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Characterization of Elapidae Snake Venom Components Using Optimized Reverse-Phase High-Performance Liquid Chromatographic Conditions and Screening Assays for α-Neurotoxin and Phospholipase A_2 Activities†

Pierre E. Bougis,* Pascale Marchot, and Hervé Rochat
Laboratoire de Biochimie, Faculté de Médecine, Secteur Nord, CNRS UA 553-INSERM U 172, 13326 Marseille Cedex 15, France

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ABSTRACT: The vast majority of Elapidae snake venoms, genus *Naja*, includes three classes of toxic polypeptides: α-neurotoxins, phospholipases A_2, and cardiotoxins. A new experimental approach using reverse-phase high-performance liquid chromatography in particular has been developed, allowing their respective resolution, identification, and quantitation from milligram quantities of venom. First, definition of optimal chromatographic conditions for *Naja mossambica mossambica* toxins has been ascertained. Different column packing and solvent systems were compared for their efficiency, with particular attention to the ionic strength of the aqueous solvent. A medium-chain alkyl support (octyl) in conjunction with a volatile ammonium formate (0.15 M, pH 2.70)/acetonitrile solvent system was found to be particularly effective. All the components known until now from this venom could be resolved in a single step, and the elution order was α-neurotoxins, phospholipases A_2, and cardiotoxins with a total recovery of absorbance and toxicity. Then, with these suitable conditions, we describe a new major cardiotoxin molecule in this venom by hydrophobic and not ionic-charge discrimination. Second, specific assays were designed to detect α-neurotoxin and phospholipase A_2 activities in chromatographic fractions: α-neurotoxin activity was determined by competition for the binding of a radiolabeled α-neurotoxin to the acetylcholine receptor of the ray electric organ, and phospholipase A_2 activity was defined by the enzymatic activity of these toxins with a fluorescent phospholipid as substrate. Finally, the applicability of these new methods to study other *Naja* snake venoms was demonstrated.

Considerable progress has been made concerning the molecular mechanisms of the pharmacology of some animal venoms and their toxins [reviewed in Bettini (1978) and Lee (1979)] as well as, more recently, the antigenic structures implicated in their immunological neutralization for an appropriate serotherapy (Boulain et al., 1982; El Ayeb et al., 1983), thereby requiring the purification and identification of a number of analogous toxins. The isolation of such polypeptidic toxins is consistently a thorny problem in biochemistry, and our laboratory has been actively engaged in this work since the 1960s (Miranda & Lissitzky, 1961; Miranda et al., 1970).

Elapidae snake venoms, genus *Naja*, can be separated into several active components. Among them α-neurotoxins, phospholipases A_2, and cardiotoxins are recognized as the main factors involved in the pharmacology and the lethality of these venoms. They are low molecular weight polypeptides, chemically very stable. Only the mode of action of α-neurotoxins is currently well-known at the molecular level. They bind to the postsynaptic AcChR[1] with a very high affinity (K_D about 0.01–1 nM). Through a competitive inhibition of the response to the acetylcholine of the neuromuscular junction, they cause a flaccid paralysis (Lee & Chang, 1966; Changeux et al., 1984). The toxicity of phospholipases A_2 is generally related to their basicity (pH_i) and to their esterase-type activity. Their mechanism of action nevertheless remains totally unknown (Karlsson et al., 1979). Cardiotoxins have been defined as such because of their pharmacological action on heart muscle, causing considerable cardiovascular depression (Sarkar, 1947).

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* Author to whom correspondence should be addressed.

[1] Abbreviations: RP-HPLC, reverse-phase high-performance liquid chromatography; AcChR, acetylcholine receptor; NTX, neurotoxin; PH, phospholipase A_2; CTX, cardiotoxin; PMSF, phenylmethanesulfonyl fluoride; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetate; TFA, trifluoroacetic acid; R_t, retention time.

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Their structural homology with α-neurotoxins is well established (Dufton & Hider, 1983). The search for a precise molecular explanation for cardiotoxin action has served above all to highlight its multiplicity (cytotoxicity and membranal enzyme inhibition; Condrea, 1974), membrane depolarization remaining the most obvious (Meldrum, 1965; Harvey et al., 1982). Then, such an unexplained mechanism of action remains in the realm of hypotheses, although a cardiotoxin–phospholipid interaction is being implicated to an increasing extent (Bougis et al., 1983).

Ideally, the complete characterization of an animal venom requires all the components to be isolated and structurally identified. However, this is currently impossible in a single chromatographic step, all the more so because the chromatographic strategy of the investigator is often prejudiced since he usually has a single type of pharmacological activity in mind. thus, it is actually at best very difficult to obtain a precise and overall idea of the composition of an animal venom. On the basis of these considerations, we decided to optimize conditions for applying recent progress in liquid partition chromatography, i.e., HPLC (Regnier, 1983), to the study of the contents of snake venoms.

Our results are a demonstration that the numerous constitutive polypeptides of Elapidae snake venoms can be resolved in a single step by the use of RP-HPLC. It is obvious that this study initially developed on *Naja mossambica mossambica* venom was facilitated in a way by our practical knowledge of this venom by means of conventional liquid chromatography (Louw, 1974a; Rochat et al., 1974; Joubert, 1977; Martin-Moutot & Rochat, 1979; Bougis et al., 1983). In spite of that fact, we are able to demonstrate the presence of a new cardiotoxin, heretofore never described in the venom of *N. mossambica mossambica*. We also designed appropriate specific tests for the screening of α-neurotoxin and phospholipase A_2 activities in RP-HPLC fractions of unknown venoms. The applicability of these new methods to study other Elapidae snake venoms was demonstrated in the cases of *Naja haje haje*, *Naja melanoleuca* and *Naja nigricollis* species.

Materials and Methods

Reagents. The water used to prepare solvents, buffers, and dialysis liquid was obtained with a Milli-Ro/Milli-Q (Millipore) system. Acetic and formic acids, as well as the other analytical grade reagents, were obtained from Merck. Acetonitrile, UV grade, was obtained from Fisons Scientific Equipment.

Venoms and Toxins. The four venoms were obtained either from a pool of animals such as *N. mossambica mossambica* (D. Muller, Northcliff, Johannesburg 2195, South Africa) and *N. haje haje* (Latoxan, 05150 Rosans, France) or from a single animal from the species in question: *N. melanoleuca* and *N. nigricollis* (Dr. J. P. Chipaux, Pasteur Institute, Abidjan, Ivory Coast).

Venom (5 mg) was preliminarily dialyzed against cold water in Eppendorf tubes closed with Spectraphore 3 (Spectrum Medical Industries) with no loss of toxic activity and then lyophilized. In addition, in the case of *N. mossambica mossambica* venom (1 g) a gel filtration was made in 0.10 N acetic acid on a 2.5 cm × 2 m column packed with Sephadex G-50 fine (Pharmacia). Elution flow rate at ambient temperature was 50 mL h^{-1}, and 5-mL fractions were collected. The elution profile was similar to that obtained by Rochat et al. (1974), except with lower resolution since we did not use recycling steps. But, as we desired, there was a better separation between nontoxic high molecular weight components (fractions FI and FII), with elution volumes close to the void volume of the column, and toxic low molecular weight ones (fraction FIII, meaning 98% of the venom toxicity and 88% of the 280-nm absorbance load).

