Eur. J. Biochem. *68*, 457–469 (1976)

Taipoxin, an Extremely Potent Presynaptic Neurotoxin from the Venom of the Australian Snake Taipan (*Oxyuranus s. scutellatus*)

Isolation, Characterization, Quaternary Structure and Pharmacological Properties

Jan FOHLMAN, David EAKER, Evert KARLSSON, and Stephen THESLEFF

Institute of Biochemistry, University of Uppsala, and Department of Pharmacology, Lund University

(Received April 23, 1976)

Taipoxin (taipan toxin), purified from the venom of the Australian taipan (*Oxyuranus s. scutellatus*) by gel filtration on Sephadex G-75 followed by column zone electrophoresis, is the most lethal neurotoxin yet isolated from any snake venom. The LD_{50} is 2 μg/kg in the mouse. The main physiological effect is a gradual reduction to complete stop of evoked and spontaneous release of acetylcholine from motor nerve terminals. Intoxicated animals die of asphyxia caused by neuromuscular blockage of the respiratory muscles.

Taipoxin is a moderately acidic sialo-glycoprotein (p*I* 5) with a molecular weight of 45600 as calculated from composition data or 46800 as determined by meniscus depletion sedimentation equilibrium. Taipoxin is a 1:1:1 ternary complex of subunits designated α, β and γ which dissociate completely at low pH and high ionic strength or in 6 M guanidine hydrochloride. The dissociation by guanidine at neutral pH is reversible, while the acid-induced dissociation is not. The α and β components consist of 120 amino acid residues cross-linked by seven disulfide bridges, whereas the γ component has 135 residues and eight disulfides.

The very basic (p$I > 10$) α component contains 13 residues of arginine and is the only subunit displaying lethal neurotoxicity (mouse $LD_{50} \approx 300$ μg/kg). The neutral β fraction was separated by ion-exchange chromatography into two iso-component , β_1 and β_2, which differ slightly in amino acid composition. The very acidic γ component contains all of the carbohydrate, which includes 4–5 residues of sialic aid. The three subunits are homologous in sequence although the γ component is eight residues longer on the N-terminus and must also contain extra amino acids elsewhere.

The taipan (*Oxyuranus scutellatus scutellatus*) is an elapid snake indigenous to tropical Australia and the southern parts of New Guinea [1]. The snake can be as long as 3.5 m and has exceptionally long fangs capable of penetrating thick leather boots [2]. The lethal potency of the venom is very high and as much as 400 mg (dry weight) has been extracted from a single adult specimen [3]. We have determined the intravenous mouse LD_{50} of the dried crude venom to be 12 μg/kg, which is the lowest value yet reported from any snake venom. This is also considerably lower than the lethal dose of any previously purified snake venom neurotoxin. In the absence of prompt treatment with specific antiserum a taipan bite is almost invariably fatal. Only two cases of recovery have been reported [2,4]. The taipan is very aggressive when disturbed and is by all accounts the most dangerous snake in the world.

The venom is strongly neurotoxic and the clinical syndrome resembles severe myasthenia gravis [4]. Tiger snake (*Notechis scutatus scutatus*) antivenin gives very little protection, which is rather remarkable, since the latter antivenin is effective against most other Australian snakes and in fact against elapid venoms in general [5].

We have reported earlier that tiger snake venom contains two pharmacologically distinct types of neurotoxins; (a) the presynaptic toxin, notexin, which inhibits the release of acetylcholine from motor nerve terminals, and (b) postsynaptic toxins which block the nicotinic acetylcholine receptor of the muscle motor end plate [6,7].

Although the direct prothrombin-activating property of taipan venom has attracted some attention no attempts to isolate and characterize the neurotoxic principle(s) have been described. Because the available immunological and toxicological data suggested the presence of a novel type of neurotoxin we decided to isolate the toxins of this venom. One of them, the taipoxin, has turned out to be the most potent snake venom toxin known.

MATERIALS AND METHODS

Venom

Dried *Oxyuranus s. scutellatus* venom was purchased from Worrell's Australian Reptile Park, P.O. Box 192, Gosford, New South Wales, Australia 2250.

Gel Filtration

Crude venom ($\approx$ 1 g) was dissolved in 10 ml 0.10 M ammonium acetate. Small amounts of insoluble material were removed by centrifugation at $20000 \times g$ for 20 min and the clarified, slightly yellow solution was applied to a column of Sephadex G-75 equilibrated with 0.10 M ammonium acetate. The fractions were screened for toxicity and lyophilized. The dried fractions were transferred to smaller tared bottles in small portions of 0.01 M ammonium acetate and lyophilized a second time. Lyophilization from the latter medium gives products which are readily soluble and easy to handle. The weights of the dried fractions were used to estimate the material balance.

Immediately before use all Sephadex columns were eluted with at least one bed volume of buffer to minimize contamination of the samples with carbohydrates leached from the gel matrix during long standing.

The buffer used in the acid gel filtration was prepared by dissolving 1 mol of NaCl in 1 l of 7.8% acetic acid/2.5% formic acid (v/v). The pH dropped from 1.9 to 1.7 upon the addition of the salt.

Column Zone Electrophoresis

The most toxic fraction was examined by column zone electrophoresis in externally cooled all-glass columns, essentially as described by Porath [8]. The column (1×86 cm) was packed with water/pyridine-extracted cellulose (Munktell cellulose powder No. 410, Grycksbo, Sweden) and equilibrated with 0.05 M *N*-ethylmorpholine buffer, pH 7.5. The preparative polypeptide chain separation was done in a similar column equilibrated with a buffer composed of 7.8% acetic acid/2.5% formic acid, v/v, pH 1.9.

To ensure electrophoretic sharpening into a narrow starting zone [9], the samples were dissolved in 1–2 ml of buffer diluted with an equal amount of distilled water. The samples were displaced downward to an appropriate starting position and runs were done at 1000 V for a time estimated from paper electrophoresis experiments in the same buffer at 440 V, wherein a mobility of $1\ \text{cm} \times \text{h}^{-1}$ usually corresponds to a migration of $0.4-0.7\ \text{ml} \times \text{h}^{-1}$ in the cellulose powder column at 1000 V [10]. After electrophoresis the column was eluted with a peristaltic pump at about $14\ \text{ml} \times \text{h}^{-1}$.

Ion-Exchange Chromatography on Sulfopropyl-Sephadex

Sulfopropyl-Sephadex (SP-Sephadex C-25, Pharmacia Fine Chemicals) was equilibrated with 0.1 M ammonium acetate buffer adjusted to pH 5.0 with glacial acetic acid. The sample was dissolved in 2.0 ml of this buffer and adsorbed onto the column. Elution was done with a 150 ml linear gradient of 0.1 M to 1.0 M ammonium acetate at pH 5.0.

Estimation of Molecular Weight

Gel Filtration in Non-Denaturing Media. The apparent molecular weights of the complex and its polypeptide chains were estimated by gel filtration on a Sephadex G-75 column, 1.02×100 cm, $V_t = 81.8$ ml, calibrated with standard proteins (immunoglobin IgG as a void volume (V_0) marker, bovine chymotrypsinogen A, $M_r = 25700$ [11], bovine ribonuclease A, $M_r = 13700$ [11], neurotoxin naja 4, $M_r = 7800$ [12] and [^{14}C]alanine for determination of the interstitial volume (V_i). The dead volume (2.3 ml) was collected as a prefraction and held constant during all the experiments. The sample was 1.0 ml, the flow rate was $10.0\ \text{ml} \times \text{h}^{-1}$, and the fraction volume was 2.0 ml. The distribution coefficient K_D was calculated as $K_D = (V_e - V_0)/V_i$ where V_i is the elution volume of [^{14}C]alanine minus V_0. A plot of $K_D^{1/3}$ vs $M_r^{1/2}$ [13] for the reference proteins gave a good straight line the equation of which was calculated by linear regression analysis.

