be recognized in the formulation of models or mechanisms of cooperative ligand binding to hemoglobin.

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References

Abraham, R. J. (1961), *Mol. Phys. 4*, 145.
Antonini, E. (1965), *Physiol. Rev. 45*, 123.
Carrell, R. W., Lehmann, H., Lorkin, P. A., Raik, E., and Hunter, E. (1967), *Nature (London) 215*, 626.
Dadok, J., Sprecher, R. F., Bothner-By, A. A., and Link, T. (1970), *Abstr. 11th Exp. NMR Conf., Pittsburgh, Pa.*
Dayhoff, M. O. (1969), Atlas of Protein Sequence and Structure, Silver Spring, Md., National Biomedical Research Foundation.
Dozy, A. M., Kleihauer, E. F., and Huisman, T. H. (1968), *J. Chromatogr. 32*, 723.
Drabkin, D. L. (1946), *J. Biol. Chem. 158*, 703.
Geraci, G., Parkhurst, L. J., and Gibson, Q. H. (1969), *J. Biol. Chem. 244*, 4664.
Glasoe, P. D., and Long, F. A. (1960), *J. Phys. Chem. 64*, 188.
Ho, C., Davis, D. G., Mock, N. H., Lindstrom, T. R., and Charache, S. (1970), *Biochem. Biophys. Res. Commun. 38*, 779.
Johnson, C. E., and Bovey, F. A. (1958), *J. Chem. Phys. 29*, 1012.
McDonald, C. C., and Phillips, W. D. (1967), *J. Amer. Chem. Soc. 89*, 6332.
McDonald, C. C., Phillips, W. D., and Vinogradov, S. N. (1969), *Biochem. Biophys. Res. Commun. 36*, 442.
Muller, C. J., and Kingma, S. (1961), *Biochim. Biophys. Acta 50*, 595.
Perutz, M. F. (1969), *Proc. Roy. Soc., Ser. B 173*, 113.
Perutz, M. F. (1970), *Nature (London) 228*, 726.
Schroeder, W. A., Shelton, J. R., Shelton, J. B., Cormick, J., and Jones, R. T. (1963), *Biochemistry 6*, 2395.
Shulman, R. G., Wüthrich, K., Yamane, T., Patel, D. J., and Blumberg, W. E. (1970), *J. Mol. Biol. 53*, 143.
Sternlicht, H., and Wilson, D. (1967), *Biochemistry 6*, 2881.
Van Gelder, B. F., and Slater, E. C. (1962), *Biochim. Biophys. Acta 58*, 593.
Zade-Oppen, A. M. M. (1963), *Scand. J. Clin. Lab. Invest. 15*, 491.

Structure–Function Relationships of Neurotoxins Isolated from *Naja haje* Venom. Physicochemical Properties and Identification of the Active Site†

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ABSTRACT: Neurotoxins I and III of *Naja haje*, like other miniproteins with a high content of disulfide bridges, present an unusually high resistance to denaturing conditions at neutral pH. Optical rotatory dispersion measurements with neurotoxin I indicate 2 conformational changes at acidic pH. One of them is controlled by the unmasking of a carboxylate side chain having an apparent pK of 2.0 at 13°. Titration, nitration, and acetylation show that tyrosine-24 is masked in the native conformation of neurotoxin I. This residue is essential for the stabilization of an active structure. Its nitration abolishes toxicity. Difference spectra and fluorescence data indicate a masking of the tryptophan-28 side chain. Formylation does not abolish toxicity. Acetylation and maleylation indicate that lysine residues are essential elements of the active site. Dansylation affects selectively 2 superreactive residues, lysines-26 and -46. The kinetics of the loss of toxicity closely parallels the covalent modification of these two lysine residues.

Snake neurotoxins are made of single peptide chains of 60–74 amino acids cross-linked internally by 4 or 5 disulfide bridges. These miniproteins are well suited for comparative sequence studies, analyses of conformational properties and determinations of active sites.

Due to the increasing use of neurotoxins to study neurotransmitters–receptors interactions (Changeux *et al.*, 1970; Miledi *et al.*, 1971) it is of prime importance to understand the structure–function properties of these proteins.

Neurotoxins I and III obtained from *Naja haje* venom have been selected for this work. The sequence of neurotoxin I which consists of 61 amino acid residues and 4 disulfide bridges has been recently elucidated (Botes and Strydom,

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1969). The sequence of neurotoxin III which contains 71 amino acids and 5 disulfide bridges is not yet known.

Parallel studies on scorpion neurotoxins are also carried out in our laboratories (Rochat *et al.*, 1970a,b; Chicheportiche and Lazdunski, 1970).

Materials and Methods

Neurotoxins and Chemicals. Neurotoxins I and III were purified from the venom of *Naja haje* as previously described by Miranda *et al.* (1970a). The proteins were shown to be homogeneous by equilibrium chromatograghy on Amberlite CG-50, starch gel electrophoresis, and amino acid composition.

Toxicity was determined according to Miranda *et al.* (1970b) by subcutaneous injection of the neurotoxins into mice. Neurotoxins I and III have an LD_{50} of 1.12 and 1.35 μg, respectively, per 20 g of body weight.

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide was obtained from the Ott Chemical Company. Dansyl chloride came from Sigma and dithioerythritol from Pierce. TPCK[1]-treated trypsin was a Worthington product. [^{14}C]Acetic anhydride, [^{14}C]maleic anhydride, and [^{14}C]glycine ethyl ester were obtained from the Commissariat à l'Energie Atomique (France).

Optical Rotatory Dispersion, Spectrophotometric Titration, and Fluorescence Measurements. Optical rotatory dispersion (ORD) measurements were carried out in a Fica Spectropol I spectropolarimeter. The cell could be thermostated between 5 and 85° ± 0.2°. Optical rotatory dispersion data are given as mean residue rotation [m], in degrees × cm^2 × decimole^{-1}.

$$[m]_\lambda = \frac{\alpha_\lambda \times MRW}{100cl}$$

The mean residue weight (*MRW*) was 114 for neurotoxin I and 113 for neurotoxin III.

Citrate (0.01 M), acetate (0.01 M), and phosphate (0.01 M) were the buffers used between pH 2 and 8 in thermal denaturations studies. They all contained 0.1 M NaCl. Dissolved gases were carefully removed from the buffers so that bubbles would not form on heating. Prior to use the solutions were filtered through Millipore filters (0.45 μ). Protein concentrations were estimated in a Zeiss PMQ II spectrophotometer. At 280 nm $\epsilon^{1\%}_{1\,cm} = 13.5$ for neurotoxin I and 11.5 for neurotoxin III (Miranda *et al.*, 1970a).

Spectrophotometric titration of the tyrosine residue of neurotoxin I was performed with a Cary 14 spectrophotometer using a 0.12 mg/ml protein solution in a 0.01 M phosphate buffer containing 0.1 M NaCl. The temperature was maintained at 20° ± 0.1° in both compartments of the spectrophotometer. The pH of the solution in the reference cell was kept at 7.0 while the pH of the solution in the other cell was constantly varied by addition of minute amounts of NaOH 1 N or 10 N. The new pH value was measured after each NaOH addition and the spectrum was recorded between 250 and 320 nm.

Fluorescence measurements (corrected excitation and emission spectra) were taken with a Fica 55 differential spectrofluorimeter. All fluorescence measurements were performed at 30°. The quantum yield was evaluated as described by Teale (1961).

Nitration. Nitration was carried out according to the general method of Sokolovsky *et al.* (1966) in a 0.1 M Na phosphate buffer at pH 8.0, 20° during 4 hr. The reaction was stopped by filtration on Bio-Gel P2 equilibrated at pH 6.9 with 0.1 M ammonium acetate. The nitrotyrosine content was measured by amino acid analysis in a Beckman 120 C analyser.

Chemical Modification of Carboxylates. Carboxylic functions were transformed into amides with glycine ethyl ester under the influence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide by the method of Hoare and Koshland (1967). The reaction was carried out at pH 4.7, 25°, with 1 M [^{14}C]-glycine ethyl ester and 0.065 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. The neurotoxin concentration was 0.33 mg/ml. The reaction was stopped after 3 hr by chromatography through a Sephadex G-25 column (3 × 30 cm) equilibrated with 0.001 M HCl. The protein peak was pooled, concentrated by partial lyophylization, and reacted with hydroxylamine (0.5 M) at pH 7, 25°, to reverse a possible covalent modification of the tyrosine residue (Carraway and Koshland, 1968). The number of [^{14}C]glycine ethyl esters incorporated was counted in a Packard scintillation spectrometer after rechromatography on the same Sephadex column.