The 11 toxic polypeptides described until the present from *N. mossambica mossambica* venom were purified in the laboratory by means of conventional gel permeation and ion-exchange chromatographies: α-neurotoxins [NTX Nmm I–III (Rochat et al., 1974)], phospholipases A_2 [PH Nmm I–III (Joubert, 1977)], and cardiotoxins [CTX Nmm I–V (Bougis et al., 1983)].

High-Performance Liquid Chromatography. A Waters Associates system was used, including two 6000A pumps, a U6K injector, a Model 660 gradient programmer, a Model 440 spectrophotometric detector, and a Data Module integrator/recorder. Analytical RP-HPLC was carried out on 4.6 × 250 mm columns prepacked with (C18) Ultrasphere ODS, 5 μm, Serial No. 4 UE 1750 N (Beckman); (C8) Lichrosorb RP-8, 5 μm, Serial No. 214647; and (C2) Lichrosorb RP-2, 5 μm, Serial No. 319011 (Hibar, Merck). Semipreparative RP-HPLC was carried out on a 10 × 250 mm column prepacked with Ultrasphere octyl, 5 μm, Serial No. 4 UE 037 N (Beckman). Column temperature was regulated at 25 °C, a temperature with no effect on the biological activity of the venom toxins, which were particularly stable. Additional details concerning the aqueous (A) or organic (B) elution solvents, elution flow rates and the form of the gradient concentration of B in A are given in the text or figure legends. The 0.15 M ammonium formate elution solvent (pH 2.70, conductivity 12 mS at 20 °C) was composed of about 2425 mL of H_2O, 75 mL of HCOOH, and 30 mL of NH_3. Only aqueous solvents were filtered (Millipore HATF, 0.45 μm) and degassed before use. Venom or toxin samples were prepared by dissolving them in elution solvent A, followed by filtration through Millex HV_4 (Millipore, 0.45 μm). Samples were injected on the column used without a precolumn in a maximum volume of 20 μL. Each time the solvent conditions were to be changed, especially in the case of return to initial ones, we systematically used gradients in order to prolong column lifetime and to obtain satisfactory reproducibility of the results. Column head pressure was kept below 3000 psi. A Redirac fraction collector (LKB Instruments) was used at detector output with glass tubes (Corning). Rather than direct dialysis, the elution solvents were eliminated by diluting the fraction one-tenth in water and lyophilizing it. Chromatograms were recorded and integrated in the following conditions: chart speed 0.3 cm min^{-1}, peak width 10, noise rejection 40000, area rejection 5. Columns were stored in a water/acetonitrile mixture (1/1) when not used.

Amino Acid Analyses. Special conditions were adopted as a result of the relatively high ammonia concentration of the ammonium formate elution solvent. HPLC samples were diluted one-tenth in water, evaporated (Speed Vac concentrator, Savant Instruments), taken up with water, dialyzed against water in Eppendorf tubes closed with M_r 1000 exclusion limit Spectrapore (Spectrum Medical Industries), and then evaporated.

Acid hydrolysis with 6 N hydrochloric acid in sealed vials was carried out on approximately 1 nmol of peptide for either 20 or 70 h at 110 °C under vacuum. The amino acid contents of the vials were determined with a Biotronik Model LC 7000 amino acid analyzer.

Preparation of Ray Electric Organ Membrane Fragments. A living specimen of *Torpedo marmorata* was supplied by the Marine Station of Arcachon (France). The method used was adapted from that of Reed and Raftery (1976) and Morel et

al. (1977). The following operations were carried out at 4 °C. Frozen fragments of the electric organ weighing 30 g were finely minced and homogenzied for 20 s with an Ultra Turax (Ika-Werk) apparatus in 190 mL of buffer (280 mM NaCl, 3 mM KCl, 1.8 mM $MgCl_2$, 3.4 mM $CaCl_2$, 300 mM urea, 5.5 mM glucose, 250 mM sucrose, 1.2 mM NaH_2PO_4/Na_2HPO_4, pH 6.7), supplemented with 0.1 mM PMSF and 3 mM NaN_3 in order to inhibit endogenous proteolytic activity. After equilibration with O_2, $NaHCO_3$ was added to bring the pH of the buffer to 7.0–7.2. The homogenate was centrifuged at 600*g* (Beckman J 2.21) for 10 min, and the supernatant was discarded. The pellet was taken up in a minimum volume of the above buffer and sonicated (Sonimasse 150 T, Ultrasons Annemasse) for 10 s. The opalescent suspension was filtered through a fine 200-μm mesh steel screen to eliminate connective tissue aggregated in long filaments. The volume was brought to 90 mL with buffer, and the preparation was centrifuged at 100000*g* (Beckman L2-75B, SW 27 rotor) for 90 min. The pellet was taken up and homogenized in a final volume of 40 mL of buffer (5 mM NaH_2PO_4/Na_2HPO_4, pH 7.4, 877 mM sucrose, 0.1 mM PMSF, 3 mM NaN_3) and centrifuged at 5000*g* (Beckman J 2.21) for 20 min. The supernatant was then diluted with an equal volume of buffer (5 mM NaH_2PO_4/Na_2HPO_4, pH 7.4) and was centrifuged at 100000*g* (Beckman L2-75B, SW 27 rotor) for 90 min. The final pellet was taken up with buffer (5 mM NaH_2PO_4/Na_2HPO_4, pH 7.4, 250 mM sucrose, 0.1 mM PMSF, 3 mM NaN_3). The membranes were stored in liquid nitrogen at a protein concentration from 6 to 8 mg mL^{-1} until use. Protein content was determined with the Bio-Rad Protein Assay with BSA as reference.

α-Neurotoxin/AcChR Binding Assay. NTX Nmm I was enzymatically iodinated with ^{125}I (Amersham), with the use of lactoperoxidase (Sigma), and then immunoprecipitated (Rochat et al., 1977). Specific radioactivity was 1500 Ci $mmol^{-1}$. ^{125}I-NTX Nmm I bound to the AcChR of the ray electric organ membrane fragments at 20 °C was determined with a Millititer filtration plate (Millipore) for 96 samples. The incubation buffer A/BSA (10 mM Tris HCl, pH 7.5, 100 mM NaCl) contained 0.1% BSA (Sigma). In each well of the STGV (Millipore) filtration plate (0.22-μm pore size) for a final incubation volume of 250 μL, the following were successively added: 175 μL of A/BSA supplemented with 2.86 mM EDTA in the case of screening assay, 25 μL of a solution of 0.66 nM ^{125}I-NTX Nmm I in A/BSA, 25 μL of an appropriate dilution in A/BSA of the sample tested, and 53 ng of membrane protein (5 fmol of binding sites) in 25 μL of A/BSA. After incubation for 1 h with agitation, the liquid in each well was simultaneously filtered under vacuum (16 mmHg) with a Millititer vacuum holder (Millipore). Each filter was washed 5 times with 250 μL of cold A/BSA. After the underside of the plate was thoroughly blotted with paper towels, the plate was dried in an incubator. All the well filters were then cut with a Millititer filter punch (Millipore), and radioactivity on the filters was determined with an Auto Gamma 500 (Packard).

