# ON THE USE OF TETRANITROMETHANE AS A NITRATION REAGENT FOR TYROSYL RESIDUES

Mordechai SOKOLOVSKY*
*Department of Biochemistry, Tel-Aviv University, Israel*

and

James F.RIORDAN
*Biophys. Research Lab., Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts, U.S.A.*

Received 29 June 1970

## 1. Introduction

Nitration with tetranitromethane has proven to be an exceptionally valuable means for studying residues of proteins [1, 2]. The agent can be employed under mild conditions i.e. room temperature at low concentrations and at pH values close to or at 8.0. The reaction is generally terminated by gel-filtration at the same pH [1]. In several reports the sum of the nitrotyrosyl and tyrosyl content of the nitrated protein was in good agreement with the tyrosyl content of the starting material. However, it was noted recently in a few cases [3–5] that the amount of nitrated tyrosine plus tyrosine was less than expected. The main difference between the original reports and the reports concerning the destruction of tyrosyl residues is in the procedure employed to terminate the reaction. In the past excess reagent was removed at pH 8.0 as initially recommended. Loss of tyrosine was observed in instances where nitration was stopped by lowering the pH. As part of a study to delineate further the specificity of TNM toward proteins [6, 7], we have now investigated the possible consequences of this latter procedure. Ribonuclease A has been used as a model since it does not contain tryptophanyl or cysteinyl residues which might add additional complications [6, 7].

* To whom inquiries should be addressed.

## 2. Materials and methods

TNM was obtained from Fluka and only the fraction that solidified at 13° was used. Ribonuclease was obtained from Worthington Biochemical Corp. Freehold, N.J.

Nitration of ribonuclease was carried out in 0.05 M tris buffer at pH 8, 25°, 0.2 M NaCl, 4–5 mg/ml protein and 8.4 mM tetranitromethane. The reaction was terminated by chromatography on a short Bio-Gel P-4 column (1.2 × 14 cm) equilibrated either with 0.05 M tris or with 0.001 M HCl. The degree of nitration was estimated from the absorbance at 425 mμ [1] and by amino acid analysis [1].

Amino acid analyses were performed with a Beckman-Unichrome amino acid analyzer. Acid hydrolysis was carried out in sealed, evacuated tubes with 6 N HCl at 110° for 22 hr. Phenol was added to prevent loss of tyrosine during hydrolysis. Nitrite was determined according to the procedure of Kolthoff and Elving [8].

## 3. Results

The absorption spectrum of nitroribonuclease in the visible region at pH 8 differs depending upon its mode of preparation (fig. 1). The absorption spectrum of the sample obtained after gel filtration at pH 8, i.e. $NO_2$ RNAase I, is very similar to that of the model

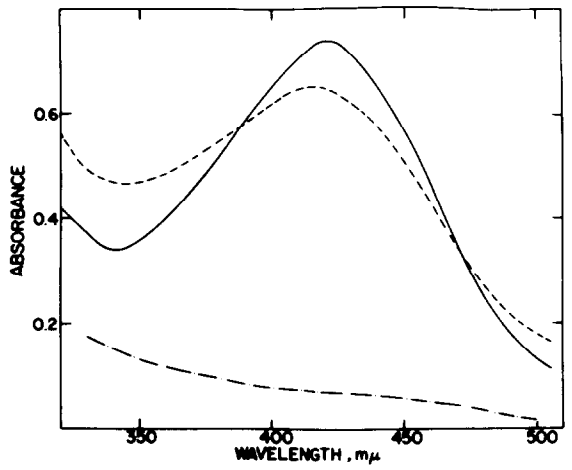


Fig. 1. Absorption spectra of $8 \times 10^{-5}$ M protein at pH 8.0 M tris-HCl. $NO_2$ RNAase I (—); $NO_2$ RNAase II (- - - -) and RNAase treated with nitrite and separated at acidic pH (-·-·-·-).

compound *N*-acetyl-3-nitrotyrosine, while that of the sample obtained after gel filtration at pH 3, $NO_2$ RNAase II, is not. Its $\lambda_{max}$ is shifted towards shorter wavelengths and the absorbance in the region 330–380 mμ is much higher. Therefore, the degree of nitration can be calculated from the absorption only for $NO_2$ RNAase I (table 1), and was found to be 2.3 nitrotyrosyl residues per molecule of protein. Amino acid analysis was also employed to quantitate the nitration of tyrosyl residues. As shown in table 1 there is a good agreement between the two methods for estimation of nitrotyrosyl content. Table 1 also reveals that there is destruction of tyrosine in

Table 2
Effect of nitrite on tyrosyl content of ribonuclease[a].

| Amino acid | After separation on Bio-Gel[b] | After separation on Bio-Gel[c] |
|---|---|---|
| $NO_2$Tyr[d] | – | 0.2 |
| Tyr[d] | 5.6 | 4.6 |
| Total | 5.6 | 4.8 |

[a] RNAase (3 μmoles/ml) was incubated with $NaNO_2$ (1.5 moles/mole protein) in 0.05 M tris-HCl pH 8.0 for 5 min.
[b] Aliquot was chromatographed on Bio-Gel P-4 equilibrated with 0.05 M tris-HCl pH 8.0.
[c] Aliquot was chromatographed on Bio-Gel P-4 equilibrated with 0.001 M HCl.
[d] Results expressed as moles of amino acid per mole of protein calculated on the basis of 3 phenylalanines/mole.