Formylation of Tryptophan. Formylation of toxin I of *Naja haje* was conducted in anhydrous formic acid saturated with gaseous HCl as described by Previero *et al* (1967). Deformylation easily occurs at pH higher than 8.5 (Previero *et al.*, 1967); it was followed at pH 9.7 in a 0.8 M sodium carbonate buffer containing 7 M urea.

The extent of tryptophan reversibly transformed into *N*-formyltryptophan was estimated spectrophotometrically at 298 nm (ϵ_M 4.88 × 10^3, Previero *et al.*, 1967) in a Cary 14 spectrophotometer. Toxicity of the modified toxin was measured after dilution of aliquots into a bovine serum albumin solution at 10 mg/ml in 0.14 M NaCl at pH 7.0. Amino acid analyses were carried out with a Technicon autoanalyser after acidic hydrolysis in 6 N HCl during 20 hr at 110°.

It was verified that neurotoxin I retained a full activity after staying in anhydrous formic acid, *or in 1 N HCl* during 100 min.

Acetylation and Maleylation. Acetylation of neurotoxins I and III was carried out at pH 8 (0.1 M Tris) and 4° by adding every 10 min 10-μl aliquots of [^{14}C]acetic anhydride to the protein solution (1–2 mg/ml). The pH was maintained constant in a Radiometer pH-Stat. The reaction was stopped after 1 hr. The mixture was then passed through a Sephadex G-25 column (2 × 30 cm), equilibrated in 0.1 M ammonium acetate, to separate radioactive acetate from the acetylated protein. This protein sample was then treated with 1 M hydroxylamine pH 7 to reverse a possible acetylation of tyrosine. After 1 hr the excess of hydroxylamine was eliminated by filtration on Sephadex G-25 as described above. The number of acetyl groups incorporated in the neurotoxin was estimated from radioactivity measurements in a Packard Tricarb scintillation spectrometer model 3375. The kinetics of tyrosine deacylation under the influence of hydroxylamine were carried out at 278 nm in a Cary 15 spectrophotometer.

Maleylation of neurotoxin I (1 mg/ml) was carried out at pH 9 according to Butler *et al.* (1969) using [^{14}C]maleic anhydride. After 1 hr, the reaction mixture was passed through a Sephadex G-25 column (2 × 20 cm) equilibrated with a 0.1 M phosphate buffer, pH 7.5. The number of maleyl groups incorporated into the neurotoxin was estimated by radioactivity measurements. Demaleylation was obtained after heating the maleylated protein at 60°, pH 2.7, for 4 hr.

[1] Abbreviation used is: TPCK, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone.

Dansylation of Neurotoxin I. Neurotoxin I of *Naja haje* (10 mg) was dissolved in 8 ml of a 0.05 M phosphate buffer at pH 8.9. The reaction was started with 0.25 ml of a 0.2 M solution of dansyl chloride in acetonitrile. The temperature of the mixture was kept at 25°. Aliquots were taken at different times to measure the extent of dansylation, as well as the physical properties and toxicity of the derivative. Aliquots of 0.6 ml were chromatographed on a small column of Sephadex G-25 (2 × 7 cm) equilibrated with 0.2 M acetic acid to separate the dansylated protein from excess of dansyl chloride. This dansylated toxin was used for amino acid analysis and for ORD measurements. Toxicity measurements (LD_{50}) were made on aliquots of 0.2 to 1 ml which were diluted with a solution of bovine serum albumin (10 mg/ml in 0.15 M NaCl) at pH 7.0 to stop the dansylation. Dansyl chloride had no toxic effect by itself after dilution of the aliquot.

Dansylated peptides were obtained in the following way. One micromole of the dansylated toxin I was dissolved in 3 ml of a Tris-acetate buffer (0.25 M) at pH 8.6 containing 8 M urea and 14 mM EDTA. Protein reduction at 40° was achieved by incubation with 0.24 mmole of dithioerythritol during 17 hr and under nitrogen. The reduced toxin was then alkylated by treatment with iodoacetic acid (0.61 mmole) at 20° for 15 min, isolated by filtration on a column of Sephadex G-10 equilibrated with 5 M acetic acid and lyophylized. Trypsin hydrolysis of the reduced and alkylated toxin was obtained at pH 8.0 and 38° in a Radiometer pH-Stat. The concentrations of the alkylated toxin and of trypsin were 1.5 mg/ml and 0.015 mg/ml, respectively.

The tryptic peptides were separated by paper chromatography using 1-butanol–acetic acid–water (4:1:5, v/v) and high-voltage electrophoresis (44 V/cm) at pH 3.6 in a pyridine–acetic acid–water buffer (1:10:289, v/v) according to Katz *et al.* (1959). The dansylated peptides were located by ultraviolet fluorescence (340 nm) and eluted with 5 M acetic acid. After desiccation, they were hydrolyzed in 6 N HCl at 110° during 20 hr. Amino acid compositions were obtained with a Beckman 120 C analyser.

Results

Conformational Properties of Neurotoxins. pH and Temperature Dependences. Figure 1 shows some optical rotatory dispersion spectra of neurotoxins I and III. The ORD spectrum of neurotoxin I at neutral pH is very atypical with a shoulder around 250 nm, a peak at 233 nm and a very small trough at 222 nm. This observation underlines once more (see for example Gratzer and Cowburn, 1969) the dangers of interpreting ORD spectra, or circular dichroism (CD) spectra, in terms of admixtures of α helix, β structure, and random coil. The spectrum of neurotoxin III presents a peak at 233 nm and a trough at 220 nm.

ORD measurements provide one of the most convenient ways for the study of conformational stabilities and denaturation equilibria. As shown in Figure 1, the ORD spectrum of neurotoxin I varies considerably with pH and temperature.

Figure 2 describes the variation of $[m]_{233}$ with pH for neurotoxin I. The simplest interpretation of this figure is that there exists 2 successive reversible structural changes in the acidic pH range. Transition between forms I and II occurs between pH 8 and 4; transition II $\rightleftharpoons$ III occurs at lower pH values. The good agreement between the experimental points and the calculated curve in Figure 2 indicates that both isomerizations I $\rightleftharpoons$ II and II $\rightleftharpoons$ III are due to the protonation of single ionizable groups with apparent pK's of 5.2 and 2.05, respectively. The group with a pK_{app} of 5.2 is masked in form I and unmasked in form II. The group with a pK_{app} of 2.05 is masked in form II and unmasked in form III. The characteristic molecular rotations of forms I, II, and III at 233 nm are $[m]_I = 1560$, $[m]_{II} = 1140$, $[m]_{III} = 0$. The conformational changes which occur at acidic pH are limited since not only form I but also forms II and III are still folded structures which can be denatured by elevating the temperature. Some typical melting curves are presented in Figure 3.

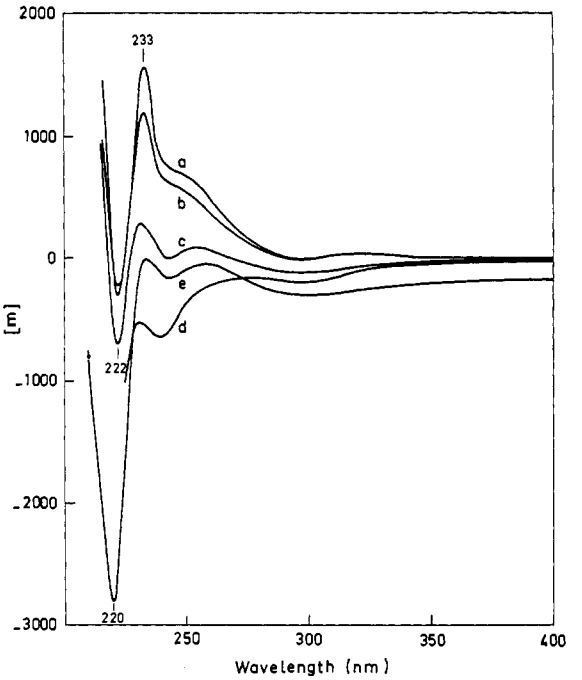

FIGURE 1: ORD spectra of neurotoxin I (c = 0.156 mg/ml) at 15°, pH 7.0 (a), pH 4.6 (b), pH 2.0 (c), and at 65°, pH 4.6 (d). Curve e represents the spectrum of neurotoxin III at 20°, pH 7.0 (c = 0.11 mg/ml). Phosphate (0.01 M) and acetate (0.01 M) buffers were used at pH 7.0 and 4.6. They contained 0.1 M NaCl.