The HPLC fractions were processed as follows. Five microliters of each RP-HPLC fraction was placed in each of the 96 wells of a Linbro microtitration plate (Flow Laboratories), lyophilized, and then taken up with a quantity of A/BSA appropriate for a dilution factor experimentally chosen. When large dilutions were necessary, successive dilutions were performed in a series of intermediate Linbro microtitration plates.

Phospholipase A_2 Assay. The substrate used was 1-palmitoyl-2-(6-pyrenylhexanoyl)-*sn*-glycero-3-phosphorylcholine (FC. 3024, KSV Chemicals, Finland). For 13 standard tests, 60 μg of pyrene lipid in toluene/ethanol (1/1) was transferred to a conical tube (Kontes). The solvent was eliminated by evaporation under a nitrogen stream, followed by a rinse with 300 μL of diethyl ether. The pyrene lipid was then dissolved in 400 μL of ethanol, and in each test 30 μL was rapidly injected (Hamilton microsyringe) in 3 mL of buffer (50 mM CH_3COONa, pH 8.0, 10 mM $CaCl_2$) for a final substrate concentration of 2 μM. Five microliters of each RP-HPLC fraction was used for the enzyme assay using an SFM 25 spectrofluorometer (Kontron) equipped with a recorder. The excitation and emission wavelengths were 337 and 400 nm, respectively, or the emission wavelength was varied when spectra were recorded. Calibration was performed with 6-pyrenylhexanoic acid (FH 4006, KSV Chemicals) up to a final concentration of 100 nM. The increment of intensity of fluorescence emission was linear within this concentration range. Enzyme activity was expressed as the number of picomoles of fatty acid released per minute.

We alternatively developed an overall test using the 96 wells of a Linbro microtitration plate (Flow Laboratories). Five microliters of each HPLC fraction was added to each well and lyophilized, and 250 μL of 10 μM pyrene lipid in the same buffer as above was added. The course of the reaction was followed after 30 min at 37 °C by photographing the plate (Polaroid type 665 film, 1-min exposure) under fluorescent illumination (Bioblock Scientific; 302 nm).

In Vivo Assay. Toxicity in vivo was tested in male NMRI white mice (Evic-Ceba, 33 Blanquefort, France) weighing 20 ± 3 g. The samples tested were taken up with 0.9% NaCl and 0.1% BSA, and 0.2-mL volumes were injected subcutaneously. The LD_{50} values were determined according to Berhens and Karber (1935) after a 48-h observation period.

RESULTS

Optimal Analytical RP-HPLC Conditions for Toxins. We initially sought for an optimal combination of elution solvents and stationary phase (column packing) leading to the best resolution of polypeptidic toxins. We have used in this study CTX Nmm II, a toxic fraction previously purified by means of conventional ion-exchange chromatography from *N. mossambica mossambica* venom.

Concerning the solvents, we first tested the aqueous solvent (A) 0.1% TFA combined with gradients of the organic solvent (B) acetonitrile (Ultrasphere ODS column). These widely used conditions are known for their efficiency in resolving mixtures of peptides and proteins (Mahoney & Hermodson, 1980; Henderson et al., 1981). Nevertheless, these assays were unsuccessful. The effect of the nature of the elution solvents on the retention time of the sample, and especially on its elution (peak height), was therefore systematically examined. These parameters reflect the efficacy of the column in defined conditions and should be maximal in the case of complex polypeptidic mixtures such as animal venoms. We have tested 0.10 M triethylamine phosphate, pH 2.30, 0.10 M ammonium trifluoroacetate, pH 2.45, and 0.10 M ammonium formate, pH 2.70, in combination with gradients of organic solvents such as ethanol, methanol, 2-propanol, and acetonitrile. The ammonium formate/acetonitrile pair was adopted for subsequent use in all chromatographies, since it was found to be the most effective among all the combinations tested.

The ionic strength of the aqueous solvent (A) was also shown to be a determining factor. The results obtained in the case of CTX Nmm II are shown in Figure 1a. Contrary to expectations, two major elution peaks were observed (Figure 1c), suggesting some heterogeneity of the CTX Nmm II fraction

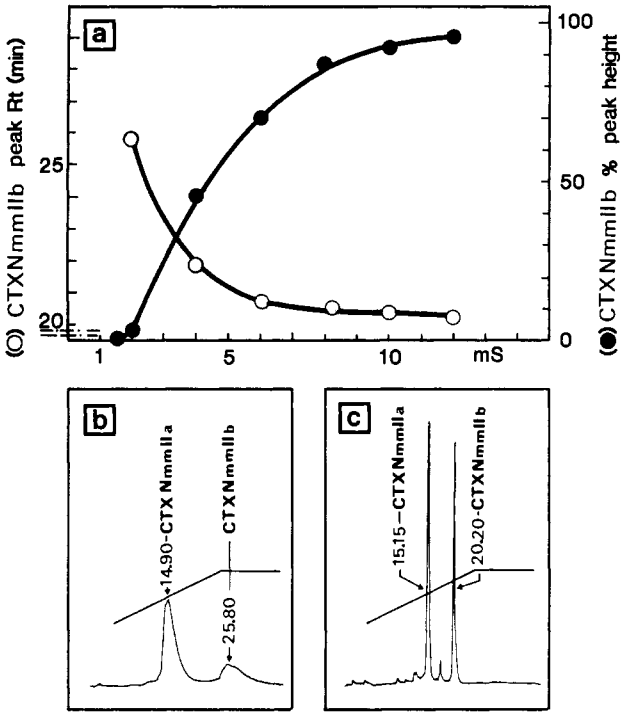

FIGURE 1: Influence of ionic strength of aqueous solvent (A) on the efficiency (peak height) of the column and R_t in the case of CTX Nmm II (actually a mixture of CTX Nmm IIa and -b). Ionic strength was expressed as the conductivity at 20 °C in millisiemens. (a) Lichrosorb RP-8 column. Solvent A: ammonium formate from 2 to 12 mS, pH from 2.60 to 2.70. Solvent B: acetonitrile. Gradient 6 from 25% to 45% B in A in 20 min, slope 1% B in A min^{-1}. Flow rate 1.5 mL min^{-1}. Sample loaded 140 μg of CTX Nmm II with 0.100 A. Full ordinate scale. (b) Recording for x = 2 mS. (c) Recording for x = 12 mS.

used, whose significance will be provided below. The increased ionic strength of solvent A, expressed by its conductivity at 20 °C in millisiemens, was a determining factor in elution peak heights, and values greater than 12 mS generated a plateau. Retention times increased at conductivity values lower than 6 mS. To illustrate this, parts b and c of Figure 1 show the chromatograms obtained in the extreme cases of 2 mS and 12 mS. In the case of only formic acid diluted in water (1.6 mS), no elution was observed, as in the case of 0.1% TFA mentioned above. The best results were obtained with solvent A as 0.15 M ammonium formate, 12 mS, pH 2.70, and solvent B as acetonitrile.