Gel Filtration in 6 M Guanidine Hydrochloride. The eluant used in this experiment was prepared by treating 2 l of 6 M guanidine hydrochloride solution (Fluka AG, purum) with 10–20 g of active charcoal followed by filtration through two layers of filter paper on a Büchner funnel and final clarification by filtration through an 0.45-μm millipore filter. This treatment reduced the $A_{280}^{1\text{cm}}$ from 0.3 to 0.07 and greatly facilitated spectrophotometric monitoring. A micromethod was developed by introducing a radioactive label in the alkylation reaction as described for the preparation of reduced and *S*-carboxymethylated derivatives. Sepharose 6B (Pharmacia Fine Chemicals) was wet-sieved to a particle size of 40–100 μm and equil-

ibrated with 6 M guanidine hydrochloride on a Büchner funnel. The slurry was poured into the column (1.03×97.8 cm, $w_t = 81.0$ g) and allowed to settle under flow with a hydrostatic head of 20 cm. The packed column was eluted with 6 M guanidine-HCl for two weeks at $\approx$ 2 ml/h before the calibration was initiated. To attain adequate precision elution positions were determined by weight (w_e) rather than volume. The flow rate was maintained at $2.0\ \text{ml} \times \text{h}^{-1}$ with an LKB perpex peristaltic pump and fractions of 1.0 g were collected with a Gilson microcol TDC 80 fraction collector. This arrangement gave excellent reproducibility; the elution weights of the marker substances agreed within ± 0.1 g in duplicate experiments. Monitoring was done spectrophotometrically and/or by liquid scintillation counting. The elution positions were determined to the nearest 0.1 g. K_D was calculated as $K_D = (w_e - w_{BD})/w_{re} - w_{BD})$ where w_{BD} and w_{re} are the elution 'weights' of blue dextran and excess reagent respectively. A plot of $K_D^{1/3}$ *vs* $n^{0.555}$ gave a good straight line [14] the equation of which was calculated by the least-squares method (n = number of amino acid residues).

Radioactive Reduced and S-Carboxymethylated Derivatives

2–3 mg of each calibration protein was dissolved in 1 ml 6 M guanidine hydrochloride containing 0.86 M Tris-HCl buffer, pH 8.6, and 0.3% EDTA. After flushing with nitrogen for 5 min, 13 µmol dithioerythritol was added in 100 µl of the same medium. Alkylation was first done with 3.4 µmol iodo[^{14}C]acetamide (specific activity 13.4 Ci/mol) for 10 min and then with 26 µmol unlabelled iodoacetate. For each calibration run, 100 µl of the calibration mixture was mixed with 100 µl of a 1% blue dextran solution and the resulting solution was applied to the column. All solutions containing iodide were protected from light and the lower part of the column was wrapped with aluminium foil.

Liquid scintillation counting was done in disposable vials containing 5 ml scintillation cocktail according to Anderson [15].

Analytical Ultracentrifugation

Long-column sedimentation equilibrium by the meniscus depletion technique was done with a Spinco model E analytical ultracentrifuge equipped with electronic speed control essentially as described by Chervenka [16]. A double-sector synthetic-boundary cell with sapphire windows was filled and placed in an An-D rotor and run at 20000 rev./min for 17 h at 17.8 °C. The rotor was allowed to reach thermal equilibrium during constant refrigeration. After the run the cell was emptied and rinsed carefully without disassembly and a water blank was then run to correct for optical aberrations. The sample was from a gel filtration fraction and the reference was taken from the void fraction. The initial protein concentration was 1.13 mg/ml in 0.1 M ammonium acetate. Rayleigh interference optics were used to analyze the concentration distribution. Five consecutive fringes were read at 0.1-mm intervals in a Nikon microcomparator all the way to the bottom. The difference between the means of the experimental and blank values was computed. A plot of ln fringe displacement *vs* r^2 was a good straight line (Fig. 3) the slope of which was determined by linear regression analysis using all points with a displacement larger than 100 µm. The partial specific volume, $\bar{v}$, calculated from the amino acid and carbohydrate composition data was 0.70 ml/g.

Test for Free Sulfhydryl Groups

0.5 mg (11 nmol) taipoxin was dissolved in 1 ml 0.86 M Tris-HCl, pH 8.6, 0.3% in EDTA and 6 M in urea. 330 mg (1.6 mmol) iodoacetic acid (sodium salt) was added in 1 ml of the same medium and the solution was allowed to stand in the dark for 1 h. The reaction mixture was then transferred to a dialysis bag (Visking 23/32 in, 18.26 mm, cellophane membrane) and dialyzed against water for two days in the dark with frequent changes of the outer solution. The contents of the dialysis bag were then transferred to a thick-walled hydrolysis tube, freeze-dried and subsequently submitted to acid hydrolysis for amino acid analysis.

Ninhydrin Analysis following Alkaline Hydrolysis

For alkaline hydrolysis appropriate aliquots were pipetted into polypropylene (Nalgene) tubes and evaporated to dryness at 110 °C before adding the alkali, since formic acid gives a high red 'blank' with ninhydrin. 0.5 ml 2.5 M NaOH was then added and the hydrolysis was done for 3 h at 110 °C. Following neutralization with 1.0 ml 1.5 M acetic acid, 1.0 ml ninhydrin reagent [17] was added and the mixture was incubated for 15 min in a boiling water bath. Each fraction was then diluted with 5 ml 50% ethanol and the absorbance at 570 nm was measured.

Analytical Isoelectric Focusing

This was done on a LKB 2117 Multiphor essentially according to the instruction manual.

Electrofocusing was run at maximally 40 W with upward readjustment of the voltage at 15-min intervals until 1000 V was reached, which usually took 1–2 h. Staining for 15 min at 60 °C with Coomassie brilliant blue R-250 and destaining was performed as

described by Vesterberg [18]. The pH gradient was measured by homogenizing small strips of the gel in distilled and deaerated H_2O.

Amino Acid Analysis

After lyophilization of an appropriate amount of protein (maximum 50 nmol of the predominant amino acid), hydrolysis was done for 24 h and 72 h with 6 N HCl containing 10 mg/ml reagent grade phenol in thoroughly evacuated tubes at 110 °C. Cysteine and methionine were determined as cysteic acid and methionine sulphone, respectively, after performic acid oxidation. Serine and threonine were calculated using the standard recovery values 0.90 and 0.96 for 24-h hydrolysates or by linear (threonine) or first-order (serine) extrapolation to zero time of the values observed after 24-h and 72-h hydrolysis. Tryptophan was estimated from ultraviolet spectra in conjunction with amino acid analysis, using molar absorptivity values of 5554 M^{-1} cm^{-1} for tryptophan, 1260 M^{-1} cm^{-1} for tyrosine and 150 M^{-1} cm^{-1} for disulfide bridges at 278 nm. Glucosamine was determined in the amino acid analysis of the 24-h hydrolysates, using a recovery factor of 54% obtained with free *N*-acetylglucosamine 'hydrolyzed' under the same conditions.

N-Terminal Analysis

Edman degradation of the reduced and *S*-carboxymethylated taipoxin subunits was done by the direct phenylisothiocyanate method as described by Iwanaga *et al.* [19]. After spectrophotometric determination of the concentration 5 nmol of the ethyl-acetate-soluble phenylthiohydantoin derivatives were identified by thin-layer chromatography on silica gel plates (Merck Fertigplatten F_{254}) in solvent V [20] and solvents II and III [21]. The spots were located visually under ultraviolet (254-nm) illumination.

The water-soluble derivatives of arginine and histidine were identified after paper electrophoresis at 440 V for 2 h at pH 6.5 in sodium phosphate buffer containing 0.015 M Na_2HPO_4, 0.03 M NaH_2PO_4, 1 g of Na_2EDTA and 5 g of soluble starch per l. The derivatives were detected as white spots on a coffee-coloured background by means of the iodine/azide reagent [22].

Carbohydrate Analysis

Carbohydrates were analyzed by gas-liquid chromatography essentially as described by Clamp *et al.* [23]. Lyophilized samples were dried in a desiccator over P_2O_5 for 24 h prior to methanolysis in anhydrous methanol containing 1.0 M HCl for 24 h at 80 °C together with an appropriate amount of mannitol as an internal standard. After neutralization with $AgCO_3$ and subsequent re-*N*-acetylation with acetic anhydride the samples were dried and trimethylsilylated. The volatile derivatives were injected onto a 3.8% silicone gum rubber SE-30 glass spiral column mounted in a Pye Unicam model 104 gas chromatograph equipped with a flame ionization detector. Peaks were integrated electronically.

Metal Analysis

A sample of taipoxin as obtained directly in the initial gel filtration step was analyzed with a Techtron atomic absorption spectrophotometer (Techtron, Ltd, Melbourne) along with an appropriate blank. The precision was estimated at ±5%. The protein concentration was determined by total amino acid analysis of another aliquot of the solution used.