Toxin I exhibits a characteristic feature found in this laboratory with other miniproteins of the same size (Chicheportiche and Lazdunski, 1970; Vincent *et al.* 1971), namely, the low cooperativity of the thermal denaturation. Melting occurs in a temperature range covering more than 30°. van't Hoff plots obtained from melting curves are perfectly linear as it has been observed in other instances (Chicheportiche and Lazdunski, 1970; Vincent *et al.* 1971). The transition temperature varies considerably with pH passing from a high value of 77.5° at neutral pH to a low value of 28° at pH 1.6 (Figure 3). Form I is considerably more resistant to thermal denaturation than forms II and III. Form III is the less stable structure. Neurotoxin III of *Naja haje* is considerably more resistant than neurotoxin I; at pH 4.8 its transition temperature is 85° as compared to 71° for neurotoxin I. This may be due to the presence in neurotoxin III of 5 disulfide bridges instead of 4 in neurotoxin I.

Figure 4 presents a typical difference spectrum obtained by thermal denaturation of neurotoxin I. Perturbations occur at 273, 278, 287, and 299 nm. There are only 2 aromatic chromophores in neurotoxin I, the side chains of tyrosine-24 and of tryptophan-28. Peaks over 290 nm have never been obtained by unfolding proteins such as insulin (Leach and Scheraga, 1960) or ribonuclease (Scheraga, 1957) which contain only tyrosine residues. This suggests strongly that the perturbation at 299 nm is due to an unmasking of tryptophan-

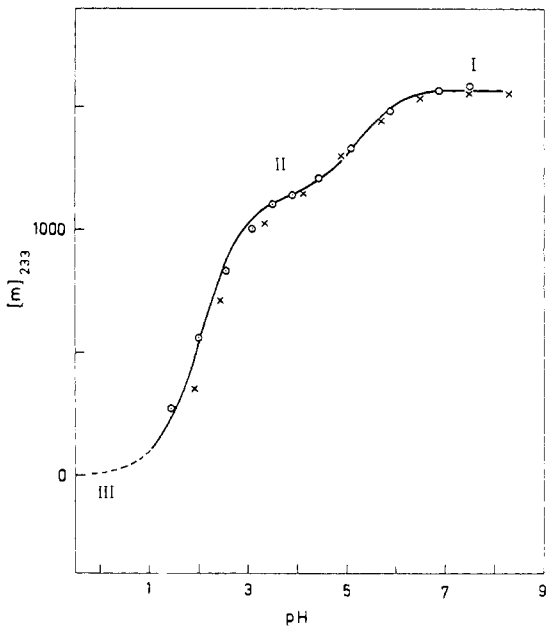

FIGURE 2: Isothermal conformational changes of neurotoxin I at acidic pH followed by the variation of $[m]_{233}$ *vs.* pH at 13° (c = 0.156 mg/ml in 0.1 M NaCl and 0.01 M acetate). The different symbols represent different sets of experiments. The transitions are perfectly reversible. I, II, and III are the different isomers identified below pH 9. The solid line is calculated according to the following equations with $[m]_{I} = 1560$, $[m]_{II} = 1140$, $[m]_{III} = 0$, $K_1 = 10^{-5.2}$ and $K_2 = 10^{-2.05}$.

$$I \underset{}{\overset{+H^+}{\rightleftharpoons}} II \overset{+H^+}{\rightleftharpoons} III$$

$$[m]_{233} = \frac{1}{1 + \frac{[H^+]}{K_1}\left(1 + \frac{[H^+]}{K_2}\right)}[m]_I + I\frac{1}{1 + \frac{K_1}{[H^+]} + \frac{[H^+]}{K_2}}[m]_{II} + \frac{1}{1 + \frac{K_2}{[H^+]}\left(1 + \frac{K_1}{[H^+]}\right)}[m]_{III}$$

28, while perturbation at 273, 278, and 287 nm which are also found for insulin or ribonuclease are due to unmasking of tyrosine-24. Difference spectra with model compounds and lysozyme also support this interpretation (Scheraga, 1961).

Conformational Properties of Neurotoxin I in Urea. ORD measurements have also been used for the study of the stability of neurotoxin I in urea (Figure 5A). The ORD spectrum of neurotoxin I in 9.8 M urea at pH 7.0 is characteristic of a folded form noted Iu. The protein which remains in a conformation close to the native form (form I), even in this high concentration of denaturing agent, can be unfolded at high temperatures (Figure 5B). The transition temperature is still high (56°) at pH 7.0 in 9.8 M urea. While the neurotoxin I in 9.8 M urea remains tightly folded at neutral pH and ambient temperature, complete denaturation occurs at pH 4 in 9.8 M urea as can be seen from the shape of the ORD spectrum and its insensitivity to changes of temperature (Figure 5). A systematic study of the pH influence upon denaturation by increasing concentrations of urea is presented in Figure 6. Denaturation is induced at a constant urea concentration by lowering the pH. The detailed theoretical treatment of the cooperativity between the denaturing agent and pH for the unfolding of proteins can be found elsewhere (Aune and Tanford, 1969a,b). At all urea concentrations the transformation of the form Iu of neurotoxin I into the denatured form, D, is described by the simple equation, Iu + $H^+ \rightleftarrows$ D. The sensitivity of neurotoxin I to urea denaturation is dependent upon the protonation of a single group. The midpoints of the curves $[m]_{233}$ *vs.* pH (pH_m) are dependent upon urea concentration. The limit of pH_m at zero concentration of urea (Figure 6) represents the value of pK_{app}, the apparent pK of the ionizable group which controls the acidic transition. This value is 2.0, very similar to that already found for isomerization II $\rightleftharpoons$ III in Figure 2.

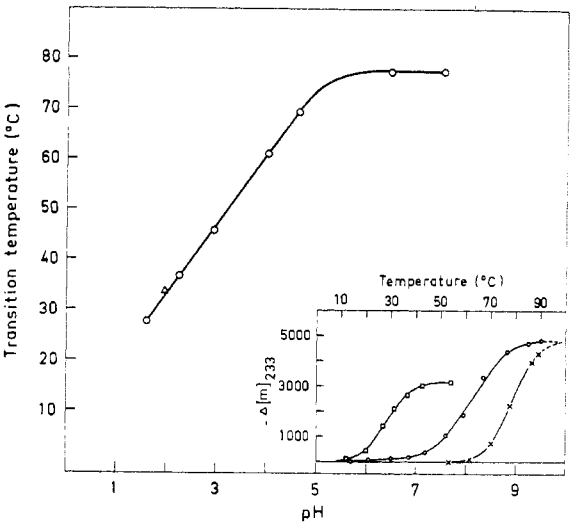

FIGURE 3: Variation of the transition temperature of neurotoxin I with pH. T_{tr} values are obtained by ORD measurements (○) or differential spectrophotometry (△). Insert: some typical melting curves at pH 1.65 (□), pH 4.0 (●), and pH 7.0 (×). All thermal transitions are perfectly reversible.

It is therefore very tempting to identify the form Iu with form II, and to describe the data in Figure 6 by the more detailed scheme

$$II \underset{+H^+}{\rightleftharpoons} III \underset{nU}{\rightleftharpoons} D$$

where n represents the number of urea molecules involved in the denaturation process. This interpretation is further substantiated by the value of $[m]_{233} = 1000$ at pH 7.0 in 9.8 M urea which is practically identical with that ascribed to form II from the results of Figure 2. $[m]_{233}$ for form D is about −3000. The above scheme indicates that form III is highly susceptible to urea denaturation while form II is absolutely resistant. The pH range in which form III is thermodynamically stable will evidently become larger when urea concentrations are increased. Transition I $\rightleftharpoons$ II was not seen in the experiments of Figure 6; it appears therefore that form I is readily transformed into form II even at low urea concentrations. The easy conversion of form I into form II at moderately acidic pH or in low urea conditions suggests that these 2 forms differ only slightly in conformation.