An elution rate of 1.5 mL min^{-1} was chosen after several tests. This led to satisfactory resoltuion for relatively short chromatographic times.

The type of stationary-phase bonding was chosen among three acyl chain lengths bearing 2, 8, or 18 carbon atoms. Intermediate length bonding (octyl) led to the best separation efficiency.

Analytical RP-HPLC of N. mossambica mossambica Toxins. Using the solvent and column conditions defined above, we undertook a thorough investigation of the RP-HPLC behavior of *N. mossambica mossambica* toxins.

We used relatively unselective but rapid conditions, i.e., a linear elution gradient with a steep acetonitrile slope (2% min^{-1}). The toxic fraction FIII obtained from the Sephadex G-50 chromatography of the crude venom (see Materials and Methods) was analyzed as shown in Figure 2a. Two main groups of components could be observed before the 45% acetonitrile step with R_ts close to 20 and 23 min following a group with R_t 9 min. An increase of the acetonitrile concentration up to 85% released tightly bound minor products

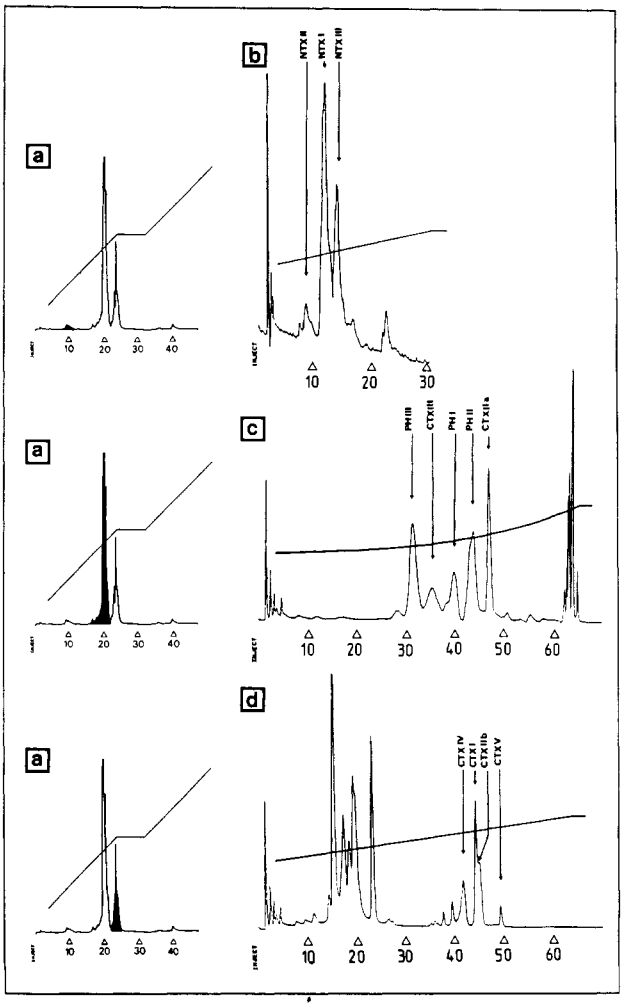

FIGURE 2: Analytical RP-HPLC for *N. mossambica mossambica* toxins. Lichrosorb RP-8 column. Solvent A: 0.15 M ammonium formate, 12 mS, pH 2.70. Solvent B: acetonitrile. Flow rate 1.5 mL min^{-1}. (a) Sample loaded 1 mg of FIII with 0.500 A. Full ordinate scale. Gradient 6 from 5% to 45% B in A in 20 min, slope 2% B in A min^{-1}, 8 min later from 45% to 85% B in A in 20 min, slope 2% B in A min^{-1}. The three main component groups observed were blackened and specifically analyzed as below. (b) Sample loaded 3 mg of FIII with 0.020 A. Full ordinate scale. Gradient 6 from 8% to 20% B in A in 30 min, slope 0.4% B in A min^{-1}. (c) Sample loaded 3 mg of FIII with 0.200 A. Full ordinate scale. Gradient 8 from 25% to 40% B in A in 60 min. (d) Sample loaded 3 mg of FIII with 0.200 A. Full ordinate scale. Gradient 6 from 25% to 40% B in A in 60 min, slope 0.25% B in A min^{-1}.

in some cases, but not systematically. So, nearly all the components detectable at a wavelength of 280 nm were eluted before 45% in acetonitrile. After RP-HPLC fractionation in these conditions, total yield of both absorbance and toxicity were of 100%. With regard to nontoxic high molecular weight components (FI and FII), all of them eluted quantitatively before the 45% acetonitrile step (data not shown).

We investigated special chromatographic conditions in order to obtain a more suitable separation of the different components of FIII. Moreover, in the present work the 11 polypeptidic toxins (3 α-neurotoxins, 3 phospholipases A_2, and 5 cardiotoxins) already purified from *N. mossambica mossambica* venom by means of conventional chromatographic methods (molecular sieving and ion exchange) have been used as standards in order to localize their homologues in the FIII elution diagrams on the basis of respective R_t values. The denomination codes of these toxins are referenced under Materials and Methods. For the α-neurotoxins, i.e., for minor components of this venom, the acetonitrile slope was decreased

Table I: Respective Percentages of Toxins in *N. mossambica mossambica* Venom[a]

name	AU	$E^{1\%}_{280}$	AU/$E^{1\%}_{280nm}$	% (w/w)		% related (w/w)		
NTX Nmm I	2863	13.70	209	1	1.17[b]	73	72[b]	
NTX Nmm II	173	14.46	12	0.1	0.07[b]	6	4[b]	
NTX Nmm III	1489	21.28	70	0.4	0.38[b]	21	24[b]	
				(1.5)		(100)		
PH Nmm I	28732	22.87	1256	7	2[c]	21	16[c]	
PH Nmm II	54127	22.87	2366	13	4[c]	40	26[c]	
PH Nmm III	53909	22.87	2357	13	9[c]	39	58[c]	
				(33)		(100)		
CTX Nmm I	26656	13.79	1933	11	7[d]	16	9[e]	37[d]
CTX Nmm IIa	37953	13.85	2740	15	6[d]	23	53[e]	31[d]
CTX Nmm IIb	17771	13.85	1283	7		11		
CTX Nmm III	25680	6.88	3732	20	2[d]	31	26[e]	11[d]
CTX Nmm IV	22688	13.67	1659	9	3[d]	14	10[e]	16[d]
CTX Nmm V	5129	8.00	641	3.5		5	2[e]	
				(65.5)		(100)		

