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Effect of N-bromosuccinimide-modification of tyrosine side chains of cardiotoxin II of the Indian cobra on biological activity

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Abstract. The essential role of tyrosine residue(s) of cardiotoxin II in the biological activity of the toxin was evaluated using N-bromosuccinimide. N-bromosuccinimide effected oxidation of the tyrosine residues in cardiotoxin II with enhancement in absorbance at 260 nm. The influence of various solvent media such as acetate-formate buffer (pH 4·0),0·01 N H_2SO_4 (pH 2·0) and Tris-HCl buffer (pH 8·5) on oxidation of tyrosine residues was exa mined. In comparison with 0·01 N H_2S O_4, acetate-formate buffer could prevent secondary oxidations as revealed by lower consumption of oxidant, N-bromosuccinimide, to achieve oxidation. In Tris-HCl buffer oxidation of tyrosine did not take place effectively. N-iodosuccinimide caused only limited oxidation as evident from minor increase in absorbance at 260 nm. N-chlorosuccinimide was completely ineffective. Oxidation of cardiotoxin II with 3·75 equivalents of N-bromosuccinimide tyrosine residue led to complete loss of lethal activity. However, the derivative retained the ability to protect bacterial protoplasts from lysis in solutions of low tonicity. Unlike cardiotoxin II oxidized with N-chlorosuccinimide (50 equivalents/mol of toxin) which retained lethal activity as well as the ability to protect protoplasts from lysis, performic acid-oxidized toxin had lost both the activities.

Keywords. Cardiotoxin II; *Naja naja;* N-bromosuccinimide oxidation; essential tyrosine; protoplast stabilization.

Introduction

Cardiotoxins are non-neurotoxic protein constituents of cobra venom. These basic toxins are known for their membrane destabilizing property (Condrea, 1974). Recently we have shown that cardiotoxin II stabilizes bacterial protoplasts against lysis under hypotonic condition (Shashidharan and Ramachandran, 1984).

Chemical modification studies on biologically active proteins are carried out in delineating amino acid side chains which are important for their structure and function. An earlier report of ours had dealt with the side-chain amino groups of cardiotoxin II for biological activity (Shashidharan and Ramachandran, 1985). In this paper observations on modification of tyrosine residues with N-bromosuccinimide (NBS) are reported. Earlier reports (Keung *et al.,* 1975; Carlsson, 1980) of modifications of tyrosine sidechains in cardiotoxins had been achieved using tetranitromethane (TNM). Cardiotoxin II is eminently suited for oxidative modification studies with NBS, because the toxin contains neither tryptophan nor histidine (Srinivasa *et al.,* 1982) whose side chains also are known to be susceptible to modifica tion by the reagent (Witkop, 1961). The usefulness of other oxidizing agents such as N-chlorosuccinimide (NCS) and N-iodosuccinimide (NIS) in effecting oxidation of tyrosine residues has also been examined.

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Abbreviations used: NBS, N-Bromosuccinimide; TNM, tetranitromethane; NCS, N-chlorosuccinimide; NI S, N-iodosuccinimide; PCMB,*p*-chloromercuribenzoate.

Materials and methods

Cardiotoxin II was purified as described earlier (Achyuthan *et al.,* 1980); *Micrococcus lysodeikticus* (lyophilised cells) and *p*-chloromercuribenzoate (PCMB) were from Sigma Chemical Co., St. Louis, Missouri, USA; egg-white lysozyme was a gift from Societa Prodotti Antibiotici, Milano; NBS from Koch Light, UK; sodium borohydride from British Drug House, UK; trinitrobenzene sulphonic acid (TNBS) and NCS were gift samples; NIS was recrystallized (dioxane-CCl_4) before use and had a m.p. 201°C (reported 201°C); dansyl chloride was from Pierce, USA and polyamide sheets were from Schleicher and Schuell, Federal Republic of Germany. All other chemicals were analytical grade commercial samples.

The measurements of light absorption were done with a Shimadzu spectrophoto meter (Model UV-150-02) or a MSE Spectro-Plus (Model MK1A) or a Coleman Junior spectrophotometer (Model 6A).

