detailing the underlying chemistry.

Tetranitromethane is highly specific and selective in nitrating tyrosyl residues of proteins. Very low molar ratios of reagent to protein can be employed; the reaction conditions are mild and do not require exposure of the protein to extremes of pH, ionic strength, or temperatures.

The mechanism of nitration of tyrosyl residues by TNM probably involves the participation of nitronium ions (Patterson, 1955) and therefore might be considered analogous to iodination or bromination. While iodination and bromination can affect tyrosyl, tryptophanyl, histidyl, sulfhydryl, and methionyl residues of proteins, nitration with TNM affects only tyrosyl and cysteinyl groups, but not the others. However, the nitration of tyrosyl residues does not proceed at pH 6 where cysteinyl residues continue to be oxidized. This variance in pH dependence of oxidation *vs.* nitration can be employed to differentiate between them.

The mildness of the TNM reaction is an important and attractive features of its use. Judicious choice of the reaction conditions, in fact, can restrict nitration to a very small number of tyrosyl residues of proteins. In this regard, we have already isolated mononitro derivatives of ribonuclease and carboxypeptidase, the properties of which will be the subject of separate communications.

In earlier modifications of proteins with TNM, the induction of color was noticed, though attempts were not made either to quantitate or to establish the chemical basis of this observation or the nature of the reaction. Thus, *e.g.*, on addition of tetranitromethane to serum, Wormall (1930) noted the appearance of a red, orange, brown, and finally, deep green color. Later Ehrenberg and co-workers (1946) reported that on reaction of TNM both with oleic acid and proteins, an absorption band with a maximum at 350 mμ appeared, and concluded that both of these materials form the same addition compound.[3] This color is now known to be due to nitroformate.

The generation in a protein of a chromophore which absorbs in the visible spectrum provides a number of

[3] TNM adds to the double bond in oleic acid and is employed as a means for the routine detection of unsaturation in organic compounds (Schmidt and Fischer, 1920).

convenient experimental approaches. If the group modified is functional, substrates, substrate analogs, and inhibitors may affect its spectral properties. Since the nitrotyrosyl residue is ionizable, it may serve to probe the microscopic environment of active center residues by means of perturbation spectra and similar methods.

This prediction has already been borne out by studies of nitrocarboxypeptidase. On addition of β-phenylpropionate, a competitive inhibitor of the enzyme, the pK of the nitrotyrosyl residues in mononitrocarboxypeptidase is shifted from 6.3 to 7.2, a very sensitive indicator of vicinal changes (Riordan *et al.*, 1966b, and in preparation).

Such a group, if optically active, might further reveal characteristic extrinsic Cotton effects as additional probes of protein conformation. Importantly, the spectral characteristics represent a guide to labeling and subsequently isolating nitrotyrosyl peptides. The existence of the nitrotyrosyl absorption peak at 428 mμ may also serve as a simple means for the recognition of naturally occurring nitrotyrosyl-containing proteins and peptides, in addition to rufomycin A (Iwasaki and Witkop, 1964).

Reduction of the substituent nitro to an amino group elicits additional potentials of nitration of proteins. Reaction should again alter the phenolic pK, the characteristics of the chromophoric group, and enzymatic activity (if the tyrosyl residue is functional). Importantly, the resultant aminophenol should have unique chemical properties which will permit even more specific chemical modifications. We have recently achieved the reduction of the nitro group of mononitrocarboxypeptidase and structural as well as functional consequences, consistent with these expectations, will be reported (M. Sokolovsky, J. F. Riordan, and B. L. Vallee, in preparation).

The absorption maximum at 428 mμ is the most direct means of monitoring and quantitating the reaction. However, the measurement of the release of hydrogen ions on the pH-Stat is particularly useful when dealing with amino acids and peptides when the separation from nitroform is not feasible by gel filtration. The numerical correspondence between protons released and nitro groups introduced verifies the occurrence of the reaction and also serves as a lead to its mechanism.