[a] Arbitrary absorbance units (AU) at 280 nm were furnished by the data module integrator/recorder in conditions described under Materials and Methods. $E^{1\%}_{280nm}$ values were taken from the literature. Numbers in parentheses represent totals. [b] Same batch of this venom (Rochat et al., 1974). [c] Venom from same species and supplier (Joubert, 1977). [d] Venom from same species and supplier (Louw, 1974a). [e] Same batch of this venom (Bougis et al., 1983).

to 0.4% min^{-1}. Thus, NTX Nmm II, NTX Nmm I, or NTX Nmm III were individualized in this elution order (Figure 2b) and appeared to constitute the first component group with a R_t of 9 min (Figure 2a). For the most tightly bound polypeptides such as phospholipases A_2 and cardiotoxins, we simultaneously increased the initial acetonitrile percentage to 25% and decreased the gradient slope to 0.25% min^{-1} (Figure 2d); we changed the gradient slope's linear form for a curved one (Figure 2c). In these experimental conditions the α-neurotoxins eluted in the void volume of the column. The elution order observed was PH Nmm III, CTX Nmm III, PH Nmm I, and PH Nmm II for the central group (20-min R_t in Figure 2a) and CTX Nmm IV, CTX Nmm I, and CTX Nmm V for the last one (23-min R_t in Figure 2a). Other elution peaks were also observed, and so up to 26 distinct components of FIII, including the 10 yet identified, could be detected by using RP-HPLC. Moreover, the component corresponding to the shoulder on the trailing edge of the elution peak of CTX Nmm I (Figure 2d), as well as that one eluted just after those of the central group (Figure 2c), has been identified as constitutive of the CTX Nmm II fraction purified by means of conventional ion-exchange chromatography on Amberlite CG-50. This very fraction previously demonstrated as heterogeneous in RP-HPLC (Figure 1c), and its two components were termed CTX Nmm IIa and -b with reference to their elution order. Their amino acid analyses were carried out and showed an accurate correspondence with those of CTX Nmm II and CTX Nmm I, respectively.

Taking into consideration the integrated data of FIII elution diagrams, the 12 above-considered toxins were found to account for 90% of the sum of the elution peak areas. The weight percentages of these polypeptidic toxins were determined either individually or by groups, taking the known extinction coefficient of each into account (Table I).

The reproductibility of such RP-HPLC elution diagrams was ±6%, determined by calculating the standard deviation of the total sum of the elution peak areas of five RP-HPLC runs carried out under identical conditions.

α-Neurotoxin Activity. We tested α-neurotoxin activity by examining its binding competition with ^{125}I-NTX Nmm I for the AcChR of the ray electric organ membrane fragments. We examined the binding of ^{125}I-NTX Nmm I vs. the concentration of NTX Nmm I (Figure 3) in order to estimate the quantity of NTX Nmm I-like polypeptide thus detectable. The half-effect was obtained for 1.12×10^{-10} M in NTX Nmm I. Under our experimental conditions the lower limit of detection (10% of the signal) appeared to be 1.12×10^{-11} M (2.8 fmol in the assay) of polypeptides related to the tracer and whose affinity for the AcChR was identical with that of the tracer.

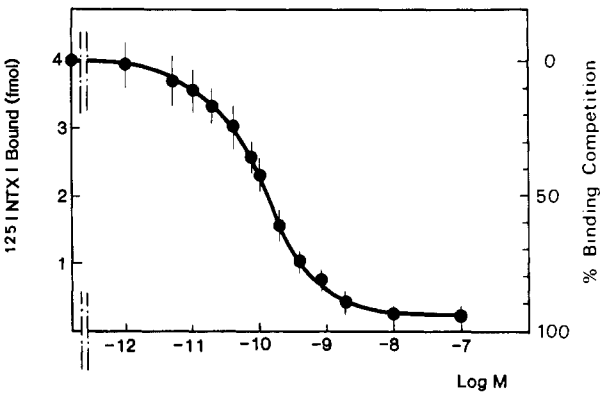

FIGURE 3: Binding competition between ^{125}I-NTX Nmm I and NTX Nmm I for AcChR. Respective concentrations of ^{125}I-NTX Nmm I and α-neurotoxin binding sites in each well of the microtitration plate assay were 60 and 20 pM in buffer A/BSA. K_D of ^{125}I-NTX Nmm I/AcChR complex was around 60 pM (unpublished personal observation). $K_{0.5}$ observed was 1.2×10^{-10} M in NTX Nmm I.

Unfortunately, the phospholipases A_2 in the tested venoms possessed some esterase activity against ray electric organ membrane phospholipids, thereby providing a positive response to the α-neurotoxin specific assay of the RP-HPLC phospholipase A_2 fractions. This interference was eliminated by adding an inhibitor of phospholipase A_2 activity (2 mM EDTA) to the incubation buffer, with no noticeable change in the level of ^{125}I-NTX Nmm I bound to AcChR.

Phospholipase A_2 Activity. Phospholipases A_2 (EC 3.1.1.4) have an esterase activity, since they hydrolyze fatty acid ester bonds of 3-*sn*-phosphoglycerides at the *sn*-2 position. This property was adopted as an assay for phospholipase A_2 activity. A fluorometric assay was developed with a pyrene lipid as substrate. After hydrolysis of the ester bond, the fluorescence spectrum of the pyrene group was considerably modified (Figure 4a). The enzymatic hydrolysis of the substrate was thus easily followed by recording the fluorescence emission signal at 400 nm as a function of time.

We initially used the overall rapid assay involving the photography of microtitration plate illuminated with UV. This assay was not quantitative but positive (fluorescence shift) from a concentration of phospholipase A_2 of at least 4×10^{-8} M

Table II: Percentage of *Naja* Venom Components Detected from Their α-Neurotoxin or Phospholipase A_2 Activities[a]

venoms			α-neurotoxin activity				phospholipase A_2 activity			
species	LD_{50} (μg/ mouse)	PH activity	name	R_t (min)	% total AU	% related AU	name	R_t (min)	% total AU	% related AU
N. haje haje	4	53 000	NTX Nhh I	18.61	14	40	PH Nhh	49.23	<1	100
			NTX Nhh II	33.96	20	60				
N. melanoleuca	40	2 300 000	NTX Nm I	53.75	2.6	63	PH Nm I	43.70	8	12
			NTX Nm II	71.53	1.5	37	PH Nm II	44.94	5	9
							PH Nm III	48.46	21	33
							PH Nm IV	49.06	24	38
							PH Nm V	56.16	5	8
N. mossambica mossambica	40	420 000	NTX Nmm II	8.07	<1	4	PH Nmm IV	48.35	2	3
			NTX Nmm I	10.84	1	63	PH Nmm III	49.04	21	43
			NTX Nmm III	13.60	<1	33	PH Nmm I	51.15	6	11
							PH Nmm II	51.72	20	42
							PH Nmm V	55.72	<1	1
N. nigricollis	65	30 000	NTX Nn	14.48	<1	100	PH Nn I	48.35	12	44
							PH Nn II	51.82	7	26
							PH Nn III	53.22	8	30

[a] PH activity is expressed as picomoles of fluorescent fatty acid released per minute per 1 absorbance unit at 280 nm. AU was arbitrary absorbance units as in Table I. R_t referred to RP-HPLC conditions of Figures 5 and 6.