Oxidation of cardiotoxin II with NCS, NBS and NIS

Appropriate amounts (0·325 mg) of cardiotoxin II were dissolved in 3 ml of acetate-formic acid buffer (0·05 M, pH 4·0), or 0·01 N H_2SO_4 or Tris-HCl buffer 0·1 M, pH 8·5. Aliquots (10 μl) of aqueous solutions of NCS or NBS or ethanolic solutions of NIS were added to samples and mixed well, so as to ultimately secure levels in excess of 1·5 equivalents of oxidant/residue of tyrosine in cardiotoxin II. The increase in absorbance at 260 nm was measured after each addition. Necessary corrections were made for dilution with appropriate blanks run simultaneously. The additions were continued until there was no further increase in absorbance at 260 nm. Based on the content of tyrosine residues in cardiotoxin II (Srinivasa *et al.,* 1982) and the increase in absorbance at 260 nm, the equivalents of NBS required to oxidize one to four tyrosine residues were determined. For further studies unless otherwise men tioned, oxidized derivatives were prepared by adding the requisite amount of oxidizing agent to known amounts of cardiotoxin II and the derivatives desalted on Sephadex G-10 columns, using 0·1 N acetic acid for elution.

Consumption of NBS due to oxidation of formate in acetate-formate buffer was followed by iodometry with buffer of strength of 0·005 M containing 0·011 M NBS. At different time intervals, 5 ml of the mixture was withdrawn, excess KI added and iodine liberated titrated against 0·01 N sodium thiosulphate.

Performic acid oxidation

To 0·1 ml of H_2O_2 (30%) was added 0·9 ml of 98% HCOOH and the same left at room temperature (30°C) for 1 h, followed by cooling to 0°C. Cardiotoxin II (1 mg) was treated with 1 ml of the above solution at 0°C, for 1 h. The sample was then diluted to 10 ml with water and lyophilised. To remove any traces of performic acid remaining, the residue was redissolved in 0·5 ml of distilled water and lyophilized again. This procedure was repeated twice.

Reversion of methionine sulphoxide generated, during the oxidation, back to methionine in the oxidized protein (0·5 mg) was achieved by treatment with 0·1 ml of mercaptoethanol, overnight, at room temperature (26–28°C). The excess of mercaptoethanol was removed by lyophilisation.

Estimation of free amino groups

Free amino groups in cardiotoxin were estimated using the method of Fields (1972). The number of free amino groups in the oxidized derivatives was calculated based on the molar absorption (E_M for TNP group 12,587) of products of reaction with TNBS. There are 10 free amino groups in cardiotoxin II (Srinivasa *et al.,* 1982).

Determination of disulphide bonds

Estimation of disulphide bonds was carried out by an indirect method. The toxin was reduced and the resulting –SH groups were estimated using PCMB as described by Boyer (1954).

The reduction of cardiotoxin II and oxidized derivatives was done using sodium borohydride. To appropriate amounts (0·1–0·4 mg) of sample, 0·5 ml of 5% sodium borohydride was added and the tubes stoppered and left at 37 ± 1°C for 2·5 h, after which excess borohydride was destroyed by the addition of 0·1 ml of 50% acetic acid. The last traces of borohydride were eliminated by wetting the sides of the tube with 2 ml of 0·33 M acetate buffer of pH 4·6, so as to avoid interference during estimation of –SH groups using PCMB. After 5 min the required amount (1·5-2·0 mol/mol of –SH group) of PCMB was added and mixed well. After 15 min at room temperature the increase in absorbance at 255 nm was recorded against appropriate blanks. The number of –SH groups was calculated using the known molar absorbance (E_M255 nm 6200).

Dansylation

Dansylation of cardiotoxin and derivatives was carried out according to Hartley (1970).

Toxicity

The toxicity of the oxidized products (NBS and NCS oxidized) was assayed by injecting weights of material corresponding to one to ten LD_{50} doses of native cardiotoxin II, in 0·5 ml of 0·9% saline, intraperitoneally, into Swiss albino mice. The animals were kept under observation for a period of 72 h thereafter.

Assay of stabilization of bacterial protoplasts: The assay was carried out as descri bed earlier (Shashidharan and Ramachandran, 1984).