Chemical Modification of Carboxyl Groups in Neurotoxin I. Physicochemical evidence was presented in the first part of this paper showing that the conformation of neurotoxin I, while remaining in a folded form (it is still vulnerable to thermal denaturation), is strongly affected at acidic pH. The

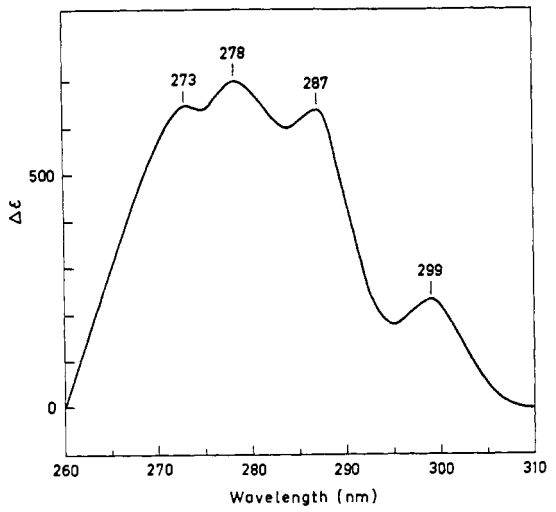

FIGURE 4: Difference spectrum induced by thermal denaturation of neurotoxin I at pH 2.0 (c = 0.171 mg/ml in 0.1 M NaCl). Neurotoxin at 70° *vs.* neurotoxin at 13° (reference cell).

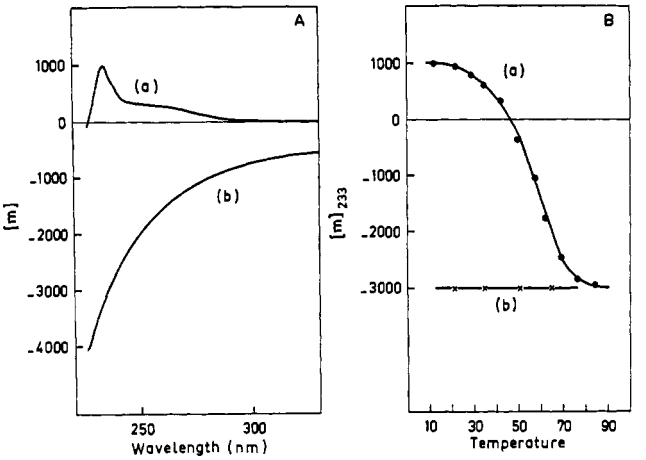

FIGURE 5: Stability of neurotoxin I in urea. (A) ORD spectra at pH 7.0 (a) and pH 4.0 (b) in 9.8 M urea at 20°; (B) the variation of $[m]_{233}$ *vs.* temperature in 9.8 M urea at pH 7.0 (a) and pH 4.0 (b). In this case

$$[m]_\lambda = \frac{n^2_W + 2}{n^2_u + 2} \times \frac{\alpha_\lambda \times MRW}{100\ cl}$$

The refractive index is n_w in water, n_u in urea solutions.

ionizable group which controls the transition with a pK_{app} of 2.0 ± 0.1 is probably a masked carboxyl group as was found to be the case for many other proteins (see for example Scheraga, 1961; Aune and Tanford, 1969a; Lazdunski *et al.*, 1970). Direct evidence for the structural importance of a carboxyl group was sought for by chemical modification. Carboxylic functions were transformed into amides with glycine ethyl ester under the influence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. Under our experimental conditions 6 residues of [^{14}C]glycine ethyl ester were incorporated in neurotoxin I. This means a modification of all the carboxylates since the protein has 2 aspartic, 3 glutamic acid residues, and 1 α-carboxylic function (Botes and Strydom, 1969). The physicochemical properties of the derivative are considerably changed when compared to those of the native neurotoxin (see Table I which summarizes the main properties of the chemical derivatives of neurotoxin I). The value of $[m]_{233}$ is strongly affected and the transition temperature is decreased from 77.5 to 28° at pH 7.0. This experiment confirms that at least one carboxyl group has an important structural role since the modified toxin is completely inactive.

Reactivity of Tyrosine-24 in Neurotoxin I. The masking of the phenol side chain of tyrosine-24, which was already shown by the experiments in Figure 4, is further demonstrated by titration, acetylation (see below the identification of the active site), and nitration. The spectrophotometric titration of the tyrosine side chain is shown in Figure 7. The apparent pK was found to be 11.9 at 20°. This value is sufficiently high as compared to normal pK's of 9.5–10.5 to indicate that the residue is masked in the native form.

The reactivity of the ortho position of the tyrosine side chain was tested with tetranitromethane. This protein reagent was first described by Vallee and coworkers (Sokolovsky *et al.*, 1966; Riordan *et al.*, 1966, 1967). It permits nitration of tyrosine side chains but gives cross-linking reactions when used with proteins having high amounts of tyrosine residues (Vincent *et al.*, 1970). In neurotoxin I this problem is eliminated since it contains a single tyrosine residue. Use of tetranitromethane not only produces nitration, but also causes oxidation sometimes. Table II indicates that the most vulnerable amino acids, arginine, lysine, and disulfide bridges, are not very significantly affected during nitrations which lead to nearly complete lost of toxic activity. Table II also indicates the extent of the modification of tyrosine as a function of tetranitromethane concentration. Nitration barely occurs at the lowest tetranitromethane concentration. It takes an increase of a hundred times in tetranitromethane concentration to reach an 80% modification. An estimation of the rate constant of nitration from the most precise results obtained with 10 mM tetranitromethane gives a value of 0.3 M^{-1} min^{-1}, much lower than the value of 6–8 M^{-1} min^{-1}

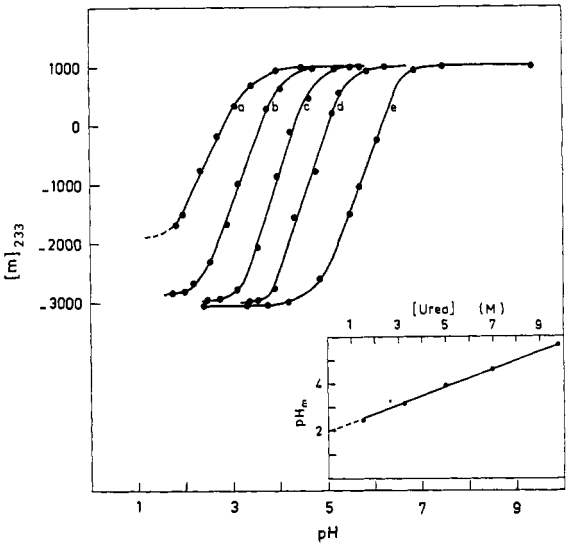

FIGURE 6: Acidic isothermal transition of neurotoxin I in increasing concentrations of urea (20°). Concentrations of urea are 1.5 M (a), 3.25 M (b), 5.0 M (c), 7.0 M (d), and 9.8 M (e). All transitions are fully reversible. Graphical transformation of the data indicates that isothermal transitions are perfectly described by the simple equation pH = pH_m + log (I_u/D) where (I_u/D) = ($[m]_{obsd}$ − $[m]_D$)/($[m]_N$ − $[m]_{obsd}$). $[m]_{obsd}$ is the observed rotation at 233 nm and at a given pH value. pH_m is the pH value for the midvariation of $[m]_{233}$. $[m]$ values were calculated according to the equation given in the legend of Figure 5.

TABLE I: Properties of Native and Modified Neurotoxins of *Naja haje*.

Toxins	Number of Substituents Incorporated	Nature and Amount of the Modified Groups	pH	ORD Spectra (20°) λ_{ext} (nm)	ORD Spectra (20°) $[m]_{\lambda}$ ext	Transition Temperature (°C)	Toxicity[a] LD_{50} (μg) (20-g Mice)
Native neurotoxin I			7	233	+1540	77.5	1.12
			4.8	233	+1200	71	
Amidated I	6	1α-COOH 2β + 3γ-COOH	7	233	−2230	28	Nontoxic
Maleylated I	7.3	1α-NH_2 6ϵ-NH_2	7	233	+1400	49	Nontoxic
Demaleylated I			7	233	+1450	69	2.9
Acetylated I	7	1α-NH_2 6ϵ-NH_2	7	233	+1560	75.5	Nontoxic
Acetylated I in 6 M Gdn·HCl	8.1	1α-NH_2 6ϵ-NH_2 1-Phenol	4.8	233	+800		Nontoxic
Dansylated I	2	2ϵ-NH_2	7	233	+1500	63	Nontoxic
Native neurotoxin III			4.8	233	0	85	1.35
				220	−2800		
Acetylated III	5	1α-NH_2 4ϵ-NH_2	4.8	220	−2600	84	Nontoxic

[a] The derivative is considered as nontoxic when an injection of at least 10 times the LD_{50} of the native neurotoxin (most of the time between 20 and 30 μg per 20 g of body weight) is unable to kill the mice.

TABLE II: Nitration of *Naja haje* Neurotoxin I.