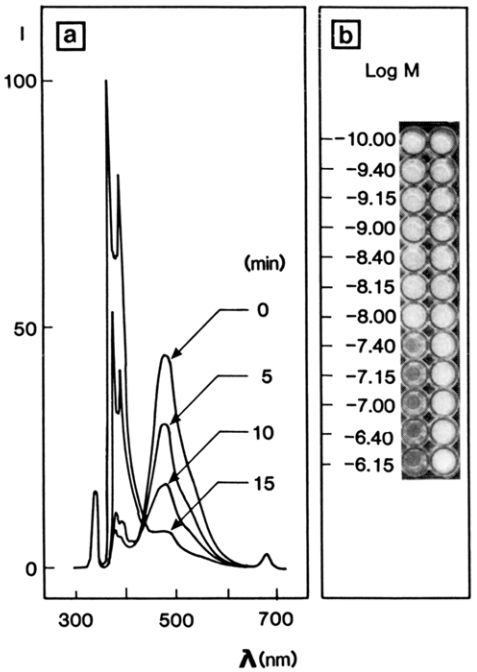

FIGURE 4: Phospholipase A_2 assay. (a) Fluorescence emission spectra of the pyrene lipid at 20 °C (0 min) and during its hydrolysis by 4 μg of PH Nmm II (5, 10, and 15 min). (b) Microtitration plate photographic assay. The entire right row of wells contained 10 μM of pyrene lipid. The left row contained 10 μM of pyrene lipid and a given concentration of PH Nmm II as indicated.

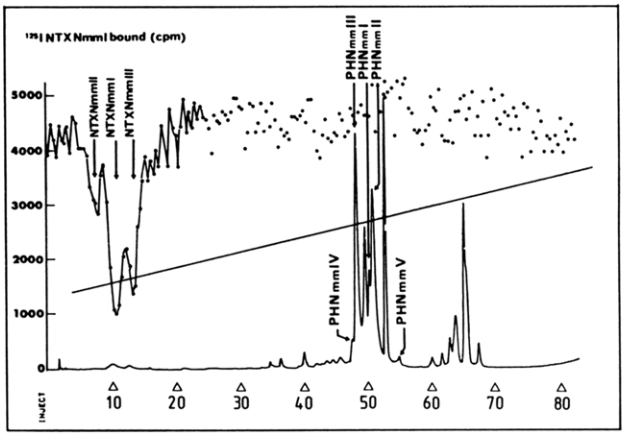

FIGURE 5: Semipreparative RP-HPLC of *N. mossambica mossambica* venom and screening for α-neurotoxin (NTX) and phospholipase A_2 (PH) activities. Ultrasphere octyl column. Solvent A: 0.15 M ammonium formate, 12 mS, pH 2.70. Solvent B: acetonitrile. Gradient 6 from 8% to 45% B in A in 82 min, slope 0.45% B in A min^{-1}. Flow rate 5 mL min^{-1}. Sample loaded 2 mg of F III with 0.200 A. Full ordinate scale.

(10 pmol in the assay) having the same activity and subtrate specificity as PH Nmm III (Figure 4b). The second step involved the kinetic spectrofluorometric assay. The minor components of venoms could be examined as a result of the higher sensitivity of this assay. In our experimental conditions, we could quantify enzyme activities on the order of 0.2 pmol min^{-1}. Thus, in the case of PH Nmm I (specific activity 1.9 μmol min^{-1} mg^{-1}), it corresponded to a PH Nmm I concentration of 7.4 pM (22 fmol in the assay).

Semipreparative RP-HPLC of Venoms. Screening for α-Neurotoxin and Phospholipase A_2 Activities. N. mossambica mossambica venom was initially chosen as a reference, since it had previously been investigated in detail by means of analytical RP-HPLC. Conditions adapted to a semipreparative fractionation of FIII were developed (Figure 5), which involved a transposition of analytical RP-HPLC to the use of a higher capacity column with a flow-rate modification by a multiplying factor of 0.7 $(D/d)^2$ (D and d the inner column diameters). In such conditions the optimal column capacity was about 20 mg of this specific venom with no notable loss in peak resolution. Each RP-HPLC fraction thus obtained was subsequently tested for its α-neurotoxin and phospholipase A_2 contents with the activity assays described above. The experimental data obtained for the α-neurotoxin activity assay are shown in Figure 5. The slight dispersion of the points was most probably due to the high sensitivity of the assay. For this reason only elution peaks defined by several consecutive RP-HPLC fractions were considered as positive. In this way, three elution peaks of α-neurotoxin activity could be detected. Their correspondences in terms of optical density showed, as expected, minor components. In the case of phospholipase A_2 activity, the combination of the photographic and kinetic assays enabled us to demonstrate five positive elution peaks (Figure 5).

This experimental strategy was applied to the venoms of other *Naja* species (six species known) from different geographical origins. Figure 6 shows the results obtained with venoms of the *N. haje haje* (Egypt), *N. melanoleuca* (Ivory Coast), and *N. nigricollis* (Ivory Coast) species. A chroma-