Similarly, measurement of the increase in absorption at 350 mμ due to the release of nitroform also has proven to be a sensitive parameter of this reaction when dealing with small molecules, *e.g.*, tyrosine or glutathione (Figure 1) (Riordan *et al.*, 1966a), but less so with proteins. With the former, the amount of nitroform produced is equal to the amount of mononitrotyrosine formed, as determined either by titration or by amino acid analysis. With proteins, however, the amount of nitroform and protons produced generally exceeds that of mononitrotyrosine formed. This phenomenon is not as yet understood precisely, though it is thought that basic groups of the protein may catalyze the breakdown of TNM. Kinetic studies as a function of pH support this idea. Hence, with proteins this mode of quantitation has not proven to be reliable.

Ultimately the chemical identification of the nitrated amino acid residue(s) is the most definitive procedure and serves as the standard of reference for the other, more inferential methods. Nitrotyrosine is stable under conditions conventional for acid hydrolysis of proteins, hence the number of nitrotyrosine residues can be determined in this manner.

In the present work, we have examined the effect of TNM on a number of polymers and proteins, among them carboxypeptidase A, ribonuclease, insulin, and pepsinogen (Table III, Figure 7). Although data on a sufficient number of proteins have not as yet been obtained to be decisive, the number of tyrosyl residues nitrated appear to approximate those known from other studies to be "free" and exposed and, hence, reactive. These data need to be extended. All of the tyrosyl residues of polymers devoid of major, secondary and tertiary structure are readily modified (Table IV), similar to the effect of acetylimidazole (Riordan *et al.*, 1965a). The effects of TNM on functional properties of enzymes to be described elsewhere (Riordan *et al.*, 1966b) are also analogous to those of acetylation (Riordan and Vallee, 1963).

Nitration with TNM extends the number of procedures available for the modification of tyrosine, providing greater experimental latitude in the study of the biological function of tyrosyl enzymes and facilitating their identification. In this regard we have found that nitration can alter the functional behavior of carboxypeptidase A (Riordan *et al.*, 1966b) and of other tyrosyl enzymes (unpublished observations). With minimal excess of TNM marked alterations in catalytic properties already occur on nitration of a single residue. Such findings give promise that this reagent will be very useful for functional as well as structural studies.

It has been emphasized that the abolition or alteration of catalysis on specific chemical modifications has been critical in the assignment of functional importance to specific residues. In fact, it is the availability of particular agents, selective for specific groups, that has led to the classification of enzymes according to their reactive residues (Riordan *et al.*, 1965a). TNM exhibits the characteristics demanded of such a reagent. While detailing the role of other groups, acylation, iodination, or diazo coupling prior or subsequent to nitration with TNM can help to specify the participation of tyrosyl residues in maintenance of structure, substrate binding, or catalysis.

Caution in the handling of the reagent is indicated. While mild in its interactions with proteins, TNM is a very hazardous chemical (Sievers *et al.*, 1947). It has been studied widely as a potential oxidant for rocket fuels and as a diesel fuel additive, and it has even received some attention as a potential war gas. It is quite volatile and extremely toxic. Its use should be restricted to fume hoods. While apparently not susceptible to detonation, it is claimed that TNM is more explosive than nitroglycerine. On mixing TNM with toluene, at least one accident resulting in multiple fatalities has been reported (Stettbacher, 1941). When proper precautions

are taken, however, no mishaps in its use have occurred.

Until recently, pepsin was the only enzyme in which tyrosyl groups were thought to play a functional role (Herriott, 1947). With the advent of acetylimidazole, however, a number of other enzymes have been added to this category (Simpson *et al.*, 1963; Plummer and Lawson, 1966). With the additional availability of TNM as a convenient and specific tyrosyl reagent, this group of enzymes and knowledge concerning their mechanism of action should increase rapidly.

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