Results

Reaction of cardiotoxin II with NCS, NBS and NIS

The course of oxidation of cardiotoxin II with increasing concentration of oxidizing agents such as NCS, NBS and NIS is shown in figure 1. The increase in absorbance

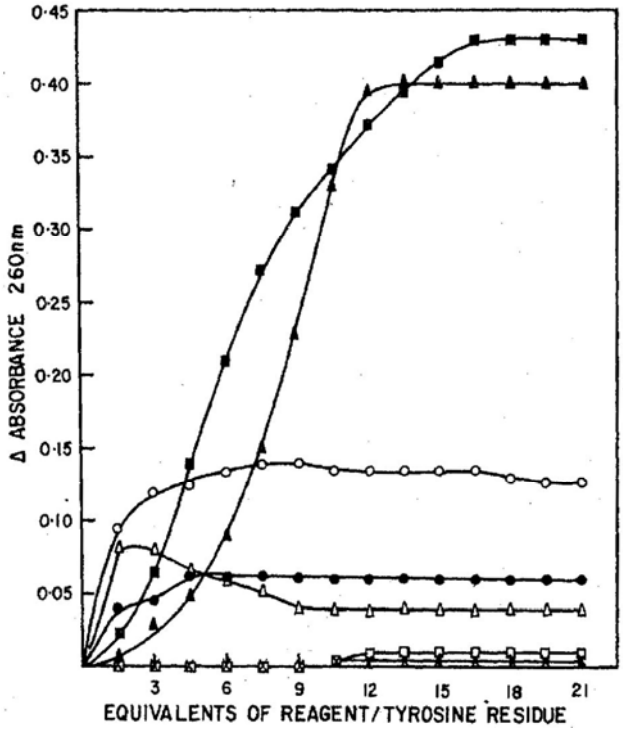
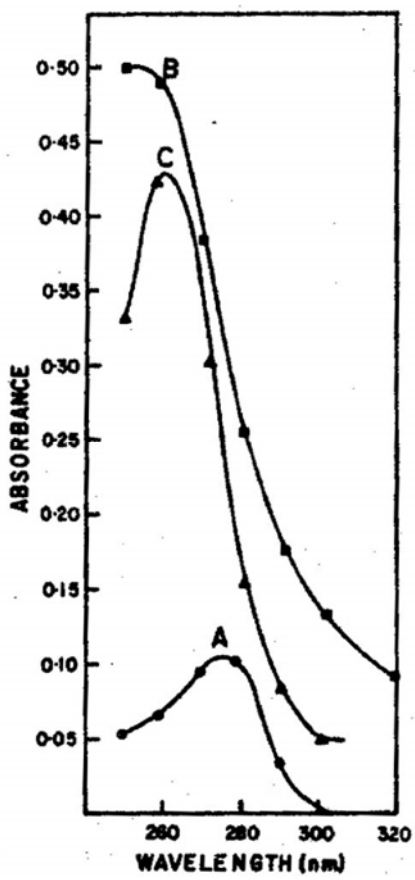


Figure 1. Oxidation of cardiotoxin II (0·0158 μmol/ml) under varying conditions with increasing concentrations of the reagents: with NBS:(▲), pH 2·0; (■), pH 4·0; (●), H8·5; with NCS: (×), pH 4·0; (□), pH 8·5; with NIS: (O), pH 4·0;(Δ), pH 8·5.

at 260 nm with increments in amount of reagent used is highest with NBS in acidic conditions. Amounts of reagent (NBS) consumed for different degrees of oxidation with acetate-formic acid buffer and 0·01 N H_2SO_4 as media are given in table 1. UV spectra of the toxin oxidized with NBS at pH 4·0 are shown in figure 2, while spectra

Table 1. Oxidation of tyrosine residues of cardiotoxin II (0·0158 μmol/ml) in acetate-formate bufer and 0·01 N H_2SO_4.