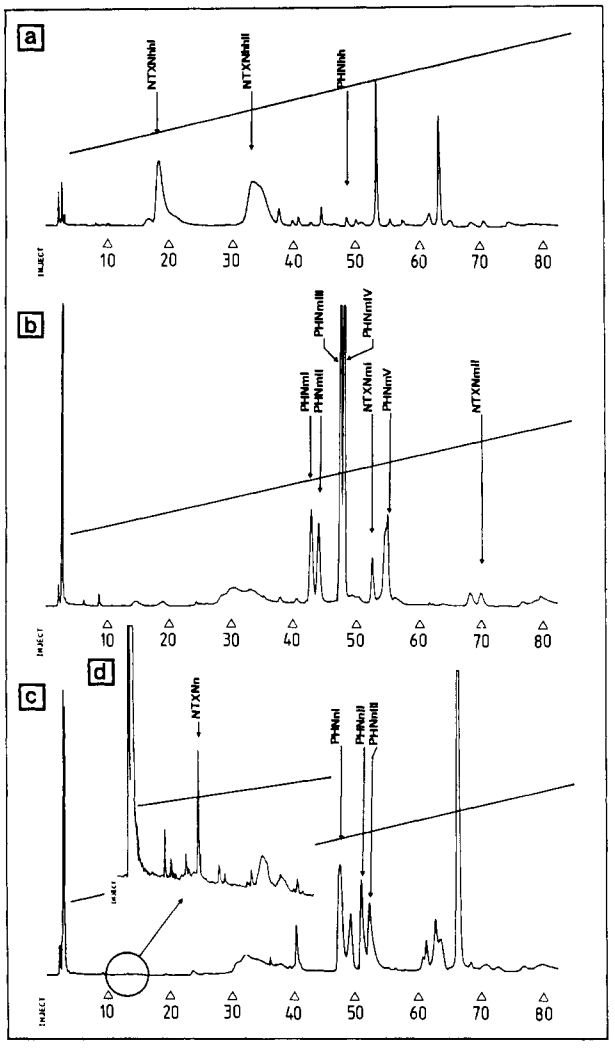

FIGURE 6: Semipreparative RP-HPLC of three different *Naja* venoms and screening for α-neurotoxin (NTX) and phospholipase A_2 (PH) activities. Ultrasphere octyl column. Solvent A: 0.15 ammonium formate, 12 mS, pH 2.70. Solvent B: acetonitrile. Gradient 6 from 8% to 45% B in A in 82 min, slope 0.45% B in A min^{-1}. Flow rate 5 mL min^{-1}. (a) *N. haje haje* venom, 1 mg loaded with 0.100 A. Full ordinate scale. (b) *N. melanoleuca* venom, 1 mg loaded with 0.100 A. Full ordinate scale. (c) *N. nigricollis* venom, 1 mg loaded with 0.100 A. Full ordinate scale. (d) *N. nigricollis* venom, 3 mg loaded with 0.010 A. Full ordinate scale, identical experimental conditions except that gradient 6 was from 8% to 20% B in A in 40 min, slope 0.30% B in A min^{-1}.

tographic examination of the latter, more resolutive for α-neurotoxins, was performed (Figure 6d). Table II summarizes the quantification, by means of the integrated data of elution diagrams, for the different α-neurotoxin and phospholipase A_2 fractions in these venoms as well as their in vivo toxicity expressed as their LD_{50} on mouse.

Discussion

The venoms of Elapidae snakes, genus *Naja*, contain numerous polypeptidic components, the majority of which being toxins (α-neurotoxins, phospholipases A_2, and cardiotoxins) responsible for the lethality of the bite. We briefly described their known or presumed molecular actions in the introduction.

The primary, secondary, and tertiary structures of a biopolymer are considered as controlling the distribution of amino acid residues on its surface. This in turn determines the number of nature of residues that can interact with the acyl chains bonded on the stationary silica phase in RP-HPLC. In the present case, the toxic polypeptides of venoms are composed of relatively short amino acid sequences (60–120 residues), highly cross-linked by a great number of disulfide bonds (4–7). Thus such polypeptides have compact three-dimensional structures (Low et al., 1976; Dufton & Hider, 1983; Dufton et al., 1983), allowing their charge but also hydrophobic characters to be chiefly expressed on the surface of the molecule. Cardiotoxins, essentially basic and hydrophobic, are well representative of this fact. So, we have selected the CTX Nmm II from *N. mossambica mossambica* to determine optimal RP-HPLC conditions. Firstly, this CTX Nmm II, previously purified as a homogenous fraction by means of conventional ion-exchange chromatography (Louw, 1974a; Bougis et al., 1983), appears to be a mixture of two polypeptides: CTX Nmm IIa and -b in a 68/32 ratio (wt %) and representing respectively 15% and 7% of the whole components of FIII (Figure 1 and Table I). Referring only to the amino acid compositions, CTX Nmm IIa seems to be the $v^{II}2$ previously purified and sequenced by Louw (1974b). Concerning CTX Nmm I and CTX Nmm IIb, having identical amino acid compositions, the minimum respective substitution of Asp-40 for Asn-40—as in the CTX Nmm III sequence—may be envisioned between these two cardiotoxins, allowing their total differentiation in ion-exchange chromatography and their almost identity in RP-HPLC (Figure 2d). If true, this occurrence underlines the efficiency of RP-HPLC in the resolution of very small polypeptide sequence differences. Secondly, it is our opinion that the unique chemical characteristics of the cardiotoxins mentioned above explain the results obtained concerning the optimal RP-HPLC conditions. Indeed, because cardiotoxins are the most tightly bound venom components, they are generally eluted at the end of the acetonitrile gradient (Figure 2). Then, it appears judicious to use short acyl chains (C8 in place of C18) bonded on the stationary silica phase to avoid excessive retention of cardiotoxins and so to obtained the best separation efficiency. Other types of bonding on the stationary silica phase have been used in the past to separate cardiotoxins from the venom of *Naja naja atra*, e.g., phenyl (Wu, 1982) and cyanopropyl (Kaneda & Hayashi, 1983), but it is obvious that total venom could not have been resolved with the experimental conditions they used. There is also an absolute necessity for the aqueous solvent (A) to have an appropriate ionic strength, i.e., a conductivity at least equal to 12 mS (Figure 1). The presence of counterions should lead to a more differential adsorption of the most basic components on the inevitably free silanol groups of the stationary silica phase and should prevent the possible disorganization of the three-dimensional structure of the polypeptides that intimately controls their biological activities as well as their adsorption properties, as we have mentioned above. The optimal solvent system accepted [(A) 0.15 M ammonium formate, 12 mS, pH 2.70/(B) acetonitrile] because of the high ammonium formate concentration is not consistent with a detection of the absorbance at the wavelength of 230 nm, but it is easily removed by lyophilization and does not inhibit the biological properties of the polypeptides we have to recover.

The analytical RP-HPLC of *N. mossambica mossambica* toxins in conjunction with the use as standards of the 11 polypeptidic toxins until now purified from this venom by means of conventional chromatographies led to the following findings. (i) All the toxic components of the venom (fraction FIII) were eluted before the 45% acetonitrile step (Figure 2a). (ii) The toxins can be classified into three distinct groups according to their increasing hydrophobicity as α-neurotoxins < phospholipases A_2 < cardiotoxins, with the exception of CTX Nmm III. This is in good agreement with the well-

known interfacial affinity of phospholipases A_2 and cardiotoxins, particularly for biological membranes and their main components, the phospholipids (Jain et al., 1982; Bougis et al., 1983), what is not the case for α-neurotoxins (Faucon et al., 1979; Bougis et al., 1981). (iii) The parameters of the acetonitrile gradient could be optimized for a better separation inside each group of toxins (Figure 2b–d), although at the expense of the others. However, RP-HPLC is not able to really differentiate between CTX Nmm I and CTX Nmm IIb (Figure 2d). One appropriate solution would involve separate pools of each of the three main groups of components after a first RP-HPLC and then a reinjection of each of them for a second RP-HPLC with more straight specific conditions. (iv) The nontoxic high molecular weight components (fractions FI and F II) are also eluted before the 45% acetonitrile step, and so we have no reason to suspect that they choke up the column. At the most, they could interfere with the fractionation of the toxins if not for the fact that they are generally very minor components of the venom. In this way, preliminary filtration on Sephadex G-50 of crude venoms may be omitted, especially in the case of semipreparative RP-HPLC.

