REACTION OF TETRANITROMETHANE WITH TRYPTOPHAN AND RELATED COMPOUNDS

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Received 4 February 1970

1. Introduction

Initial studies on the use of tetranitromethane (TNM) as a reagent for the nitration of proteins had shown that low molar excesses of the reagent selectively nitrates a unique tyrosyl residue of carboxypeptidase A under very mild conditions [1, 2, 3]. A fourfold molar excess of TNM at pH 8.0 and 20° was adopted for examining the specificity toward other amino acid residues. In addition to tyrosine only the sulfhydryl group of cysteine and several other model thiols was found to react with TNM under these conditions. Since that time TNM has become accepted widely as a reagent for modifying tyrosyl though it has been extended to a variety of conditions [4]. As part of a study to delineate further the specificity of TNM over a broader range of reaction parameters and applications, we first investigated the possible oxidation reaction pathways with sulfhydryl groups [5]. The major products were found to be a mixture of disulfide and sulfinic acid. In addition, there was preliminary evidence that histidine, tryptophan and methionine could also undergo oxidation with TNM under certain conditions [5]. Hence, we have continued these specificity studies and have now examined a series of substituted indoles and tryptophan analogs.

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2. Experimental procedure

Materials. TNM was obtained from Fluka, 5 and 6-methyltryptophan and L-tryptophan were obtained from Calbiochem, and 3-indolepropionic acid and Alumina chromagram sheets (6063) from Eastman Organic Chemicals. *N*-Acetyltryptamine and *N*-carbobenzoxytryptophan amide were gifts from Dr.M.Wilcheck and 1-benzylindole-3-carboxylic acid was a gift from D. A.Kalir.

Methods. Determination of tryptophan: Carbobenzoxytryptophan amide was dissolved in dimethylformamide and diluted with 0.001 M tris-HCl, pH 8.0 to a final concentration of 2% DMF and 0.25 mg/ml of the tryptophan derivative. To a 20 ml solution of tryptophan derivative, a 40-fold molar excess of TNM was added. The reaction mixture was stirred with a magnetic bar and the pH was maintained constant with 0.1 N NaOH using a Radiometer TTT1A pH stat. Aliquots of 2 ml were removed for alkaline hydrolysis while 1 ml aliquots were pipetted into the reaction mixture for the determination of tryptophan by the method of Spies and Chambers [6].

Alkaline hydrolysis: Aliquots (2 ml) were dried under reduced pressure (excess of TNM is also removed). To the dry sample, 0.7 ml of H_2O containing 20 μmoles of phenol was added followed by 0.3 ml of 50% KOH. The tubes (Pyrex) were sealed and incubated for 16 hr

at 110°. The solution was then neutralized by adding 0.95 ml of 6 N HCl and the sample was diluted to 5 ml with 0.2 M citrate buffer, pH 2.2 and centrifuged. One ml of sample was chromatographed on the short column of the Beckman Unichrome amino acid analyser. The recovery of tryptophan from carbobenzoxytryptophan amide by this procedure was 70–74%.

3. Results and discussions

The nitroformate ion, a product of the reaction of TNM, absorbs intensely at 350 nm (ϵ = 14,400) and, hence, its formation can be employed to detect and to follow the course of the reaction [1, 2]. By this criterion the reaction of 3-indolepropionic acid with increasing concentrations of TNM results in the formation of up to one equivalent of nitroformate per mole of acid.

Table 1
Reaction of TNM with indolepropionic acid[a]: Production of nitroformate.

Molar excess of TNM/mole indole	moles of Nitroformate formed/mole indole
1	0.05
5	0.15
10	0.21
40	0.90
100	0.95

[a] The reaction was carried out at pH 8.0 in 0.01 M tris-Cl buffer at room temperature for 2 hr. Nitroformate was determined from the absorbance at 350 nm. No corrections were made for absorbance due to the modified indole.