Tyrosine oxidized no. of residues	Solvent: Acetate-formate buffer pH 4·0 — Absorbance (260 nm)	Solvent: Acetate-formate buffer pH 4·0 — Eqs. of NBS per tyrosine residue	Solvent: 0·01 N H_2SO_4 — Absorbance (260 nm)	Solvent: 0·01 N H_2SO_4 — Eqs. of NBS per tyrosine residue
1	0·107	3·75	0·10	6·30
2	0·215	6·25	0·20	8·40
3	0·322	9·00	0·30	10·05
4	0·430	16·87	0·40	13·50



Figured 2. UV spectra of native and foully NBS-oxidized cardiotoxin II (0·0158 μmol/ml). (A), native cardiotoxin II; (B), cardiotoxin II oxidized in acetate-formate buffer 0·05 M, pH4·0; (C), cardiotoxin II oxidized in 0·01 N H_2SO_4.

obtained at pH 8·5 and for oxidations with NCS and NIS in different solvent media are not shown.

The molar absorbance (E_M260) of an oxidized tyrosine residue in cardiotoxin was found to be 7288 in acetate-formate buffer and 6780 in 0·01 H_2SO_4. Reported values in the range 10,000-11,000 for dibromodienone spirolactone derivative of tyrosine are taken as basis for calculations (Ramachandran and Witkop, 1967). The spectrophotometric data would correspond to oxidation of 2·65-2·85 mol of tyrosine/mol of toxin.

Destruction of NBS by solvent system

Within 20 min after addition of the reagent (NBS) more than 50% of the reagent was destroyed in 0·01 M acetate-formate bluffer (pH 4·0) of one-tenth the molarity used in routine experiments in which consumption of reagent in oxidation of formate would be more rapid. The rate of destruction of NBS in original buffer could not be follo wed owing to a nearly instantaneous destruction of reagent. In 0·01 N H_2SO_4 there was no destruction of the reagent.

Intactness of free amino groups in cardiotoxin

Table 2 presents data on number of free amino groups in cardiotoxin II and oxidized derivatives.

Table 2. The free amino groups in cardiotoxin II and oxidized cardiotoxin II as determined with TNBS.

Sample	Molar absorbance* (E_M 420 nm)	No. of free amino groups
Native cardiotoxin II	1,25,874	10·00
Cardiotoxin II with one Tyr oxidized	1,23,786	9·80
Cardiotoxin II with two Tyr oxidized	1,20,145	9·50
Cardiotoxin II with three Tyr oxidized	1,17,718	9·18
Cardiotoxin II with four Tyr oxidized	87,378	6·94
Cardiotoxin II with performic acid	1,25,600	9·9

*Concentration of toxin and the derivatives in the range 0·412–0·429 × 10^{-5} M were used.

Intactness of disulphide bonds

Table 3 provides data on the integrity of disulphide bonds. No cleavage of disulphide bonds occurs on oxidation at NBS levels of 3·75 eqs/tyrosine residue (S. No. 2). With excess of reagent i.e., 6·25, 9·0 and 16·87 eqs of NBS/tyrosine residue (S. Nos. 3, 4, 5), 0·8, 1·51 and 2·05 disulphide bonds are, respectively, cleaved oxidatively per mol of cardiotoxin.

Table 3. Number of – SH groups generated on reduction of cardiotoxin II and its NBS-oxidized derivatives with sodium borohydride.

Sample	Conc. of sample used for–SH measurement (M)	Molar absorbance change in the PCMB reaction (E_M 255 nm)	No. of–SH groups generated on borohydride reduction
Native cardiotoxin II	$0·296 \times 10^{-5}$	50,000	8·06
Cardiotoxin II with one Tyr oxidized	$0·425 \times 10^{-5}$	48,717	7·85
Cardiotoxin II with two Tyr oxidized	$0·596 \times 10^{-5}$	39,705	6·40
Cardiotoxin II with three Tyr oxidized	$0·568 \times 10^{-5}$	31,092	5·01
Cardiotoxin II with four Tyr oxidized	$0·948 \times 10^{-5}$	24,369	3·90
Cardiotoxin II oxidized with performic acid	$0·948 \times 10^{-5}$	0	0

Cleavage of peptide bonds

The release of new terminal amino groups as monitored by dansylation of oxidized products showed that there was no detectable new amino terminal residues released on oxidation of cardiotoxin II with NBS. Dansylation and hydrolysis did not reveal the presence of O-dansyl tyrosine in hydrolyzates of the oxidized derivatives. It was

however present as expected in the hydrolyzate of dansylated native protein. But performic acid-oxidized cardiotoxin on further oxidation with NBS (16·87 eqs/ tyrosine residue) contained 3 new amino terminal residues (not identifier), indicating peptide bond cleavage under such conditions.