Polyacrylamide Gel Electrophoresis

100 µg protein in 50 µl 20% sucrose solution was layered on top of 6% polyacrylamide gels with 5% cross-linking. Electrophoresis was run at 20–50 V, 2–4 mA for 2–10 h using the discontinuous buffer system described by Davis (quoted in [24]): gel buffer pH 8.9, electrode buffer pH 8.3. Staining was done with Coomassie brilliant blue R-250 in 12.5% trichloroacetic acid [25].

Toxicity

Intravenous LD_{50} doses were determined by the method of Reed and Muench [26] using 3 or 4 mice at each dose level. The injections were done in 100 µl physiological saline. The protein concentrations were estimated spectrophotometrically using the $A_{278}^{1\,mg/ml}$ values determined in conjunction with amino acid analysis. In the case of the crude venom the solutions were made up by weight.

Physiological Techniques

Isolated phrenic nerve-hemidiaphragm muscles of mice were mounted in a constant temperature bath which was continuously perfused with an oxygenated fluid maintained at pH 7.2–7.4 and at 37 °C. To study neuromuscular transmission the phrenic nerve was stimulated by bipolar platinum electrodes using square-wave pulses of supramaximal voltage and 0.1-ms duration, and the resulting twitch response of the muscle was recorded isometrically. Taipoxin, or one of its polypeptide chains, were added to the bathing fluid.

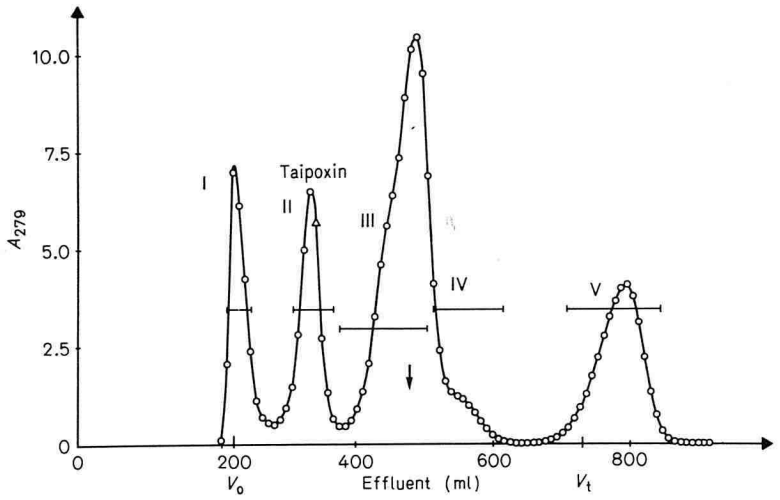

Fig. 1. *Gel filtration of 1113 mg crude taipan venom on a 3.2 × 92-cm column of Sephadex G-75 equilibrated with 0.1 M ammonium actate.* Sample volume: 10 ml. Flow rate during sample application 12.1 ml × h^{-1} and thereafter 34.5 ml × h^{-1}. 3 ml of fraction 17 (in triangle) was used for ultracentrifugation analysis using effluent void fraction as blank. The arrow indicates the elution position of cytochrome *c*

RESULTS

Isolation of Whole Taipoxin

Gel Filtration of the Crude Venom on Sephadex G-75. The four protein fractions I–IV and the non-protein fraction V pooled as indicated in Fig. 1 accounted for 21, 17, 47, 10 and 5%, respectivcly, of the crude venom as estimated from the weights of the lyophilized material. Fraction I developed a precipitate upon standing overnight and appears to contain the direct prothrombin activator (*i.e.*, clots citrated plasma without addition of calcium) discovered in taipan venom by Anderson [27]. The mouse lethal dose of fraction I is greater than 500 µg/kg. Fraction III is lethal to mice at a dose level of 100 µg/kg and appears to be paralytic. Fraction IV causes respiratory paralysis in mice and has a lethal dose of 100 µg/kg. On the basis of this and its elution position (estimated molecular weight ≈8000) we conclude that taipan venom contains curarimimetic neurotoxins of the type(s) found in all other elapid and hydrophid venoms examined so far. The non-protein fraction V eluting after the total bed volume of the column contains conjugated amino acids, but probably consists mainly of nucleosides, since $A_{260} > A_{280}$. Fraction V also has the pigment responsible for the slightly yellow colour of the crude venom. Fraction II has a LD_{50} of only 2 µg/kg mouse as compared to 12 µg/kg for the crude venom and was therefore selected for further examination.

Column Zone Electrophoresis of Fraction II. Preparative electrophoresis of fraction II in a column of cellulose powder at pH 7.5 revealed three minor components, as shown in Fig. 2. The single major component, which we have called taipoxin (taipan toxin), accounted for 95% of the applied sample.

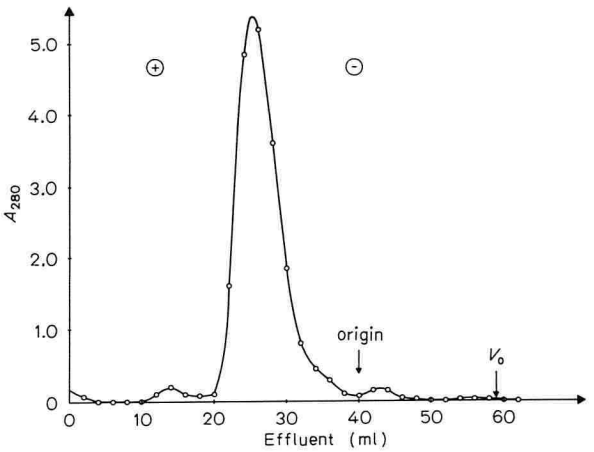

Fig. 2. *Column zone electrophoresis of 30 mg taipoxin (peak II in Fig. 1) on a 1 × 96-cm column of cellulose powder in 0.05 M N-ethylmorpholine/acetate buffer, pH 7.5.* The sample was applied in 2 ml 0.025 M buffer and displaced downward 20 ml to the starting position (arrow). The total free liquid volume of the bed (V_0) as measured with norleucine was 59 ml. The run was done at 10–12 °C for 15 h at 1000 V, 7.5 mA

The taipoxin thus obtained was electrophoretically homogeneous in polyacrylamide gels at pH 8.9. Upon isoelectric focusing in a slab of polyacrylamide in the LKB Multiphor the taipoxin showed four sharp, evenly spaced bands in the interval between pH 4.9 and 5.3 and a faint band at pH 7.1. At lower pH values the taipoxin dissociates into subunits which behave very differently from each other and from intact taipoxin in electrophoresis, gel filtration, and isoelectric focusing, as discussed later below.

Molecular Weight Estimation of Whole Taipoxin

By Gel Filtration. At neutral pH in 0.1 M ammonium acetate taipoxin eluted from a calibrated

Table 1. *Calibration data for the analytical Sephadex G-75 column* The elution positions of immunoglobulin and [^{14}C]alanine were taken as V_0 and ($V_i + V_0$) respectively. Columns dimensions: 1.02 × 100 cm. Equation of data: $K_D^{1/3} = -0.00213 \times M_r^{1/2} + 1.051$

Substance	V_e	K_D	$K_D^{1/3}$	M_r	$M_r^{1/2}$
	ml				
Immunoglobulin	26.8	0			
Chymotrypsinogen A	45.2	0.359	0.711	25666	160.2
Ribonuclease A	53.0	0.513	0.800	13683	117.0
N.n. naja neurotoxin 4	59.8	0.645	0.864	7807	88.4
[^{14}C]Alanine	78.0	1			

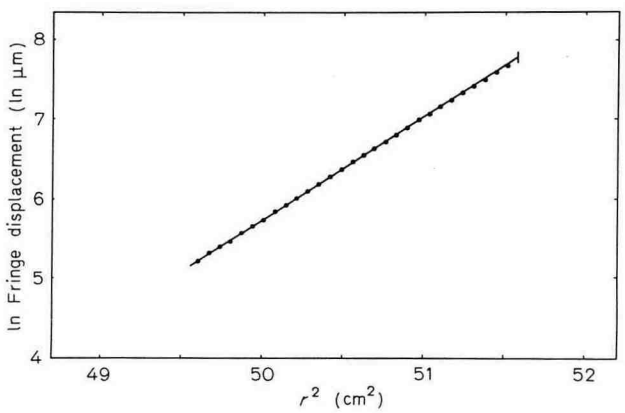

Fig. 3. *Plot of ln fringe displacement* vs r^2 (r = *distance to the center of rotation*) *obtained after 17-h long-column meniscus-depletion sedimentation equilibrium run at 20002 rev./min.* The initial concentration of taipoxin was 1.13 mg/ml. The position of the cell bottom is indicated by a vertical bar

Sephadex G-75 column with a K_D of 0.207, which upon insertion into the calibration equation (Table 1) yields a molecular weight of 46700.