Molar Ratio TNM/Toxin	Loss of Toxicity (%)	Amino Acids Residues/Mole of Toxin: Nitrotyrosine	Tyrosine	Lysine	Arginine	½ Cystine
0	0	0	1.0	5.91	3.90	7.80
4[a] (1 mM TNM)	16	0.19	0.75	5.84	3.83	7.98
40[a] (10 mM TNM)	65	0.54	0.49	5.48	3.65	8.05
400[b] (55 mM TNM)	84	0.83	0.21	5.42	3.57	7.87

Neurotoxin concentration[a] 1.7 mg/ml and[b] 0.9 mg/ml, pH 8.0, 4 hr at 20°.

found for readily available tyrosine side chains in other proteins at pH 8 (Vincent *et al.*, 1970). This is another indication of the masking of tyrosine-24. Table II shows that the loss of toxic activity follows closely the nitration of the internal residue.

Fluorescence and Formylation of Tryptophan-28 in Neurotoxin I. Fluorescence spectra of neurotoxin I in aqueous solution are presented in Figure 8A. The spectrum remains unchanged between pH 4 and pH 7. The quantum yield is $Q = 0.075$, and the position of the maximum at 344 nm is typical of the fluorescence of the tryptophan side chain (Cowgill, 1963; Teale, 1961). The spectrum is only slightly affected at neutral pH in 10 M urea; the intensity is increased probably because of the change of viscosity (Teale, 1961) but the maximum remains at 344 nm. However, when denaturation occurs, at pH 4, the position of the peak is shifted to 350 nm. Such red shifts to 350 nm are commonly observed upon denaturation of proteins containing buried tryptophans (Teale, 1961). Consequently the data in Figure 8A suggest that the side chain of tryptophan-28 is buried, or at least partially buried, in the native form of neurotoxin I while it is unmasked after denaturation in 10 M urea at acidic pH.

Formylation of tryptophan-28 has been carried out as described in Figure 8B. Under these conditions the indole side chain is quantitatively formylated after 90 min ($t_{1/2}$ = 15 min). The modified toxin has a residual toxicity of about 50%. Deformylation can be carried out easily and quantitatively (Figure 8B). No activity is regained when the indole side chain is completely regenerated from *N*-formylindole. These data indicate that the loss of activity is not related to formylation of tryptophan-28. It is due to a side reaction which has not been identified since the amino acid analyses of the unsubstituted and the formylated neurotoxins are identical after acid hydrolysis. These results suggest that tryptophan-28 is not a part of the active site of neurotoxin I. Identical results have been obtained in our laboratories with toxin α of *Naja nigricollis*.

Acetylation of Neurotoxins I and III. The importance of lysines for the toxic activity of neurotoxins I and III was studied using acetylation, maleylation, and dansylation.

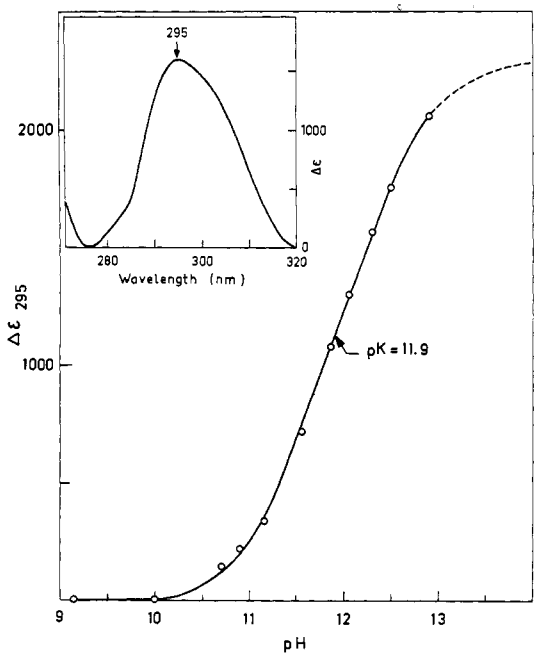

FIGURE 7: Spectrophotometric titration of tyrosine-24 in neurotoxin I. Titration curve followed by the variation of $\Delta\epsilon_{295}$ *vs.* pH. Full reversibility has been observed. Insert: difference spectrum at alkaline pH (toxin, pH 12.3/pH 7.0 (reference cell), c = 0.12 mg/ml, 20°).

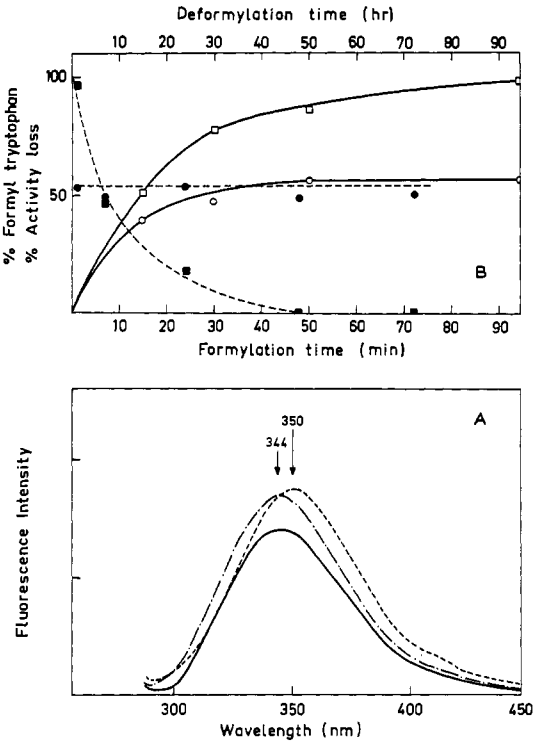

FIGURE 8: Properties of tryptophan-28 in neurotoxin I. (A) Fluorescence spectra of neurotoxin I at 30°, 0.1 M NaCl, pH 7.0, and pH 3.2 (—); 0.1 M NaCl, 10 M urea, pH 7.0 (—·—·) and pH 4.2 (- - - -). c = 0.185 mg/ml. Excitation wavelength, 280 nm. (B) Kinetics of formylation (□) and of deformylation (■) were followed by spectrophotometric evaluation of *N*-formyltryptophan. The toxic activity decreases during formylation (○) but is unaffected by deformylation (●) at pH 9.7.

At pH 8 and 4°, acetylation is known to be specific of amino and phenol groups (Riordan and Vallee, 1967a,b); 7.0 acetyl groups were found to be bound to native neurotoxin I after treatment with [^{14}C]acetic anhydride. The tyrosine side chain is masked at pH 8 as we have seen earlier. Therefore the groups which have reacted are the 6 lysine side chains and the α-amino group. This is confirmed by the fact that the ninhydrin reaction is absolutely negative with the acetylated neurotoxin. Acetylation was also carried out on unfolded neurotoxin I, in 5 M guanidine hydrochloride. In this case 8.1 acetyl groups were incorporated; that is 1.1 groups in excess of what was found with the folded protein. The difference was due to acetylation of the tyrosine side chain in the denatured form. Acetylation of tyrosine-24 was reversed by hydroxylamine treatment. The deacylation kinetics have been followed and the extent of deacylation was evaluated spectrophotometrically (Riordan and Vallee, 1967b). It corresponds to 1.07 tyrosines, in agreement with the expected value. Acetylation of the amino groups of native neurotoxin I does not change the physicochemical properties of the protein but completely abolishes the toxicity (Table I).

Acetylation followed by hydroxylamine treatment of neurotoxin III results in the quantitative modification of the four ϵ-amino groups and of the α-amino group of the protein. The acetylated neurotoxin III lost its toxic activity, without modification of the conformational properties (Table I).

Maleylation of Neurotoxin I. Maleyl groups (7.3) were incorporated in neurotoxin I at pH 9 and 4° after treatment with [^{14}C]maleic anhydride. This means again a quantitative modification of the 6 lysines and of the α-amino group.

Maleylation which replaces positive charges by negative charges is known to reduce drastically protein stability because of electrostatic repulsions between negative charges (Butler *et al.*, 1969). This results in a transition temperature decrease from 77.5° for the native protein to 49° for the maleylated toxin (Table I). Maleylation of amino groups can be reversed at acidic pH (Butler *et al.*, 1969). After a treatment at 60°, pH 2.7 for 4 hr, the physicochemical properties of the native neurotoxin were largely recovered (Table I); the transition temperature resumed a high value of 69°.

Maleylation abolishes the toxicity (Table I) and demaleylation allows a recuperation of 40% of the initial activity. This result together with the acetylation data is considered to show convincingly that one or several amino groups are part of the active site of neurotoxin I.