Toxicity of oxidized toxin

The derivative obtained on oxidation with 3·75 eqs of NBS/tyrosine residue was found to be completely lacking in lethal potency. The derivative was inactive at a level corresponding to 10 LD_{50} doses of native cardiotoxin II . That oxidation of cardiotoxin II at a level of 8 eqs NCS/mol of cardiotoxin II did not result in any inactivation of the lethal potency which was revealed by its ability to induce death in mice at one LD_{50} dose. A sample oxidized with 50 eqs NCS/mol of cardiotoxin was also found to cause death in mice but with amounts corresponding to 10 LD_{50} doses of native cardiotoxin II. Performic acid oxidized toxin and the sample in which methionine sulphoxides formed on performic acid oxidation were reverted to methionine lacked lethality even at such high dose levels.

Ability of oxidized derivatives to stabilize bacterial protoplasts from lysis

The lysis of bacterial cells in the presence of various derivatives as followed by decrease in turbidity is illustrated in figure 3. The effect of NBS-oxidized derivative on bacterial protoplast stabilization and the ability to cause the initial expected increase in absorption of the bacterial cell suspension are shown in figure 4. The derivatives with one or two tyrosine residues oxidized, and the derivative from oxidation with 50 eqs NCS/mol of cardiotoxin were as efficient as native toxin. With 3 and 4 tyrosine residues of cardiotoxin oxidized, a decrease of 7 and 50% in stabilization ability was observed. Performic acid oxidized cardiotoxin II and the same derivative in which the methionine was regenerated were not at all active as stabilizing agents. On comparison of the ability of the derivatives to enhance absorbance of bacterial cell suspensions, it was found that loss of 1 or 2 tyrosine residues caused a fall of 36% in this ability, while loss of 3 and 4 tyrosines, caused a fall of 50 and 87% respectively. On the other hand the NCS-oxidized sample retained 100% of this ability, and the performic acid-oxidized toxin and the sample in which the methionine sulphoxide formed on performic acid oxidation was reverted back to methionine which exhibited a 100% loss of this ability.

Discussion

The NBS-oxidation of tyrosine side chains in cardiotoxin II has been examined in the present study in the medium 0·05 M acetate-formic acid buffer of pH 4·0, a buffer credited with the ability to afford protection to protein from secondary oxidations (Patchornik *et al.,* 1960). NBS decomposes to CO_2, hydrobromic acid and succinimide in the above medium instantaneously (Barakat *et al.,* 1955). For reasons which are therefore evident, reagent consumption for oxidation of cardiotoxin was relatively high (table 1), compared to say in a medium such as acetic acid which is unsuited for