By Ultracentrifugation. Under the same conditions taipoxin is also monodisperse in the ultracentrifuge, as evidenced by the strictly linear plot of ln c *vs* r^2 shown in Fig. 3 for a sedimentation equilibrium run done by the long-column meniscus depletion technique of Chervenka [16]. The slope is 1.278, which together with a value of 0.70 ml/g for the partial specific volume as calculated from amino acid and carbohydrate composition data corresponds to a molecular weight of 46800, in excellent agreement with the gel filtration result.

Chemical Composition of Whole Taipoxin

The amino acid and carbohydrate composition data for whole taipoxin are given in the last column of Table 2. The data are normalized to a formula weight of 45600 for reasons given below.

As determined by atomic adsorption spectrophotometry taipoxin contains 0.7 mol Ca^{2+}/mol, but no significant amounts of magnesium or zinc.

No trace of carboxymethylcysteine was observed in hydrolysates of taipoxin that had been treated with iodoacetate in 6 M urea without prior reduction, indicating the absence of free −SH groups. Apparently all of the cysteine is present in disulfide form.

Toxicity of Whole Taipoxin

Taipoxin has an LD_{50} of 2 µg/kg mouse. At doses near the LD_{50} symptoms of intoxication appear about 2 h following the injection and begin with flaccid paralysis of the hind limbs. At this stage the animals typically lie with the back legs splayed out caudally. The paralysis then proceeds to other muscle groups, finally reaching the respiratory musculature and thereby causing death by asphyxia within 10–15 h. At higher dose levels the symptoms appear sooner and progress faster to the final stage. Mice that survive appear to be completely normal after a few days, indicating that sublethal intoxication is reversible. The dose-response curve for taipoxin is bilogarithmic [28].

The toxicity data for taipoxin and its subunits are given in Table 3, along with some physicochemical parameters.

The lethality of fraction III (Fig. 1) is due to 'phospholipase' components which are related to, but distinctly different from the α and β subunits of taipoxin.

Separation and Properties of the Subunits

At acid pH taipoxin dissociates and three distinct subunits designated α, β, and γ are separable by column zone electrophoresis at pH 1.9 (Fig. 4). The fourth peak with a maximum at 37 ml between the β and γ components represents undissociated αγ complex, as evidenced both by amino acid composition data and other results described below. The 'sluggish' dissociation of the αγ complex during the electrophoresis is also evidenced by the presence of some α component in the β peak. A more serious drawback of this separation system is that the prolonged exposure to pH 1.9 affects the γ subunit in some way such that the subunits will not recombine to form the taipoxin complex.

Taipoxin dissociates completely in 6 M guanidine hydrochloride, and the γ component can be separated quantitatively from the α and β components by gel filtration on Sepharose 6 B in neutral solutions of 6 M guanidine-HCl, as shown in Fig. 5. Following removal of the guanidine by dialysis, the α and β components can be separated by electrophoresis at pH 1.9, and there is no trace of either the γ component or the residual αγ complex observed in the run with whole taipoxin

Table 2. *Amino acid and carbohydrate composition of taipoxin and its α, β, and γ subunits*

Amino acid	α	β_1	β_2	γ	$\alpha + \frac{\beta_1 + \beta_2}{2} + \gamma$	Taipoxin
Asparagine/aspartate	13.02 13	13.98 14	13.90 14	22.45 22	49	50
Threonine	6.02 6	6.04 6	7.00 7	7.05 7	19.5	20
Serine	5.19 5	4.39 4	2.89 3	8.87 9	17.5	19
Glutamine/glutamate	7.71 8	13.55 14	12.60 13	14.73 15	36.5	35
Proline	4.86 5	4.81 5	4.19 4	6.76 7	16.5	16
Glycine	9.42 9	9.20 9	8.98 9	10.63 11	29	29
Alanine	8.99 9	10.16 10	9.98 10	8.35 8	27	28
Cysteine	13.85 14	13.39 14	14.10 14	15.92 16	44	42
Valine	4.74 5	5.93 6	5.96 6	2.02 2	13	12
Methionine	1.96 2	2.35 2	1.70 2	2.68 3	7	8
Isoleucine	2.07 2	3.10 3	2.98 3	6.83 7	12	13
Leucine	4.01 4	4.52 5	6.87 7	7.05 7	17	17
Tyrosine	7.80 8	6.57 7	5.64 6	7.90 8	22.5	21
Phenylalanine	4.67 5	4.69 5	4.89 5	4.02 4	14	12
Histidine	1.93 2	3.10 3	3.72 4	1.84 2	7.5	7
Lysine	6.20 6	5.97 6	5.26 5	4.86 5	16.5	16
Tryptophen	3	1	1	0	4	5
Arginine	12.70 13	6.12 6	7.00 7	2.10 2	21.5	22
Total residues	119	120	120	135	374	372
Formula weight	13750	13457	13473	18354	45569	
Carbohydrate						
Fucose	0	0	0	0.9 1		1.1
Manose	0	0	0	2.2 2		2.2
N-Acetyl-D-glucosamine	0	0	0	4.2 5		4.3
Galactose	0	0	0	3.5 4		3.7
N-Acetyl-neuraminic acid	0	0	0	3.8 4		2.5
$\bar{v}$ (calculated)						0.70 ml/g

Table 3. *Physicochemical and toxicity data for taipoxin and its α, β, and γ subunits*
The number of amino acid residues was estimated by molecular sieve chromatography of reduced and *S*-carboxymethylated derivatives on Sepharose 6B in 6 M guanidine hydrochloride. The apparent molecular weight was estimated by molecular sieve chromatography of native proteins on Sephadex G-75 and by the analytical ultracentrifuge

Parameter	Taipoxin	α	β	γ
Number of amino acid residues	–	115	115	167 (142)[a]
Apparent molecular weight				
by molecular sieve chromatography	46700	14600	16300	26900
by analytical ultracentrifuge	46800	–	–	–
Molar absorption at 278 nm	63000	31300	15000	11000[b]
$A_{278}^{1\,mg/ml}$	1.5	2.3	1.1	0.67[b]
LD_{50} µg/kg mouse	2.1	300	>2000	>2000

[a] After prolonged exposure to pH 1.9 (partial removal of carbohydrate moiety).
[b] At 276 nm.

(Fig. 4). Likewise, the 'γ peak' obtained in the guanidine-Sepharose 6B separation is electrophoretically homogeneous at pH 1.9. The electrophoretically isolated β component is a mixture of two isoforms (β_1 and β_2) which were separated by ion exchange chromatography on sulfopropyl-Sephadex as shown in Fig. 6.