Dansylation of Neurotoxin I. Dansylation of neurotoxin I is very selective for amino groups. The results of a typical amino acid analysis carried out after 60 min of dansylation are shown in Table III. Only the lysine content and the N-terminal leucine residue were found to be affected. After dansylation for 90 min, 2 lysines are modified together with 0.2 residue of leucine. The chemical modification results in a 92% loss of toxicity.

The kinetics of lysine dansylation and the loss of activity are presented in Figure 9. The loss of toxicity follows very closely the dansylation of 2 lysines. The kinetics is first order, with a rate constant of 52 M^{-1} min^{-1}. Dansylation of α-amino groups is usually faster than that of ϵ-amino groups at pH 9 (Gros and Labouesse, 1969). The situation is reversed for 2 of the 6 ϵ-amino groups in neurotoxin I.

The location of the 2 dansylated lysines was determined by fingerprinting analysis (Figures 10 and 11). After a dansylation of 1 hr followed by reduction, alkylation, and trypsin hydrolysis, the dansylated peptides were located on the peptide map by their fluorescence. Only 3 peptides were dansylated. Their amino acid composition is given in Table III.

TABLE III: Amino Acid Composition of the Dansylated Neurotoxin (I) and of the Dansylated Tryptic Peptides.[d]

Amino Acid	Dansylated neurotoxin I	Peptide 1 Lys_{26}–Arg_{27}	Peptide 2 Leu_1–Lys_{15}	Peptide 3 Gly_{39}–Lys_{47}	Peptide 4[a] Gly_{33}–Arg_{38}
CM-cysteine	7.06 (8)		0.93 (1)	0.99 (2)	
Aspartic acid	6.95 (7)		1.19 (1)		
Threonine	6.55 (7)		1.95 (2)		0.89 (1)
Serine	3.73 (4)		1.94 (2)	1.11 (1)	1.01 (1)
Glutamic acid	7.12 (7)		4.20 (4)		1.03 (1)
Proline	4.12 (4)		2.16 (2)	*c* (1)	
Glycine	5.00 (5)			1.95 (2)	0.96 (1)
Valine	1.11 (1)			0.98 (1)	
Cysteine	0 (0)				
Isoleucine	2.85 (3)				0.93 (1)
Leucine	0.79 (1)		0.23 (0)[b]		
Tyrosine	0.92 (1)				
Lysine	4.13 (6)	0.28 (0)[b]	1.01 (1)	1.18 (1)[b]	
Histidine	1.94 (2)		1.01 (1)		
Arginine	3.99 (4)	1.0 (1)			1.03 (1)
Yield (%)		100	83	33	79

[a] Nonfluorescent reference peptide. [b] Theoretical values assuming that no regeneration of dansyllysines or of dansylleucine occurs during acid hydrolysis. In fact the table shows that a regeneration amounting to 20–30% always occurs. [c] Amount difficult to evaluate because of too small quantities of peptide. [d] Theoretical values expected from Botes and Strydom's sequence are indicated in parentheses.

The acidic hydrolysis of peptide 1 gives a composition of 1 arginine and 0.28 lysine. Peptide 1 corresponds to the sequence Lys_{26}–Arg_{27}; Lys_{26} seemed to be dansylated preferentially. The small quantity of free lysine found after amino acid analysis of peptide 1 is due to the well-known regeneration of lysine from dansyllysine by acidic hydrolysis (Gros and Labouesse, 1969). No dansylated peptide was found with the sequence Thr_{16}–Lys_{26} or Thr_{16}–Arg_{27}. It is worthwhile noting that Lys_{26}, which is in a cluster of positive charges, Lys_{25}-Lys_{26}-Arg_{27}, is quantitatively modified while Lys_{25} is not dansylated at all. The amino acid analysis of peptide 2 corresponds exactly to the sequence Leu_1–Lys_{15}. Dansylation occurs on the α-amino group of Leu_1. The small quantity of free leucine found after amino acid analysis is also due to acidic regeneration. The amino acid composition of peptide 3 corresponds to the sequence Gly_{39}–Lys_{47}. Lys_{46} is the dansylated residue. Lys_{47} was not modified since trypsin hydrolysis cannot take place at the level of a dansylated lysine. The yield of peptide Gly_{39}–Lys_{47} is only 33% while the yield of peptide Gly_{39}–Lys_{46}, obtained by trypsin digestion of the native protein, is 100% (Botes and Strydom, 1969). This may reflect the fact that after dansylation of Lys_{46}, the bond Lys_{47}–Gly_{48} becomes more resistant to trypsin hydrolysis. In fact even with the native protein, yields are not of

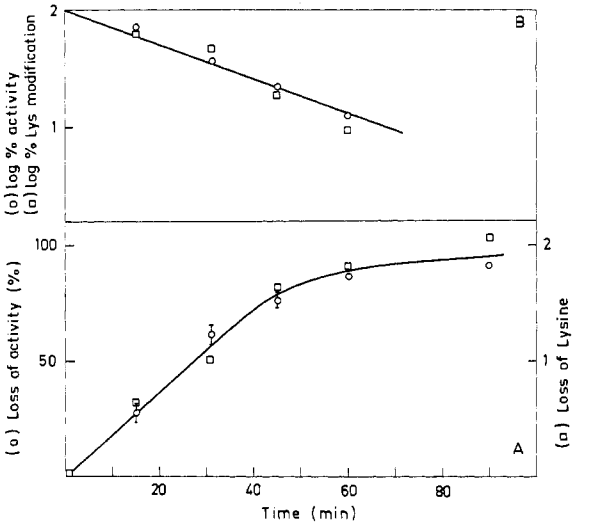

FIGURE 9: Kinetics of dansylation of neurotoxin I at pH 8.9, 20°. (A) The loss of activity (○) parallels the loss of lysines (□). (B) Pseudo-first-order representation of the previous data.

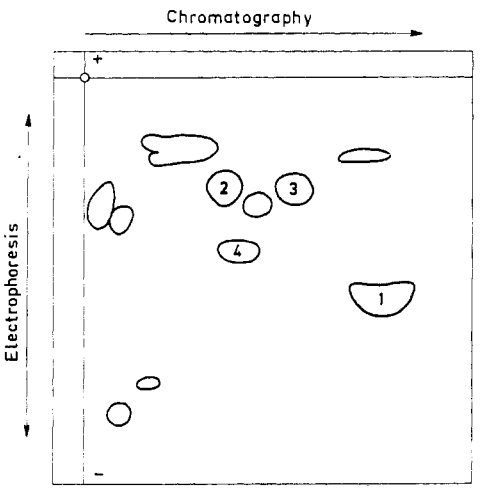

FIGURE 10: Peptide map of the tryptic hydrolysate of the dansylated and S-carboxymethylated neurotoxin I. Fluorescent dansylated peptides are noted 1, 2, and 3. Peptide 4 is the reference peptide (Table III).