The possibility to determine, in one RP-HPLC step, the weight percentages of the *N. mossambica mossambica* toxins enables their relative amounts to be easily investigated in great detail (Table I). It appears that α-neurotoxins are minor components (1.5%) in comparison to phospholipases A_2 (33%) and cardiotoxins (65%). The comparison of our results with published data is very satisfying in the case of α-neurotoxins but at variance with some authors for phospholipases A_2 and cardiotoxins. This divergence is probably originating from the low resolution of conventional chromatographies and the operating strategy used until now.

To be used in combination with HPLC we designed two appropriate assays for screening the α-neurotoxin and phospholipase A_2 activities in RP-HPLC fractions. These specific assays are based on the molecular activities related to the pharmacology of the toxins. Thus, α-neurotoxin activity is defined as the binding competition between these toxins for their biological target, i.e., the AcChR. The sensitivity of this assay is proved to be sufficiently high (concentration detected in the picomolar range; Figures 3 and 5). However, due to differences in the α-neurotoxin affinities for the AcChR (K_D may vary from 0.01 to 1 nM), a direct quantification from the binding competition experiments is not to be considered. Concerning the detection of phospholipases A_2, their enzyme activity is the most appropriate parameter. The sensitivity of the test obviously depends on the specific activity of the enzyme, i.e., both its substrate specificity and turnover. In our case, the use of a fluorescent substrate (pyrene lipid) seems to be the best compromise in terms of both simplicity and sensitivity. The kinetic assay is very sensitive (concentration detected in the picomolar range) and also quantitative in terms of enzymatic activity, but it is time-consuming. Alternatively, the photographic assay is very rapid but not so sensitive (concentration detected in the nanomolar range; Figure 4b).

A cardiotoxin activity assay would have been useful and complementary for our investigation, but without an unambiguously known molecular action of cardiotoxins, we did not attempt to design such an assay.

We have extended our knowledge of *N. mossambica mossambica* venom by demonstrating two additional phospholipases A_2, quantitatively minor components (Figure 5; Table II). Other minor components remain to be identified. The systematic study of α-neurotoxin and phospholipase A_2 contents of the different *Naja* venoms we have investigated enable us to make the following observations (Figures 5 and 6; Table II). (i) There is an obvious heterogeneity of these venoms. *N. haje haje* includes relatively few major components but contains the highest percentage of α-neurotoxins and the highest toxicity. The other include not far from 20 distinct components and have the highest phospholipase A_2 content and the lowest toxicity (*N. mossambica mossambica*, *N. melanoleuca*, *N. nigricollis*). This is largely consistent with the lethality of the Elapidae venoms and with our knowledge of the effects of bites by the Egyptian cobra *Naja haje*, powerful and predominantly neurotoxic, and by the spitting cobras of savannahs *N. mossambica mossambica* and *N. nigricollis*, weakly neurotoxic but highly cytotoxic (Tilbury, 1982). (ii) Confirming our previous findings about the elution order of the *N. mossambica mossambica* toxins, it can be observed that the α-neurotoxins are eluted before the phospholipases A_2 except for the two ones from *N. melanoleuca*, eluted as hydrophobic components of the venom. This original behavior can result from the presence of one or more hydrophobic residues on a protruding part of the molecule according to its compact three-dimensional structure. More generally there is no simple correlation between the R_t of the polypeptidic toxins and their overall hydrophobicity as there is for unstructurated polypeptides (Meek, 1980; Wilson et al., 1981).

Finally, the present study may be very helpful for the isolation and characterization of new toxins available in less than 1 mg of an animal venom. In this view, HPLC should be considered as the highly appropriate tool, especially for the toxins that are (for most of them) chemically very stable. Thus, the development of such a set of new biochemical techniques would be a hopefully promising line in the future, particularly for correlating the taxonomy with the venom contents and allowing the development of an adequate seroterapy.

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Registry No. Phospholipase A_2, 9001-84-7.

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Interaction of Fully Liganded Valency Hybrid Hemoglobin with Inositol Hexaphosphate. Implication of the IHP-Induced T State of Human Adult Methemoglobin in the Low-Spin State†

Isao Morishima,* Mitsunobu Hara, and Koichiro Ishimori
Division of Molecular Engineering, Graduate School of Engineering, Kyoto University, Kyoto 606, Japan
Received January 29, 1986; Revised Manuscript Received May 27, 1986

ABSTRACT: To gain further insight into the quaternary structures of methemoglobin derivatives in the low-spin state, the interaction of fully liganded valency hybrid human hemoglobins with IHP was studied by proton NMR spectroscopy. Upon addition of IHP to $(\alpha_{CO}\beta^{+}_{N_3^-})_2$, the same resonances as the previously reported IHP-induced NMR peaks for azidomethemoglobin $(\alpha^{+}_{N_3^-}\beta^{+}_{N_3^-})_2$ appeared, whereas the binding of IHP did not significantly affect the NMR spectra for $(\alpha^{+}_{N_3^-}\beta_{CO})_2$. The binding of IHP also brought about more pronounced spectral changes for $(\alpha_{CO}\beta^{+}_{Im})_2$ and $(\alpha_{CO}\beta^{+}_{H_2O})_2$ than for $(\alpha^{+}_{Im}\beta_{CO})_2$ and $(\alpha^{+}_{H_2O}\beta_{CO})_2$. Therefore, the IHP-induced NMR peaks for azidomethemoglobin are attributed to the β heme methyl group. Such IHP-induced β heme methyl resonances were also observed for $(\alpha_{NO}\beta^{+}_{N_3^-})_2$, which undergoes quaternary structural change, analogously to the R–T transition by the binding of IHP. From the above results, it was suggested that the IHP-induced heme methyl resonances for azidomethemoglobin and $(\alpha_{CO}\beta^{+}_{N_3^-})_2$ may also be associated with the quaternary structure of these Hbs, implying the presence of the IHP-induced "T-like" state in low-spin metHb A.

A variety of physicochemical properties of hemoglobin tetramer have been often understood on the basis of two quaternary conformational states: the T and R states, normally associated with deoxy- and oxyhemoglobin structures and also characterized by low and high oxygen ligand binding affinity, respectively (Monod et al., 1965). These two quaternary states have been defined in terms of some detailed protein and heme environmental structures (Shulman et al., 1975; Baldwin, 1975). The X-ray structural analysis revealed that the iron is out of the heme plane in deoxyhemoglobin (Fermi, 1975) but planar in oxyhemoglobin (Shaanan, 1982). On the basis of these structural differences, Perutz (1970) has proposed that this movement of iron displacement into or out of the heme plane, linked to the spin state of heme iron, exerts an influence on the globin structures of the protein that promote the T to R structural transition upon ligand binding. In support of such

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* Author to whom correspondence should be addressed.

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