$NO_2$ RNAase II. The sum of tyrosine plus nitrotyrosine accounts for only 4.4 residues, 1.2–1.3 less than that found for either $NO_2$ RNAase I or native RNAase.

One of the reasons for the above differences might be the formation of nitrite during the nitration reaction. On exposure to acidic pH, the resultant nitrous acid may cause other reactions. Hence, the method of Kolthoff and Elving [8] was used in order to detect the presence of nitrite in the reaction mixture before separation on the Bio-Gel column. Indeed, nitrite ca. 0.6 mole per mole of nitrotyrosyl could be detected as a product or by-product of the reaction. Therefore we have investigated the effect of nitrite or RNAase through the same procedures used in the nitration reaction. RNAase was incubated with 1.5 moles/mole of sodium nitrite at pH 8.0, 25° and thereafter the

Table 1
Tyrosyl and nitrotyrosyl content of ribonuclease after reaction with TNM[a].

| Amino acid | Native RNAase | $NO_2$ RNAase I | $NO_2$ RNAase II |
|---|---|---|---|
| $NO_2$Tyr (from $A_{428}$) | – | 2.3 | – |
| Amino acid analysis[b] | | | |
| $NO_2$Tyr | – | 2.2 | 2.0 |
| Tyr | 5.7 | 3.4 | 2.4 |
| Total | 5.7 | 5.6 | 4.4 |

[a] Nitration was carried out with 8.4 mM TNM at pH 8.0, 0.05 M tris-HCl, 0.2 M NaCl 25° for 120 min. The pH was readjusted whenever dropped below 7.9. The reaction was terminated by chromatography on Bio-Gel P-4 (1.2 × 14 cm) equilibrated with 0.05 M tris pH 8.0 (third column) or with 0.001 M HCl (fourth column).
[b] Results expressed as moles of amino acid per mole of protein calculated on the basis of 3 phenylalanines/mole.

mixture was chromatographed on Bio-Gel P-4 column equilibrated with 0.001 M HCl. The absorption spectrum of the product extends into the visible regions (fig. 1). Similarly, amino acid analysis (table 2) reveals the destruction of tyrosine with the formation of small amounts of amino acid assumed to be nitrotyrosine (table 2) since it elutes from the amino acid analyzer at the same position of nitrotyrosine.

## 4. Discussion

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 small number of tyrosyl residues of proteins [9]. However the reaction conditions have been widely varied and it has been shown that high molar excesses of TNM when in use for long periods of time [7], or performing the reaction at pH values higher than 8 [6] will affect the specificity of the reagent. Furthermore, as shown in this report another complication can be generated during the reaction of TNM with ribonuclease. One of the by-products of the reaction appears to be nitrite. Quenching the reaction at acidic pH values, stops the nitration but simultaneously forms nitrous acid. Eventhough the nitrous acid which is formed is in contact with the protein for a short time, it is apparently enough to modify amino acid side chains of the protein. As a result, the spectrum is perturbed (fig. 1) leading to erroneous results if nitrotyrosine is determined spectroscopically, and in addition tyrosine is destroyed, so that the sum of tyrosine plus nitrotyrosine is less than expected (table 1). It should be emphasized that even if suitable precautions are taken other side reactions are still possible. The fact that nitrite was formed during the reaction of TNM and RNAase may be related to the findings of Bruice et al. [10]. Thus, cross-linking via the phenolic groups might occur but if the tyrosyl residues are sufficiently separated from each other in the folded protein, the cross-linking most probably will be minimized.

## Acknowledgement

We would like to thank Mrs. Bella Gertner for her excellent assistance.

## References

[1] M. Sokolovsky, J.F.Riordan and B.L.Vallee, Biochemistry 5 (1966) 3582.
[2] B.L.Vallee and J.F.Riordan, Ann. Rev. Biochem. 39 (1969) 733.
[3] J.Bello, Biochemistry 8 (1969) 4535.
[4] J.P.Vincent, M.Lazdunski and M.Dellaage, European J. Biochem. 12 (1970) 250.
[5] R.W.Boesel and F.H.Carpenter, Biochem. Biophys. Res. Commun. 38 (1970) 678.
[6] M. Sokolovsky, D. Harell and J.F. Riordan, Biochemistry 8 (1969) 4740.
[7] M. Sokolovsky, M. Fuchs and J.F. Riordan, FEBS Letters 7 (1970) 167.
[8] I.M.Kolthoff and P.J.Elving, in: Treatise on analytical chemistry, Vol. 5, Part II (Interscience, New York, 1961) p. 304.
[9] J.F.Riordan, M.Sokolovsky and B.L.Vallee, Biochemistry 6 (1967) 3609.
[10] T.C.Bruice, M.J.Gregory and S.L.Walters, J. Am. Chem. Soc. 90 (1968) 1612.