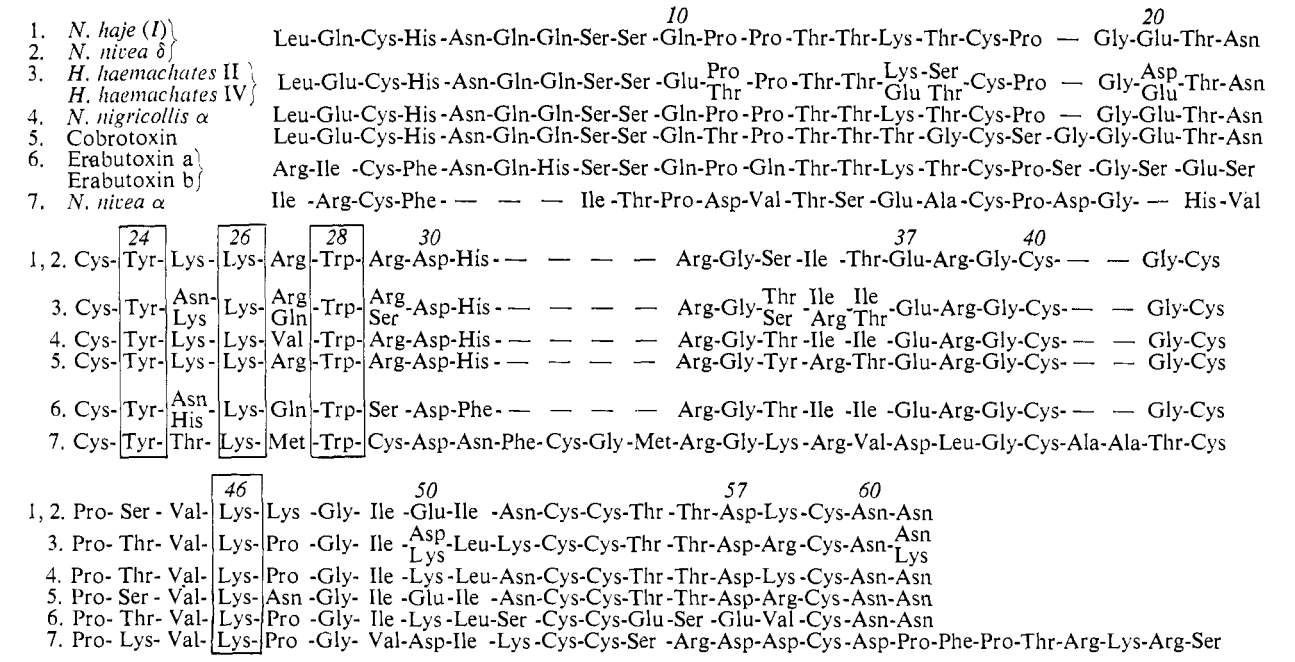

FIGURE 11: A comparison of amino acid sequences of snake neurotoxins according to Botes and Strydom (1969) (1), Botes *et al.* (1971) (2), Strydom and Botes (1971) (3), Eaker and Porath (1967) (4), Yang *et al.* (1969) (5), Sato and Tamiya (1971) (6), and Botes (1970) (7). — indicates missing amino acid.

100% for all tryptic peptides (Botes and Strydom, 1969); this is a result well known to all sequence specialists.

The ORD spectra of neurotoxin I before and after dansylation of Lys_{26} and Lys_{46} are very similar (Table I) but the chemical substitution produces a decrease of 14° in the transition temperature.

Discussion

Neurotoxins I and III of *Naja haje* like other miniproteins with a high content of disulfide bridges such as the basic trypsin inhibitor (Vincent *et al.*, 1971) or scorpion neurotoxins (Chicheportiche and Lazdunski, 1970) were found to present an unusually high resistance to denaturing conditions at neutral pH. They remain tightly folded at fairly high temperatures and in concentrated solutions of urea.

Two successive and reversible isomerizations occur in neurotoxin I at acidic pH, I $\rightleftharpoons$ II $\rightleftharpoons$ III. The most important structural rearrangement II $\rightleftharpoons$ III appears to be controlled by the protonation of a carboxylic group with an apparent pK of 2.0. The acidic isomer, form III, is much more vulnerable to urea or thermal denaturation than the 2 other forms. The buried carboxyl group which is essential for maintaining the native structure of neurotoxin I might be either the side chain of Glu_{20} which is common to most sequences of land-snake neurotoxins (Chang and Yang, 1971) or more likely the side chains of Asp_{30}, Glu_{37}, or Asp_{57} which are common to all sequences of sea-snake and land-snake neurotoxins determined up to now (see Figure 11).

Probably the most interesting result of this work is the identification of the residues which form the active site of *Naja haje* neurotoxins. Acetylation, maleylation, and dansylation have proven the essentiality of lysine residues for the toxic activity. Dansylation was shown to affect selectively 2 "super-reactive" lysines, Lys_{26} and Lys_{46}. Both lysines are present in all sequences of sea- and land-snake neurotoxins (Figure 11). These unique properties suggest that both Lys_{26} and Lys_{46} are essential for the biological activity of snake neurotoxins. However the data in Figure 9 would be perfectly explained if only one of these lysines were part of the active site. The definite answer will have to await the isolation of the toxin receptor(s) and the identification of the lysine(s) residue(s) which are masked to dansylation in the neurotoxin–receptor complex.

Snake neurotoxins are known to act at neuromuscular junctions by interfering with the binding of acetylcholine to its membranar receptor (Changeux *et al.*, 1970; Miledi *et al.*, 1971; Tamiya and Arai, 1966; Sato *et al.*, 1970). Therefore, by analogy with the acetylcholine structure, one would have thought to find monomethyl-, dimethyl-, or trimethyllysines in the active site of neurotoxins. Such methylated lysine residues have been found in other proteins (Glazer *et al.*, 1967; Paik and Kim, 1967; Delange *et al.*, 1969) but they are not present in snake neurotoxins.

The positive charge(s) of the ϵ-ammonium group(s) of the essential lysine(s) residue(s) probably forms a salt bridge with the anionic site of the receptor which recognizes the quaternary ammonium ion of acetylcholine. Such ion-pair interactions are certainly not sufficient to explain the nearly irreversible binding of neurotoxins to acetylcholine receptors. For that reason other interactions must exist and the properties of tyrosine-24 and tryptophan-28 appear to deserve a special investigation. Both amino acids are among the invariant residues which appear in Figure 11. Both of them are in the vicinity of lysine-26.

Titration, nitration, and acetylation have shown that tyrosine-24 was masked in the native neurotoxins of *Naja haje*. For that reason, this residue is not believed to participate directly in the array of interactions which stabilize the association of the toxin with the receptor. Its essential structural role is shown by the loss of toxic activity which follows nitration. Independent studies carried out on cobrotoxin (Chang *et al.*, 1971) have also shown that tyrosine-24 was resistant to nitration in the native structure; the activity was

completely lost after modification in guanidine hydrochloride.

Difference spectra and fluorescence data indicate that the side chain of tryptophan-28 is masked or patially masked in the hydrophobic interior of neurotoxin I. A number of chemical modifications of this tryptophan residue have been carried out recently on other toxins obtained from sea and land snakes (Chang and Hayashi, 1969; Seto *et al.*, 1970; Tu *et al.*, 1971; Tu and Toom 1971). Ozonation in formic acid or reaction with *N*-bromosuccinimide at acidic pH or in denaturing solvents selectively modified tryptophan and involved a total loss of activity. For that reason tryptophan-28 was considered to be one of the essential components of the active site. Considering that formylation of neurotoxin I is without effect on the toxic activity, it appears unlikely that tryptophan-28 takes part in the noncovalent interactions which stabilize the association with the receptor. It is probably more reasonable to think that the indole side chain, like the phenol part of tyrosine-24, is only involved in the stabilization of an adequate geometry for the active site.

Scorpion neurotoxins also form a family of homologous proteins (Rochat *et al.*, 1970b) with molecular properties analogous to those of snake neurotoxins (Rochat *et al.*, 1970b; Chicheportiche and Lazdunski, 1970). Scorpion neurotoxins provoke a spastic paralysis of the mouse (Tazieff-Depierre, 1966, 1968) while snake neurotoxins provoke a flaccid paralysis. For that reason, scorpion neurotoxins probably do not interfere with the binding of acetylcholine to its membrane receptor. We found that acetylation of the α- and ϵ-amino groups of neurotoxin II of *Androctonus australis* led to a complete loss of toxic activity suggesting that in that case also lysines are essential residues. However, no "superreactive" residues were found with dansyl chloride. Work is presently being carried out to obtain a more precise identification of the essential amino acids in the active site of scorpion neurotoxins.