Re-formation of Taipoxin

The separation shown in Fig. 5 is completely reversible. The two peaks were pooled together and dialyzed against several changes of 0.1 M *N*-ethylmorpholine/acetic acid buffer, pH 7.4, for 30 h in the cold to remove the guanidine and lyophilized. The re-

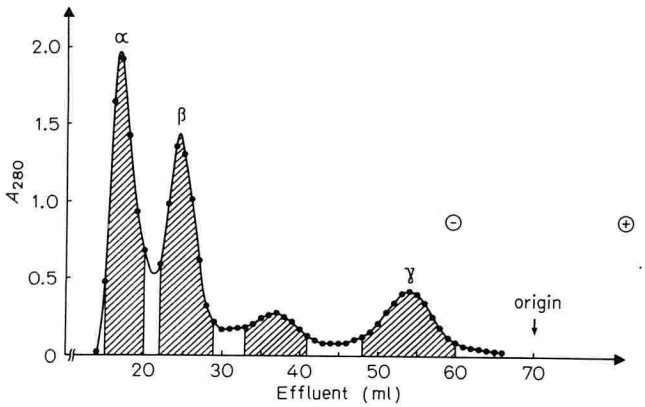

Fig. 4. *Column zone electrophoresis of 12.7 mg taipoxin on a 1 × 99-cm cellulose column equilibrated with 7.8% acetic acid, 2.5% formic acid (v/v), pH 1.9.* The sample was dissolved in 0.5 ml 3.9% acetic acid, 1.25% formic acid and displaced downward 5 ml. The run was done for 12.5 h at 1000 V, 14.5 mA, 10–12 °C. The arrow indicates the starting position

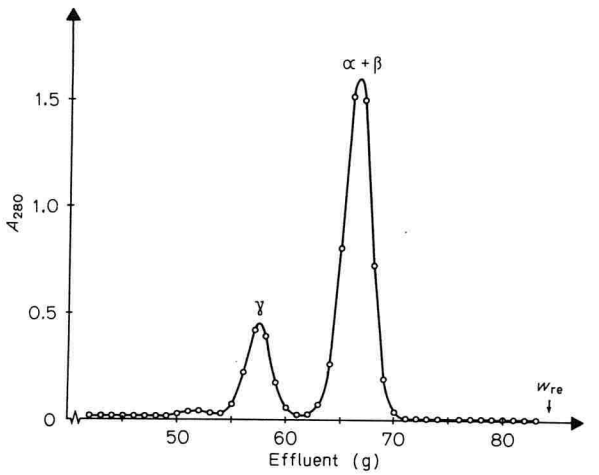

Fig. 5. *Gel filtration of 4.5 mg unreduced taipoxin on a 97 × 1.03-cm column of Sepharose 6B in 6 M guanidine hydrochloride.* The sample was applied in 0.2 ml of the same medium and elution was done at a flow rate of 2 ml × h^{-1}

dissolved material was identical to the original taipoxin with regard to elution position on Sephadex G-75 at neutral pH and toxicity.

Estimation of Peptide Chain Length

To estimate the lengths of the polypeptide chains, the completely reduced and *S*-carboxymethylated derivatives of taipoxin and its separated subunits were subjected to molecular-sieve chromatography on Sepharose 6B in 6 M guanidine hydrochloride according to the method of Fish *et al.* [14]. The calibration data for the column are given in Table 4. Since all of the subunits of taipoxin are rich in half-cystine, the use of iodo[^{14}C]acetate for the alkylation permitted peak detection at the picomolar level.

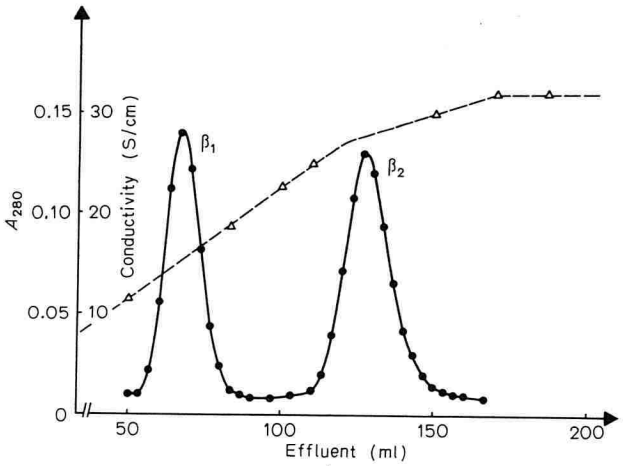

Fig. 6. *Ion-exchange chromatography on a 3 × 2-cm sulfopropyl-Sephadex column at pH 5.0 of 4.3 mg fraction β from the column electrophoresis at pH 1.9 (Fig. 4).* The sample was applied in 2 ml 0.1 M ammonium acetate, pH 5.0 to the column, equilibrated with the same buffer. A 150-ml linear gradient of 0.1–1.0 M ammonium acetate at pH 5.0 was run. (●——●) A_{280}; (Δ----Δ) conductivity

Table 4. *Calibration data for 1.03 × 97.8-cm analytical Sepharose 6B column in 6 M guanidine hydrochloride*
w_e = elution weight, K_D is defined in the Methods section, and n is the number of amino acid residues in the peptide chain. Equation of data: $K_D^{1/3} = -0.014 \times n^{0.555} + 0.993$

Substance	w_e	K_D	$K_D^{1/3}$	n	$n^{0.555}$
	%				
Blue dextran	27.4	0			
Bovine serum albumin	34.9	0.133	0.511	579	34.1
Chymotrypsinogen A	46.1	0.332	0.693	245	21.2
Ribonuclease A	54.0	0.473	0.779	124	14.5
N.n. naja neurotoxin 4	61.9	0.613	0.849	71	10.7
Excess reagent	83.7	1			

The elution pattern observed with the reduced and *S*-carboxymethylated taipoxin looked just like that shown in Fig. 5 for the unreduced protein except that both peaks were of course less retarded. The earlier-eluting γ peak and the later-eluting peak containing both the α and β chains represented K_D values of 0.423 and 0.504, corresponding to chain lengths of 167 and 115 residues, respectively, as estimated from the calibration data. Prolonged exposure of the γ subunit to pH 1.9 reduced the apparent chain as determined in the above fashion to 142 residues, probably owing to partial loss of the carbohydrate moiety.

Chemical Composition of Subunits

The amino acid and carbohydrate composition data for the individual subunits are given in Table 2.

The α and β subunits appear to contain 119–120 residues, in good agreement with the estimate made by molecular-sieve chromatography of the reduced and *S*-carboxymethylated derivatives in 6 M guanidine hydrochloride, and each contains 14 half-cystine residues apparently forming seven disulfide bridges.

The γ subunit has a longer peptide chain (135 residues) and an eighth disulfide bridge (16 half-cystine residues), and also contains all of the carbohydrate present in the taipoxin complex. The formula weight of 18354 given in Table 2 for the γ subunit is only two-thirds of the apparent molecular weight (26900) estimated by molecular sieve chromatography of the intact (*i.e.*, unreduced) γ subunit in neutral ammonium acetate. The separation shown in Fig. 5 for the unreduced taipoxin in 6 M guanidine hydrochloride would indicate that the γ subunit is about twice the size of the α and β components. Since for reasons discussed below we believe that the γ subunit, despite its greater chain length, is structurally homologous to the α and β subunits, we attribute the early elution of the γ subunit to the eccentricity conferred by the large carbohydrate moiety. The carbohydrate also contributes to the apparent length of the random coil of the reduced and *S*-carboxymethylated derivative in 6 M guanidine hydrochloride, as estimated by a procedure which is strictly valid only for unbranched chains [14].

The most striking difference among the subunits is in their charge. The α subunit contains 13 residues of arginine and is extremely basic. It precipitates near the cathode upon isoelectric focusing in 3–10 Ampholine and probably has an isoelectric point above pH 10. The β_1 and β_2 isosubunits are isoelectric at about pH 7, and are very poorly soluble at pH 6–9 and perhaps even higher. The γ subunit is very acidic with an isoelectric point of about 2.5, owing in part to its four sialic acid residues.

The extreme charge difference between the α and γ subunits rules out any possibility that taipoxin might be some sort of mixture rather than a definite complex. At pH 7.5, where whole taipoxin is moderately anodic (Fig. 2), the α subunit is strongly cathodic, the γ subunit is strongly anodic, and the β subunits are insoluble.

Alternate Modes of Dissociation of Taipoxin and the Stoichiometry of the Complex

Attempts to separate the subunits of taipoxin under various conditions have revealed something about the manner in which the α, β, and γ subunits interact with each other. When taipoxin was gel-filtered on Sephadex G-75 at pH 3 in 1 M propionic acid or 0.1 M acetic acid the β subunits dissociated off and eluted in a separate peak behind the peak of αγ complex. The separation was never really 'clean'

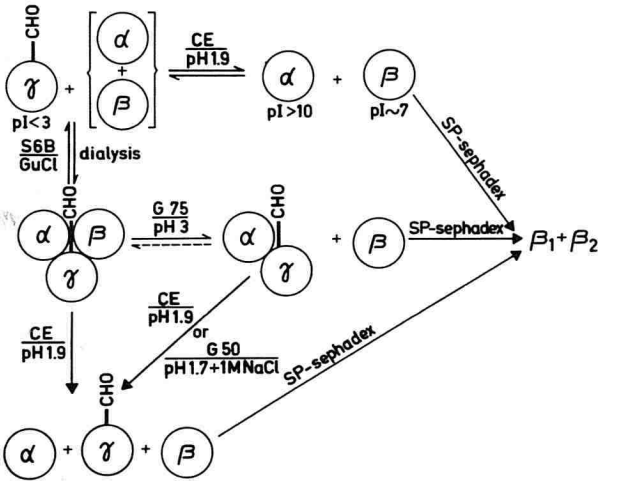

Fig. 7. *Summary of separation of the taipoxin subunits.* CE = column electrophoresis; GuCl = 6 M guanidine hydrochloride; S 6B = Sepharose 6B; G-50, G-75 = Sephadex G-50, G-75; SP-Sephadex = sulfopropyl-Sephadex

because α also dissociates off to some extent and interacts with β, and both γ and αβ elute between αγ and β. At pH 3 the γ subunit is reasonably stable and the dissociation of the complex is therefore partly reversible upon raising the pH.