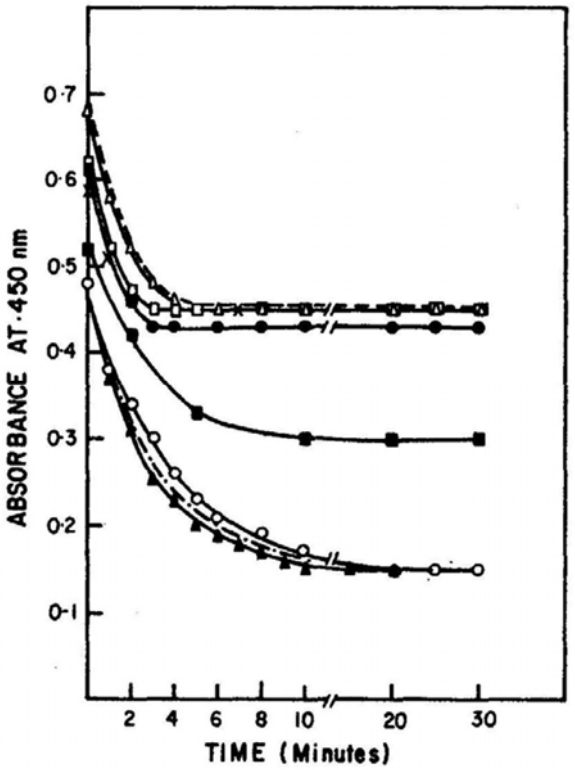


Figure 3. Time course of bacterial cell lysis inhibition by cardiotoxin II (0·026 μmol/ml) and its oxidized derivatives (0·026 μmol/ml): (O), lysozyme control; (Δ), native cardiotoxin II; (□), cardiotoxin II with 1 Tyr oxidized; (x), cardiotoxin II with 2 Tyr oxidized; (●), cardiotoxin II with 3 Tyr oxidized; (■), cardiotoxin II with 4 Tyr oxidized; (Δ), cardiotoxin II oxidized with performic acid, cardiotoxin II oxidized with performic acid and methionine sulphoxide reverted to methionine; (– – –), cardiotoxin II oxidized with NCS (see text for details).

spectrophotometric observations at the required wavelengths. The solvent 0·01N H_2SO_4 in which the reagent is stable did not seem to offer any special advantage as reagent consumption to effect the same degree of oxidation as in acetate-formic acid buffer was found by us to be much higher, for lower levels of oxidation, presumably due to low accessibility of tyrosine residues at the lower pH.

At pH 8·5 NBS and NIS give rise to product spectra with peak absorption at 310 nm which is attributable to *o*-dihalogenation of tyrosine residues, but not con version to 3,5-dihalogenodienone (λ_{max} 260 nm) at the alkaline pH (spectra not shown). At pH 4·0 and 8·5, NCS did not affect the absorption spectrum of cardiotoxin II, whereas NIS at pH 4·0 gave rise to a peak of absorption at 290–300 nm, diminu tion in the normal phenolate absorption and enhanced absorption at 260 nm and lower wavelengths (spectra not shown). Under acidic conditions, only NBS was found to be best suited for oxidation of tyrosine residues of cardiotoxin II.

The lower molar absorption obtained (7288 and 6780 in formate buffer and 0·01 N H_2SO_4, respectively) for the toxin oxidized with NBS could be due to oxidation of some or all of the tyrosines to intermediates other than the dibromodienone lactone expected from studies on NBS oxidation of much simpler (small) model substrates

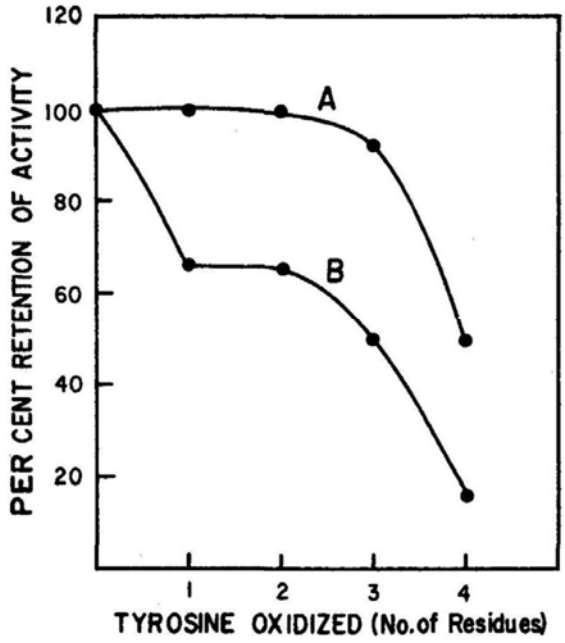


Figure 4. Effect of oxidation of tyrosine residues in cardiotoxin II with NBS on lysis inhibition of *M. lysodeikticus* cells and increase in initial absorbance of bacterial cell suspension. (A), lysis inhibition; (B), increase in absorbance.

(peptides). Even ribonuclease which contains 6 tyrosine residues (Hirs *et al.,* 1960) in its structure, and therefore not ideally suited for comparison with cardiotoxin, cleavage of tyrosyl bonds was achieved by using great excess of oxidant (46 eqs NBS/mol of ribonuclease and 30 eqs/mol) with carboxymethyl-ribonuclease (Wilson and Cohen, 1963). Based on the spectrophotometric data they showed oxidation of only 4·6 tyrosine residues. In the oxidation of cardiotoxin II 2·85 and 2·65 (approx. 3) tyrosine residues appeared to have been oxidized in formate buffer and 0·01 N H_2SO_4, respectively, based on the extinction expected of dienone (E_M 260–10,200).