Acknowledgments

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References

Aune, K. C., and Tanford, C. (1969a), *Biochemistry 8*, 4579.
Aune, K. C., and Tanford, C. (1969b), *Biochemistry 8*, 4586.
Botes, D. P. (1970), Communication at the 2nd International Symposium on Animal and Plant Toxins, Tel Aviv, Israel.
Botes, D. P., and Strydom, D. J. (1969), *J. Biol. Chem. 244*, 4147.
Botes, D. P., Strydom, D. J., Anderson, C. G., and Christensen, P. A. (1971), *J. Biol. Chem. 246*, 3122.
Butler, P. J. G., Harris, J. I., Hartley, B. S., and Leberman, R. (1969), *Biochem. J. 112*, 679.
Carraway, K. L., and Koshland, D. E. (1968), *Biochim. Biophys. Acta 160*, 272.
Chang, C. C., and Hayashi, K. (1969), *Biochem. Biophys. Res. Commun. 37*, 841.
Chang, C. C., and Yang, C. C. (1971), *Biochem. Biophys. Res. Commun. 43*, 429.
Chang, C. C., Yang, C. C., Hamaguchi, K., Nakai, K., and Hayashi, K. (1971), *Biochim. Biophys. Acta 236*, 164.
Changeux, J. P., Kasai, M., and Lee, C. Y. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 1241.
Chicheportiche, R., and Lazdunski, M. (1970), *Eur. J. Biochem. 14*, 549.
Cowgill, R. W. (1963), *Biochim. Biophys. Acta 75*, 272.
Delange, R. J., Glazer, A. N., and Smith, E. L. (1969), *J. Biol. Chem. 244*, 1385.
Eaker, D. L., and Porath, J. (1967), *Int. Congr. Biochem. 7th, 3*, 499.
Glazer, A. N., Delange, R. J., and Martinez, R. J. (1967), *Biochim. Biophys. Acta 188*, 164.
Gratzer, W. B., and Cowburn, D. A. (1969), *Nature (London) 222*, 426.
Gros, C., and Labouesse, B. (1969), *Eur. J. Biochem. 7*, 463.
Hoare, D. G., and Koshland, D. E. (1967), *J. Biol. Chem. 242*, 2447.
Katz, A. M., Dreyer, W. J., and Anfinsen, C. B. (1959), *J. Biol. Chem. 234*, 2897.
Lazdunski, M., Delaage, M., Abita, J. P., and Vincent, J. P. (1970), *in* Structure-Function Relationships of Proteolytic Enzymes, Desnuelle, P., Neurath, M., and Ottesen, M., Ed., Copenhagen, Munksgaard, p 42.
Leach, S. J., and Scheraga, M. A. (1960), *J. Amer. Chem. Soc. 82*, 4790.
Miledi, R., Molinoff, P., and Potter, L. T. (1971), *Nature (London) 229*, 554.
Miranda, F., Kupeyan, C., Rochat, H., Rochat, C., and Lissitzky, S. (1970a), *Eur. J. Biochem. 17*, 477.
Miranda, F., Kupeyan, C., Rochat, H., Rochat, C., and Lissitzky, S. (1970b), *Eur. J. Biochem. 16*, 514.
Paik, W. K., and Kim, S. (1967), *Biochem. Biophys. Res. Commun. 27*, 479.
Previero, A., Coletti-Previero, M. A., and Cavadore, J. C. (1967), *Biochim. Biophys. Acta 147*, 453.
Riordan, J. F., Sokolovsky, M., and Vallee, B. L. (1966), *J. Amer. Chem. Soc. 88*, 4104.
Riordan, J. F., Sokolovsky, M., and Vallee, B. L. (1967), *Biochemistry 6*, 358.
Riordan, J. F., and Vallee, B. L. (1967a), *Methods Enzymol. 11*, 565.
Riordan, J. F., and Vallee, B. L. (1967b), *Methods Enzymol. 11*, 570.
Rochat, H., Rochat, C., Miranda, F., Lissitzky, S., and Edman, P. (1970a), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 10*, 349.
Rochat, H., Rochat, C., Miranda, F., Lissitzky, S., and Edman, P. (1970b), *Eur. J. Biochem. 17*, 262.
Sato, S., Abe, T., and Tamiya, N. (1970), *Toxicon 8*, 313.
Sato, S., and Tamiya, N. (1971), *Biochem. J. 122*, 453.
Scheraga, H. A. (1957), *Biochim. Biophys. Acta 23*, 196.
Scheraga, H. A. (1961), *in* Protein Structure, New York, N. Y., Academic Press, p 217.
Seto, A., Sato, S., and Tamiya, N. (1970), *Biochim. Biophys. Acta 214*, 483.
Sokolovsky, M., Riordan, J. F., and Vallee, B. L. (1966), *Biochemistry 5*, 3582.
Strydom, A. J. C., and Botes, D. P. (1971), *J. Biol. Chem. 246*, 1341.
Tamiya, N., and Arai, H. (1966), *Biochem. J. 99*, 624.
Tazieff-Depierre, F. (1966), *C. R. Acad. Sci. 263*, 1785.
Tazieff-Depierre, F. (1968), *C. R. Acad. Sci. 267*, 240.
Teale, F. W. J. (1961), *Biochem. J. 76*, 381.
Tu, A. T., Hong, B. S., and Solie, T. N. (1971), *Biochemistry 10*, 1295.

Tu, A. T., and Toom, P. M. (1971), *J. Biol. Chem. 246*, 1012.

Vincent, J. P., Chicheportiche, R., and Lazdunski, M. (1971), *Eur. J. Biochem. 23*, 401.

Vincent, J. P., Lazdunski, M., and Delaage, M. (1970), *Eur. J. Biochem. 12*, 250.

Yang, C. C., Yang, H. J., and Huang, J. S. (1969), *Biochim. Biophys. Acta 188*, 65.

Inhibition of Nicotinamide Phosphoribosyltransferase by Pyridine Nucleotides†

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ABSTRACT: The ability of various pyridine bases (nicotinamide nucleoside) and various NAD analogs to inhibit rat liver nicotinamide phosphoribosyltransferase was determined. All pyridine nucleotides in the oxidized state containing nicotinamide bound to ribose in the β configuration were found to be strong noncompetitive inhibitors of nicotinamide phosphoribosyltransferase when nicotinamide was the variable substrate. Under the same conditions, all the pyridine bases examined, nicotinamide riboside and β-$NMNH_2$, were found to be competitive inhibitors. Pyridine dinucleotide derivatives which are reduced, exist in the anti form of geometric distribution between the pyridine ring and the D-ribofuranose, or lack the β-ribosyl linkage, were found to be weak inhibitors of nicotinamide phosphoribosyltransferase activity. These kinetic data are correlated with known molecular configuration and geometric distributions of the inhibiting compounds. The nicotinamide and PRPP binding sites appear to be adjacent to one and the other since β-NMN, the product of the reaction, appears to occupy both sites simultaneously. The binding of the 5′-phosphate of the second substrate, PRPP, or the product, NMN, is apparently essential since loss of this binding group, as in the case of nicotinamide riboside, results in loss of "PRPP site" affinity but retention of "nicotinamide site" affinity. The configuration of the ribose of the pyridine nucleotide is critical since changes in configuration of the ribose, as occurs during pyridine nucleotide reduction, result in distinct changes in the ability to bind at the nonnicotinamide portion of the active site as measured by the K_i intercept. The geometric disposition between the pyridine base and the D-ribofuranose ring is also critical since compounds which exist in the anti form apparently do not bind at the nonnicotinamide portion of the active site yet have affinity for the nicotinamide binding site as measured by changes in K_i intercept and K_i slope, respectively.

Nicotinamide phosphoribosyltransferase (EC 2.4.2.12) has been purified from rat liver (Dietrich *et al.*, 1966). The enzyme requires ATP in addition to 5-phosphoribosyl 1-pyrophosphate (PRPP), the role of ATP being apparently that of a modifier (Powanda *et al.*, 1969). The reaction is as follows: In the absence of ATP, the apparent K_m for nicotinamide is 0.1 M (L. S. Dietrich, unpublished data) as compared to an apparent K_m for nicotinamide of 1×10^{-6} M in the presence of ATP (Dietrich *et al.*, 1966). Furthermore, ATP addition results in a 12-fold increase in V_{max} (Dietrich, unpublished data). These observations, together with those indicative that NAD as well as the product of the reaction, NMN, inhibit enzymatic activity (Dietrich and Muniz, 1966), led to an evaluation of the nature of the inhibition produced by these compounds. A preliminary report of these studies has appeared (Dietrich and Muniz, 1967).

$$\text{nicotinamide} + \text{PRPP} \xrightarrow{\text{ATP}} \text{NMN} + \text{PP}_i \qquad (1)$$

Materials and Methods

Enzymatic Material. A rat liver preparation (fraction B) reported previously (Powanda *et al.*, 1969) was used throughout. The preparation had a specific activity of *ca.* 0.18 μmole of NMN formed per hr per mg of protein, was free of inorganic pyrophosphatase, and contained no detectable NADase, NAD kinase, NAD pyrophosphorylase, nicotinic acid phosphoribosyltransferase, nicotinamide deamidase, or ATPase. No enzymatic degradation of NMN or PRPP could be observed under the conditions in which the experiments were carried out. The preparation was obtained by DEAE-cellulose chromatography of the $(NH_4)_2SO_4$ fraction described by Dietrich *et al.* (1966), and represented around a 300-fold purification. Recent studies utilizing enzymatic preparations having 20–30 times the specific activity of the preparation employed here have yielded identical results in all cases. Enzymatic activity was assayed as previously described (Powanda *et al.*, 1969). The assay involves the chromatographic separation in paper of the product, [^{14}C]NMN, from the substrate, [^{14}C]nicotinamide, and the subsequent quantitation of the product employing liquid scintillation spectrometry. This procedure is very reproducible. Duplicate values are normally within 1% of each other at all levels of substrate and inhibitor employed and values that varied more than 2% were generally discarded.

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