In an earlier report [7] on the nature of taipoxin, the earlier-eluting (*i.e.*, αγ) of the two peaks obtained by gel filtration of taipoxin at pH 3 was called the α fraction, and the subunits which we now call α and γ were separated only in the form of their reduced and *S*-carboxymethylated derivatives, which were designated α_p and α_s, respectively.

In the formic acid/acetic acid buffer at pH 1.9 the separation by gel filtration is no better than that obtained at pH 3, and is moreover essentially irreversible. Apparently the strong α-γ interaction is partly electrostatic, since the inclusion of salt in the pH-1.9 buffer dissociates the complex completely, and gel filtration on Sephadex G-50 in 1 M NaCl at pH 1.9 thus affords the same separation as was obtained in 6 M guanidine hydrochloride (Fig. 5).

The α and β subunits are the same size and cannot be separated from each other by molecular sieve chromatography. Attempts to separate the α and β subunits from each other by cation-exchange chromatography or electrophoresis at pH 5 or by electrophoresis at pH 7 in 6 M urea have revealed that they interact quite strongly. In fact, the only method that we have found so far for the separation of α and β is electrophoresis at pH 1.9, and even this does not work well at high sample concentrations. In Fig. 7 we illustrate very schematically the various means by which we have been able to separate the subunits of taipoxin. Since we do not yet know whether β interacts directly with γ or whether it is held in the complex only by interaction with the α subunit, no significance

	-8	-7	-6	-5	-4	-3	-2	-1	1	2	3	4	5	6	7	8	9	10	11
Notexin									Asn	Leu	Val	Gln	Phe	Ser	Tyr	Leu	Ile	Gln	Cys
N. nig. Plase									Asn	Leu	Tyr	Gln	Phe	Lys	Asn	Met	Ile	His	Cys
Taipoxin α									Asn	Leu	Leu	Gln	Phe	Gly	Phe	Met	Ile	Arg	Cys
Taipoxin β									Asn	Leu	Val	Gln	Phe	Gly	Phe	Met	Ile	Gln	Cys
Taipoxin γ	Ser	Glu	Leu	Pro	Gln	Pro	Ser	Ile	Asp	Phe	Glu	Gln	Phe	Ser	Asn	Met	Ile	Gln	Cys
Pork ProPLase		Glu	Glu	Gly	Ile	Ser	Ser	Arg	Ala	Leu	Trp	Gln	Phe	Arg	Ser	Met	Ile	Lys	Cys

Fig. 8. *Amino-terminal sequences of the three taipoxin polypeptide chains showing homology with notexin [29], a basic phospholipase from* Naja nigricollis *[30] and pancreatic prophospholipase A [31]*

Table 5. *Stoichiometry of the taipoxin complex*

Manner of separation and analysis	Proportion of		
	α	β	γ
1. Column zone electrophoresis at pH 1.9 (Fig. 4) and total amino acid analysis	0.89	1.27[a]	1.00
2. Separation on Sephadex 6B in 6 M GuCl of reduced and *S*-[^{14}C]carboxymethylated derivatives. Calculated from counting data and known cysteine content		2.01	1.00
3. Separation on Sepharose 6B in 6 M GuCl of unreduced taipoxin (Fig. 5) and total amino acid analysis after dialysis		1.86	1.00
4. Separation on Sephadex G-50 in 1 M NaCl at pH 1.9 and ninhydrin color following alkaline hydrolysis of effluent fractions		1.86	1.00

[a] High value reflects some 'trailing' of α into the β peak (see Fig. 4).

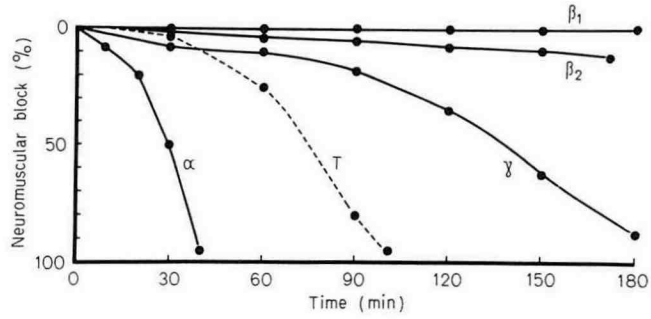

Fig. 9. *Curves showing the time course of the neuromuscular block caused in the phrenic-hemidiaphragm preparation by taipoxin (T) at 5 μg/ml and the polypeptide chains α and γ at a concentration of 50 and 100 μg/ml respectively.* The iso-chains β_1 and β_2 failed to block neuromuscular transmission at a concentration of 50 μg/ml

should be attached to the manner in which we have represented the ternary complex. However, we suspect that the carbohydrate of the γ subunit might play some direct role in the α-γ interaction.

The stoichiometry of the taipoxin complex, as determined in four different systems in which the total recovery of protein was quantitative, is summarized in Table 5. The γ, α and β subunits are clearly present in the ratio 1:1:1.

Amino-Terminal Sequences

The amino-terminal sequences of the α, β, and γ subunits as determined by 11, 11, and 19 consecutive cycles of Edman degradation, respectively, on the reduced and *S*-carboxymethylated derivatives are compared in Fig. 8 with the amino-terminal sequences of notexin from the venom of the Australian tiger snake [29], a basic phospholipase A from *N. nigricollis* venom (T. K. Obidairo, S. Tampitag & D. Eaker, unpublished results) and the proenzyme of porcine pancreatic phospholipase A_2 [31]. Residue 10 in the α subunit was not identified with complete confidence but appears to be arginine. The sequences of β_1 and β_2 are identical at least through residue 11.

The six sequences are clearly homologous, showing identity in four of eleven positions, and residues 1, 2, and 8 are highly conserved. According to the data in Table 2 the γ subunit is 15–16 residues longer than the α and β subunits, and eight of these 'extra' residues seem to be accounted for at the amino-terminal end. This amino-terminal 'tail' shows some similarity to the activation peptide of the porcine pancreatic proenzyme and might even have a related function.

Neurophysiological Characteristics

Taipoxin causes an irreversible block of neuromuscular transmission beginning after a latent period, the length of which is inversely related to the toxin concentration and the rate of nerve excitation [28]. Fig. 9 illustrates the time course of the neuromuscular block in the mouse phrenic-hemidiaphragm preparation stimulated at 0.1 Hz caused by whole taipoxin (T) and its constituent subunits. At a concentration of 5 μg/ml whole taipoxin causes a complete block in about 100 min. At 10-fold higher concentration the α subunit produces a complete block in about 40 min, and at 20-fold higher concentration the γ subunit blocks in about 3 h. The β subunits are essentially inactive at 50 μg/ml. The fact that the α subunit is the only one showing appreciable blocking activity at the nerve terminals of the hemidiaphragm preparation

correlates with the observation that it is also the only one of the subunits which is highly lethal.

DISCUSSION

The elution position and mouse assay activity of venom fraction IV (Fig. 1) suggest that this fraction contains postsynaptic curarimimetic neurotoxins of the type(s) found in all other elapid and hydrophid venoms examined so far. The presence of such toxins was expected on the basis of Minton's observation [5] that taipan antiserum effectively neutralized all of the cobra venoms tested. However, the content of postsynaptic toxins cannot exceed 5% of the total venom protein. The genes for the postsynaptic toxins thus persist and are expressed despite the emergence of the considerably more potent weapon taipoxin.