Destruction of side chain amino groups of lysozyme by NBS is known (Ramachandran and Witkop, 1959). This loss in side chain amino groups was also noted in cardiotoxin II when treated with excess of NBS (6·25 eqs/tyrosine residue). At concentrations of NBS less than this, which is sufficient to cause oxidation equi valent to that of two tyrosine residues, there was no loss of amino groups.

Cleavage of peptide bonds was ruled out in the present oxidative modification studies based on the absence of formation of any new reactive N-terminal residues by dansylation which offers a sensitive probe. On the contrary, oxidation of per-formic acid oxidized cardiotoxin with NBS appears to cause some cleavage of peptide bonds. Three extra spots of dansyl amino acids, other than those expected, were seen on the chromatogram relative to the control. O-Dansyltyrosine spots were not detected on the chromatograms of hydrolyzates of oxidized protein indicating that tyrosine residues had undergone modification.

Present observations on cardiotoxin II revealed that at lower levels of NBS used, adequate for oxidation of two tyrosine residues, there was no damage to disulphide

bonds as judged from data on–SH groups generated on reduction with $NaBH_4$ of intact –S–S– left untouched by NBS in the modified derivative. However, there was oxidative cleavage of disulphide bonds (table 3) with use of larger amounts of NBS required to oxidize two more of the tyrosine residues present in the toxin.

Carlsson and Louw (1978) had selectively modified methionine residues in cardiotoxin of *Naja melanoleuca* with NCS (8 eqs/mol of toxin) leading to loss in lethal activity. In our studies, toxin treated with 50 eqs of NCS/mol still retained its letha lity to mice. Structural differences in the two toxins perhaps account for the difference in susceptibility to loss of biological activity on what is presumed to be oxida tion of the two methionine residues in the toxin. On the basis of reactivity (Schechter *et al.,* 1975) to NCS, the only two methionines in cardiotoxin II may be considered as partly, if not foully, buried. Two methionines (24 and 26) are considered invariant in the cardiotoxins (Dufton and Hider, 1983).

It seems reasonable to conclude that the observed loss of lethality of cardiotoxin II, on limited NBS oxidation, results from the oxidation of a single tyrosine residue, whose location in the structure will remain to be ascertained.

The ability of the toxin to confer stability to bacterial protoplasts was not affected when oxidized with lower amounts of NBS. However, a fully oxidized derivative exhibited a retention of only about 50% of such activity. Loss of the remaining 50% of the activity occurs only when the two left-over tyrosine residues, 3 amino groups and two disulphide bonds are oxidatively modified. Marked differences that were observed in the ability of derivatives in bringing about initial increase in absorbance of bacterial cell suspensions may be due to involvement of different structural elements in the toxin in the two functions.

Three-dimensional structural integrity associated with disulphide bonds seems to be important for the protoplast stabilization activity of the toxin. Performic acid oxidized toxin is thus inactive, even though all 10 amino groups remain intact. The basic property of the molecule as such seems inadequate, although with compounds such as spermine and related polyamines, which stabilize protoplasts under hypotonic conditions, this property has been invoked to account for the phenomenon (Tabor, 1962). With fully NBS-oxidized cardiotoxin, which still retains two intact disulphide bonds and 7 of the original 10 amino groups, 50% retention of the ability of the native toxin to protect protoplasts is observed.

Earlier studies using tetranitromethane (Keung *et al.,* 1975; Carlsson, 1980) did not fully establish the importance of tyrosine residues in the biological activity of the cardiotoxins studied, since modified derivatives retained atleast partial biological activity, such as lethal potency, haemolytic activity and cytolytic activity. In contrast, the use of the NBS in oxidation of the tyrosine residues in cardiotoxin of the Indian cobra reveals the importance of a tyrosine residue in relation to lethal activity. The tyrosine residue that is involved could be the equivalent of the invariant residue (Tyr 22) which had been noted in cardiotoxins from species other than *Naja naja* (Joubert, 1976; Ryden *et al.,* 1973). This tyrosine residue, however, seems unimportant for protecting bacterial protoplast membranes against lysis under hypotonic conditions.

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