The reaction of TNM can also be followed by titrating the release of protons (or, alternatively, the uptake of hydroxide ions) [1, 2]. The addition of a 40-fold molar excess of TNM to 5 ml of 2 X 10^{-3} M 3-indolepropionate, *N*-carbobenzoxytryptophan amide, or *N*-acetyltryptamine at pH 8 in 0.001 M tris-Cl, results in the appearance of about one equivalent of proton per mole of indole (fig. 1) consistent with the reaction stoichiometry indicated by nitroformate production. The time course for production of nitroformate ion

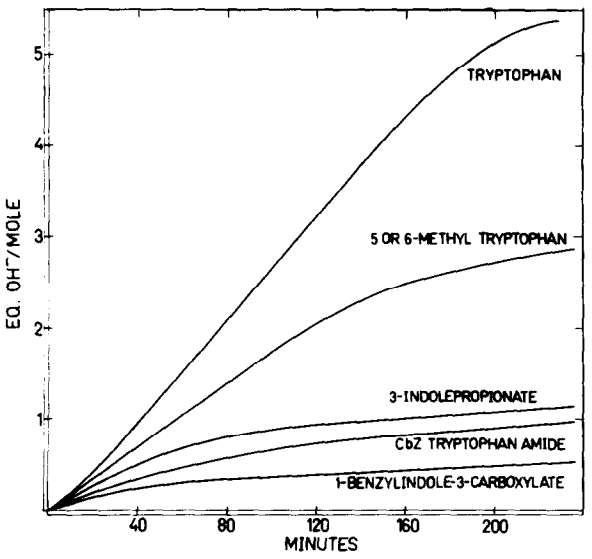

Fig. 1. Reaction of substituted indoles with TNM. A 40-fold molar excess of TNM was added to 5.0 ml of 2 X 10^{-4} M indole derivative in 0.001 M tris-Cl, pH 8.0, 20°, with constant stirring. The pH was maintained by titration with 0.1 N NaOH using a pH stat.

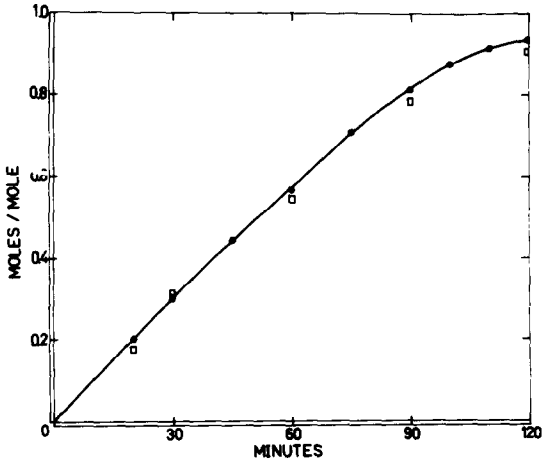

Fig. 2. Production of nitroformate (□) and uptake of base (●) on reaction of 5 ml of 2 X 10^{-4} M indolepropionate with a 40-fold molar excess of TNM as described for fig. 1. Nitroformate production was determined by measuring the absorbance at 350 nm on aliquots removed at intervals and diluted 10 times with the same buffer.

and that for the release of protons when 3-indolepropionic acid or carbobenzoxytryptophan amide reacts with a 40-fold molar excess of TNM at pH 8.0 are almost identical (fig. 2). With tryptophan more than 5 protons and for 5- and 6-methyltryptophan about 3 protons per molecule were titrated. Less than one proton per molecule was titrated when the reaction was carried out with 1-benzylindole-3-carboxylic acid.

The greater release of protons (or uptake of hydroxide ions) with compounds having a free α-amino group may be due in part to base catalyzed hydrolysis of TNM [2]. The low value observed with the *N*-substituted indole derivative might suggest that some reaction of TNM occurs at the nitrogen atom but clearly this cannot be the only site of modification.

The rate of release of protons reflecting the reaction of indole derivatives with TNM is markedly dependent on pH. It proceeds most rapidly above neutrality, but hardly at all at pH values below 7 [5]. This is most important with regard to modification of tyrosyl residues in proteins, especially in those instances where tyrolsyl nitration still occurs at pH 7. Under such conditions it might be possible to avoid difficulties due to reactions with tryptophan. For most proteins, however, tyrosine modification occurs most readily at pH 8 [2]. The observed specificity of TNM for tyrosyl residues in proteins could be due to the fact that tryptophanyl residues are not exposed to the ambient environment but are buried in the hydrophobic interior of the molecule.