Taipoxin is isolated in at least 95% pure form simply by gel filtration of the crude venom on Sephadex G-75 in neutral ammonium acetate. It is important to point out here that taipoxin cannot be isolated by an ordinary ion-exchange chromatography, since either cation or anion exchangers dissociate the complex. The column electrophoretic step (Fig. 2) removes traces of 'impurities' some of which might in fact be dissociated subunits. The most difficult tasks in the characterization of taipoxin were: (a) to establish the stoichiometry of the complex, and (b) to accomplish the subunit separation in such a way that the separated units could recombine to form the fully active complex.

Problem (a) has been a difficult one because, firstly, we have not arrived at any means to resolve the subunits completely in a single run, and secondly, the presence of the carbohydrate branch(es) in the γ subunit introduced a large uncertainty in the estimation of the length of the peptide chain. Since the present estimate of 135 residues is probably correct to within 1 or 2 residues the carbohydrate moiety(ies) contribute(s) about 30 residues to the apparent peptide chain length as estimated by the method of Fish *et al.* [14].

With regard to problem (b), considerable trial-and-error effort was expended before we found any means to separate γ and α that did not involve prolonged exposure to quite low pH, which destroys the integrity of the γ subunit and prevents re-formation of the taipoxin complex. The acid-induced damage diminishes the apparent chain length of the molecule as estimated by the method of Fish *et al.* [14], and since the amino acid composition is not affected we believe that the damage involves the carbohydrate moiety(ies). At any rate, a completely reversible separation of the γ subunit from the α and β components was finally accomplished by gel filtration of taipoxin in moderately acidic (pH 5–6) solutions of 6 M guanidine hydrochloride.

The quantitative data presented in Tables 2 and 5 clearly indicate that taipoxin is a stoichiometric ternary complex in which each of the three types of subunits makes some important, albeit as yet unknown, contribution to the manifestation of the very high lethal activity. Although each subunit has its own special properties, they appear to be homologous in structure to each other and to all other phospholipase A enzymes of vertebrate origin for which sequence data are available [32], particularly those from elapid venoms [33]. The α subunit is very basic and is the only one of the subunits which is appreciably toxic on its own. In both respect the α subunit thus resembles notexin [6, 29, 34]. The γ subunit is very acidic, owing at least in part to its four sialic acid residues, and the integrity of the carbohydrate moiety(ies) may be necessary for the formation of the complex. The neutral β subunit is represented by at least two very similar iso forms which we have designated β_1 and β_2, and which appear to be completely interchangeable in the complex.

The inhomogeneity of the β subunit, together with some small amount of dissociation, probably accounts for the four bands observed in the isoelectric focusing of taipoxin in polyacrylamide gels. Attempts to perform preparative isoelectric focusing in sucrose gradient or in columns of cellulose powder have revealed that taipoxin is slightly dissociated even in the vicinity of its isoelectric pH ($\approx$ pH 5). Because γ and $\alpha\beta$ move rapidly in opposite directions the dissociation eventually goes to completion, leaving no complex at the pH-5 position.

The neuromuscular block produced by taipoxin has been studied in detail by both electrophysiological [28] and electron-microscopic techniques [35]. Kamenskaya and Thesleff [28] showed that the block of neuromuscular transmission was pre-synaptic and was characterized by gradual reduction to complete stop of transmitter release from the motor nerve terminals. The ultrastructural studies [35] showed that the poisoned terminals were depleted of vesicles and that the degree of depletion paralleled the reduction of transmitter release. Completely blocked terminals were devoid of vesicles and were instead filled with granular material. The entire neurolemma was covered with omega-shaped indentations having the appearance of vesicles frozen in a state of fusion with the terminal membrane either in the process of release or reformation.

These observations suggest that taipoxin does not interfere with the transmitter release mechanism itself, since the release of acetylcholine from preexisting vesicles (*i.e.*, those already present in the nerve terminal before the toxin is introduced) appears to proceed in normal fashion even after the toxin has become irreversibly 'fixed' to the nerve terminal. Possibly the toxin, after being fixed to the nerve

membrane, enters the terminal as a result of the endocytotic process connected with the re-formation of synaptic vesicles, making the rate of toxin entry proportional to the frequency of transmitter release [36]. Once inside the terminal the toxin could hydrolyze the membrane of synaptic vesicles, granular material being formed in the process. Transmitter release ceases altogether when the supply of functional vesicles has been depleted. Taipoxin does not accelerate spontaneous release of transmitter except in the final stages of poisoning immediately prior to complete shutdown.

Some other protein toxins of snake venom origin which affect transmitter release from cholinergic nerve terminals are crotoxin from the venom of the South American rattlesnake *Crotalus durissus terrificus*, β-bungarotoxin from the venom of the many-banded krait *Bungarus multicinctus*, and notexin from the venom of the Australian tiger snake *Notechis scutatus scutatus*.

Although crotoxin acts mainly on the nerve terminal, it appears to have some postsynaptic action as well [37]. However, crotoxin is not a single substance but is rather a non-stoichiometric 'mosaic' of several constituents of which the proportions vary from one preparation to another [32,34,39], and the post-synaptic effects might be due to different substances than those affecting transmitter release. The reasonably high lethality and presynaptic toxicity of crotoxin is apparently due to a specific complex between a basic phospholipase A with a molecular weight of about 16300 and a smaller acidic protein called crotapotin with a molecular weight of about 9500 [39]. The basic phospholipase itself is slightly toxic, but crotapotin alone shows neither toxicity nor enzymatic activity.

β-Bungarotoxin [40] appears to be exclusively presynaptic in action [41], but like crotoxin and in contrast to both notexin and taipoxin it does accelerate spontaneous transmitter release initially, producing 'bursts' of miniature end plate potentials. β-Bungarotoxin is said to be composed of two subunits with molecular weights of 12400 and 8800 [42], and since it does have phospholipase A activity [43,44] which is greatly enhanced in the presence of deoxycholate [45] we assume that the larger subunit must be a basic phospholipase A related to all the others described above.

Given that these four presynaptic neurotoxins either are, or contain as an indispensable part, a very basic phospholipase A, the question is whether the catalytic activity plays any direct role in the neurotoxic action. It is tempting to propose mechanisms that involve the phospholipase A activity, since either the fatty acid or the lysolecithin liberated by the action of phospholipase A on 3-*sn*-phosphoglycerides can disrupt the function and/or structural integrity of elements (*e.g.*, mitochondria, vesicles, nerve-terminal membrane) that are certainly important at some stage for the release or re-formation of transmitter. Wernicke *et al.* [43] and Howard [44] have proposed that the presynaptic neurotoxicity of β-bungarotoxin is due to the liberation of long-chain fatty acids which depress mitochondrial oxidative phosphorylation, thereby abolishing the energy supply of the nerve terminal. Strong *et al.* [45] have suggested that the effects of β-bungarotoxin on transmitter release might be due to local liberation of lysophosphatides which perturb in some way the fusion of vesicles with the terminal membrane.

Indirect evidence for the involvement of the phospholipase A activity in the presynaptic action of these 'phospholipase A' type neurotoxins has been obtained with β-bungarotoxin [45] and notexin [46]. Making use of the fact that the calcium requirement for synaptic transmission at the myoneural junction can be satisfied by strontium, whereas the calcium requirement for the phospholipase activity could not, Strong *et al.* [45] were able to show that β-bungarotoxin had no effect on transmitter release in the absence of calcium, *i.e.*, in the absence of catalytic capability.

Halpert *et al.* [46] reduced both the catalytic activity and lethality of notexin about 500-fold by specific chemical modification of an apparently essential histidine with *p*-bromophenacyl bromide, a reaction originally demonstrated by Volverk *et al.* [47] with the porcine pancreatic enzyme. Some caution is warranted in the evaluation of these two experiments because they might not be as complementary or independent as they at first seem. The histidine residue modified by *p*-bromophenacyl bromide appears to be near one of the ligands for the binding of the essential calcium ion [47], and although calcium is certainly required for catalytic activity it has not been proved that the bound calcium plays any direct role in the catalytic process. The effect of calcium might rather be to produce or permit a necessary conformational change in the protein, and the absence of calcium or the lack of ability to bind it properly almost certainly has important structural consequences.

We stress this point because a highly specific binding to some structure in the nerve terminal must be invoked to account for the potency of these presynaptic neurotoxins even if the catalytic activity is the ultimate cause of the damage, and there is no reason to suppose that the target for such binding should be the substrate itself. The recognition site or binding site for the attachment of the toxin to the nerve terminal membrane might thus be independent of the catalytic site but still dependent on calcium for its structural integrity. If two sites are involved we can be sure in the case of notexin that both can reside

on the same molecule because notexin is unquestionably a single peptide chain [29].

The roles of crotapotin and the γ and β subunits of taipoxin in the potentiation of the neurotoxic activities of the basic phospholipase constituents of crotoxin and taipoxin, respectively, remain to be elucidated. Since notexin is self-sufficient there is no reason to suppose that such potentiators, when present, have any important function at the target level. Probably they function as 'chaperones', sharpening the specificity and increasing the stability of the toxic protein so as to minimize distraction and destruction *en route* to the proper site of action.

We thank Mr Sven Centring and Mr Ragnar Thorzelius for expert technical assistance with the amino acid analyzers, Dr Hans Bennich at the Uppsala University Biomedical Centre for carbohydrate analysis, Dr Lilly Gustafsson at the Analytical Department of the Institute of Chemistry of Uppsala University for performing the metal analyses, Mr Gösta Forsling for valuable help with the figures and Miss Pia Carlsson for skilful technical assistance with isoelectric focusing. Dr Lars Rydén is acknowledged for valuable criticism and stimulating discussions. This work was partly financed by the Swedish Natural Science Research Council, dnr. 2859-008.

REFERENCES

1. Garnet, J. R. (1968) *Venomous Australian Animals Dangerous to Man*, p. 66, The Commonwealth Serum Laboratories, Melbourne.
2. Reid, C. D. & Flecker, H. (1950) *Med. J. Aust. 1*, 82–146.
3. Campbell, C. H. (1967) *Med. J. Aust. 1*, 735–738.
4. Trinca, J. C. (1969) *Med. J. Aust. 1*, 514–516.
5. Minton, S. A. (1967) *Toxicon, 5*, 47–55.
6. Karlsson, E., Eaker, D. & Rydén, L. (1972) *Toxicon, 10*, 405–413.
7. Eaker, D., Halpert, J., Fohlman, J. & Karlsson, E. (1976) in *Animal, Plant and Microbial Toxins*, vol. 2, Ohsaka, A., ed., pp. 27–45, Plenum, New York.
8. Porath, J. (1956) *Biochim. Biophys. Acta, 22*, 151–175.
9. Hjertén, S. (1959) *Biochim. Biophys. Acta, 32*, 531–534.
10. Arnberg, H., Eaker, D., Fryklund, L. & Karlsson, E. (1974) *Biochim. Biophys. Acta, 359*, 222–232.
11. Dayhoff, M. O. (1969) *Atlas of Protein Sequence and Structure*, vol. 5, Silver Spring: National Biomedical Research Foundation.
12. Karlsson, E., Arnberg, H. & Eaker, D. (1971) *Eur. J. Biochem. 21*, 1–16.
13. Andrews, P. (1970) in *Methods of Biochemical Analysis* (Glick, D., ed.) pp. 1–53, John Wiley & Sons, New York.
14. Fish, W. W., Mann, K. G. & Tanford, C. (1969) *J. Biol. Chem. 244*, 4989–4994.
15. Anderson, L. E. & McClure, W. O. (1973) *Anal. Biochem. 51*, 173–179.
16. Chervenka, C. H. (1970) *Anal. Biochem. 34*, 24–29.
17. Spackman, D. H., Stein, W. H. & Moore, S. (1958) *Anal. Chem. 30*, 1190–1206.
18. Vesterberg, O. (1972) *Biochim. Biophys. Acta, 257*, 11–19.
19. Iwanaga, S., Wallén, P., Gröndahl, N. J., Henschen, A. & Blombäck, B. (1969) *Eur. J. Biochem. 8*, 189–199.
20. Jeppson, J.-O. & Sjöquist, J. (1967) *Anal. Biochem. 18*, 264–269.
21. Brenner, M., Niederweiser, A. & Pataki, G. (1962) in *Dünnschichtchromatographie* (Stahl, E., ed.) pp. 443–445, Springer-Verlag, West Berlin.
22. Edman, P. (1970) in *Protein Sequence Determination* (Needleman, S. B., ed.) pp. 211–255, Springer-Verlag, West Berlin
23. Clamp, J. R., Bhatti, T. & Chambers, R. E. (1972) *The Examination of Carbohydrates in Glycoproteins by Gas-Liquid Chromatography* in *Glycoproteins* (Gottschalk, A., ed.) pp. 300–380, Elsevier Publishing Co., Amsterdam.
24. Maurer, H. R. (1971) in *Disc Electrophoresis*, pp. 44–45, Walter de Gruyter, Berlin.
25. Chrambach, A., Reisfeld, R. A., Wychoff, M. & Zaccari, J. (1967) *Anal. Biochem. 20*, 150–154.
26. Reed, L. J. & Muench, H. (1938) *Am. J. Hyg. 27*, 493–497.
27. Andersson, L. (1971) *Coagulation, 4*, 309–317.
28. Kamenskaya, M. A. & Thesleff, S. (1974) *Acta Physiol. Scand. 90*, 716–724.
29. Halpert, J. & Eaker, D. (1975) *J. Biol. Chem. 250*, 6990–6997.
30. Reference deleted.
31. De Haas, G. H., Slotboom, A. J., Bonsen, P. P. M., van Deenen, L. L. M., Maroux, S., Puigserver, A. Y., Desnuelle, P. (1970) *Biochim. Biophys. Acta, 221*, 31–53.
32. Breithaupt, H., Omori-Satoh, T. & Lang, J. (1975) *Biochim. Biophys. Acta, 403*, 355–369.
33. Eaker, D. (1975) in *Peptides: Chemistry, Structure, and Biology* (Walter, R. & Meienhofer, J., eds) pp. 17–30, Ann Arbor Publishers, Ann Arbor.
34. Harris, J. B., Karlsson, E. & Thesleff, S. (1973) *J. Pharmacol. Exp. Ther. 184*, 339–345.
35. Cull-Candy, S. G., Fohlman, J., Gustafsson, D., Lüllmann-Rauch, R. & Thesleff, S. (1976) *Neuroscience 1*, 175–180.
36. Heuser, J. & Reese, T. S. (1973) *J. Cell Biol. 54*, 315–344.
37. Brazil, O. V. (1972) *J. Formosan Med. Assoc. 71*, 394–400.
38. Hendon, R. A. & Fraenkel-Conrat, H. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 1560–1563.
39. Breithaupt, A. H., Rübsamen, K. & Habermann, E. (1974) *Eur. J. Biochem. 49*, 333–345.
40. Lee, C. Y., Chang, S. L., Kau, S. T. & Luh, S. H. (1972) *J. Chromatog. 72*, 71–82.
41. Chang, C. C., Chen, T. F. & Lee, C. Y. (1973) *J. Pharmacol. Exp. Ther. 184*, 339–345.
42. Kelly, R. B. & Brown, F. R. (1974) *J. Neurobiol. 5*, 135–150.
43. Wernicke, J. F., Vanker, A. D. & Howard, B. D. (1975) *J. Neurochem. 25*, 483–496.
44. Howard, B. D. (1975) *Biochem. Biophys. Res. Commun. 67*, 58–65.
45. Strong, P. N., Goerke, J., Oberg, S. G. & Kelly, R. B. (1976) *Proc. Natl Acad. Sci. U.S.A. 73*, 178–182.
46. Halpert, J., Eaker, D. & Karlsson, E. (1976) *FEBS Lett. 61*, 72–76.
47. Volwerk, J. J., Pieterson, W. A. & de Haas, G. H. (1974) *Biochemistry, 13*, 1446–1454.

J. Fohlman, D. Eaker, and E. Karlsson, Institutionen för Naturvetenskaplig Biokemi, Universitetet i Uppsala, Box 576, S-75123 Uppsala, Sweden

S. Thesleff, Farmakologiska Institutionen, Lunds Universitet, Sölvesgatan 10, S-22362 Lund, Sweden

Copyright of European Journal of Biochemistry is the property of Blackwell Publishing Limited and its content may not be copied or emailed to multiple sites or posted to a listserv without the copyright holder's express written permission. However, users may print, download, or email articles for individual use.