The modification of tryptophanyl residues was also examined by amino acid analysis. Tryptophan was treated with a 40-fold molar excess of TNM at pH 8.0. Aliquots were extracted four times with benzene to remove excess TNM and then chromatographed on the amino acid analyzer. As shown in fig. 3A, disappearance of tryptophan occurs progressively over a period of three hours. No other peaks could be detected on either column of the amino acid analyzer. Similarly, the disappearance of the tryptophanyl residues of carbobenzoxytryptophan amide could be followed either chromatographically after alkaline hydrolysis or by reaction with *p*-dimethylaminobenzaldehyde [6] (fig. 3B). Both procedures give identical results.

At least three yellow derivatives could be detected in the TNM-3-indolepropionate reaction mixture by thin-layer chromatography on alumina sheets using *n*-propanol-ammonia (4:6) as solvent (fig. 4). All of

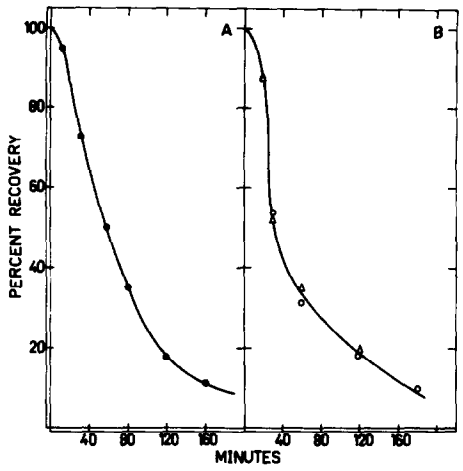

Fig. 3. A. Loss of tryptophan on reaction with TNM as described under Methods and as determined on the amino acid analyzer.

B. Loss of tryptophan on reaction of carbobenzoxytryptophan amide with TNM as described under Methods and as determined by amino acid analysis after alkaline hydrolysis (○) or by the method of Spies and Chambers [6] (△).

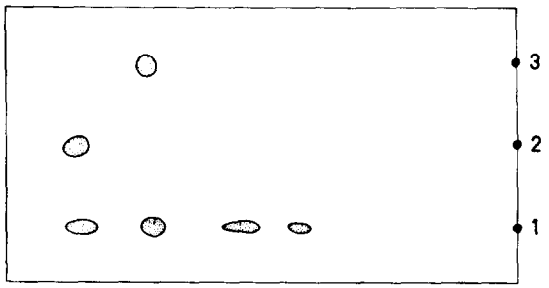

Fig. 4. Thin-layer chromatography of the reaction mixture of indolepropionic acid with TNM (conditions as for fig. 1). Solvent system: *n*-propanol-ammonia (40:60). 1, reaction mixture; 2, nitroformate; 3, indolepropionate. The shaded areas were detected with an ultraviolet lamp.

the compounds have absorption maxima near 330 nm which are insensitive to changes in pH and, for two of them, to the addition of sodium dithionite. Mass spectroscopic analysis of each of these compounds yielded mainly starting material, indole propionate, and trace amounts of higher molecular weight products. This is probably due to the thermal decomposition of the samples in the mass spectrometer. Degradation studies to characterize these products are now in progress.

The reaction of TNM with proteins is generally specific for tyrosyl residues except where free sulfhydryl groups are present and in some enzymes like carboxypeptidase A [3] certain tyrosyl residues are exceptionally reactive even at very low molar excesses of reagent. However, there have been at least two instances in which tryptophan modification has been reported. The spectral properties of the modified tryptophanyl residue of staphylococcal nuclease treated with a 60-fold molar excess of TNM [7] are similar to those observed for one of the products obtained in this study. The inactivation of papain by TNM has also been thought to be due to tryptophan modification [8]. However, oxidation of the active center cysteinyl residue of this enzyme to a sulfinic acid is an equally likely possibility under the conditions employed and has not been ruled out.

Even though in most instances studied thus far, modification by TNM of residues other than tyrosine and cysteine has not been observed, the possibility of tryptophan oxidation must always be considered. Certain residues because of their particular protein environment might be especially sensitive to TNM. Tryptophan residues tend to exist in hydrophobic regions of proteins and while this may in some cases preclude any reaction, in others it may prove very favorable. In this regard, TNM has quite recently been reported to undergo an unusual reaction with skatole in diethyl ether to form a 2-dinitromethylene-3-nitro derivative [9]. Alternate reaction pathways were thought to occur in aqueous solvents. Comparisons between the products of tryptophan nitration obtained in organic vs. aqueous solvents are now in progress.

Acknowledgement

We would like to thank Moshe Moldovan for his excellent assistance and B.L.Vallee for his advice and encouragement.

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