(1977) J. Biol. Chem. 252, 7440–7443.
Watterson, D. M., Harrelson, W. G., Keller, P. M., Sharief, F., & Vanaman, T. C. (1976) J. Biol. Chem. 251, 4501–4513.
Watterson, D. M., Sharief, F., & Vanaman, T. C. (1980) J. Biol. Chem. 255, 962–971.
Weber, K., & Osborne, M. (1969) J. Biol. Chem. 244, 4406–4412.

Purification and Pharmacological Properties of Eight Sea Anemone Toxins from *Anemonia sulcata*, *Anthopleura xanthogrammica*, *Stoichactis giganteus*, and *Actinodendron plumosum*†

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ABSTRACT: Eight different polypeptide toxins from sea anemones of four different origins (*Anemonia sulcata*, *Anthopleura xanthogrammica*, *Stoichactis giganteus*, and *Actinodendron plumosum*) have been studied. Three of these toxins are new; the purification procedure for the five other ones has been improved. Sea anemone toxins were assayed (i) for their toxicity to crabs and mice, (ii) for their affinity for the specific sea anemone toxin receptor situated on the Na^+ channels of rat brain synaptosomes, and (iii) for their capacity to increase, in synergy with veratridine, the rate of $^{22}Na^+$ entry into neuroblastoma cells via the Na^+ channel. Some of the toxins are more active on crustaceans, whereas others are more toxic to mammals. A very good correlation exists between the toxic activity to mice, the affinity of the toxin for the Na^+ channel in rat brain synaptosomes, and the stimulating effect on $^{22}Na^+$ uptake by neuroblastoma cells. The observation has also been made that the most cationic toxins are also the most active on mammals and the least active on crustaceans. Toxicities (LD_{50}) to mice of the most active sea anemone toxins and of the most active scorpion toxins are similar, and sea anemone toxins at high enough concentrations prevent binding of scorpion toxins to their receptor. However, scorpion toxins have affinities for the Na^+ channel which are ~60 times higher than those found for the most active sea anemone toxins. Three sea anemone toxins appear to be more interesting than toxin II from *A. sulcata* (the "classical" sea anemone toxin) for studies of the Na^+ channel structure and mechanism when the source of the channel is of a mammalian origin. Two of these three toxins can be radiolabeled with iodine while retaining their toxic activity; they appear to be useful tools for future biochemical studies of the Na^+ channel.

Sea anemone toxins are among the most interesting tools for analysis of the properties of the voltage-dependent Na^+ channel. The sequences of five of these polypeptide toxins are currently available (Wunderer et al., 1976a; Wunderer & Eulitz, 1978; Béress et al., 1977; Martinez et al., 1977; Tanaka et al., 1977; Norton et al., 1978) and the structure–function relationships of toxin II from *Anemonia sulcata* (AS_{II})[1] have been recently established (Barhanin et al., 1981). Sea anemone toxins selectively slow down the closing (inactivation) of the Na^+ channel. They do not interfere with the binding of tetrodotoxin or saxitoxin near the selectivity filter of the Na^+ channel (Romey et al., 1976; Jacques et al., 1978). Their binding site is different from those of other toxins which also act on the gating system of the Na^+ channel like veratridine, batrachotoxin, aconitine, grayanotoxin, and pyrethroids (Vincent et al., 1980). Sea anemone toxins have been shown to interact with a large variety of excitable membranes including myelinated and nonmyelinated axons (Rathmayer & Béress, 1976; Romey et al., 1976; Bergman et al., 1976), neuronal cells in culture (Jacques et al., 1978), cardiac and skeletal muscle cells in culture (De Barry et al., 1977; Romey et al., 1980), and nerve terminals (Abita et al., 1977).

We describe in this paper the purification of eight toxins obtained from four different sea anemone species. The activity of each toxin was followed by measuring its toxicity on crabs and mice, its affinity for the specific sea anemone toxin receptor that has been characterized in rat brain synaptosomes (Vincent et al., 1980), and its stimulating effect on the rate of $^{22}Na^+$ uptake by neuroblastoma cells in culture.

Materials and Methods

Materials. Sephadex G-50 fine, G-25 fine, and G-10, SP-Sephadex C-25, CM-Sephadex C-25, and QAE-Sephadex A-25 were purchased from Pharmacia Fine Chemicals. Veratridine was obtained from Aldrich Chemical Co. Dulbecco's modified Eagle's medium and fetal calf serum were from Gibco. Buffers and salts were analytical grade products

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[1] Abbreviations used: AS_I, AS_{II}, AS_{III}, and AS_V, toxins I, II, III, and V from the sea anemone *Anemonia sulcata*, respectively; $[^{125}I]AS_{II}$, radiolabeled monoiodo derivative of AS_{II}; AX_I and AX_{II}, toxins I and II from the sea anemone *Anthopleura xanthogrammica*, respectively; SG_I and SG_{II}, toxins I and II from the sea anemone *Stoichactis giganteus*, respectively; AP_I, toxin I from the sea anemone *Actinodendron plumosum*; AaH_{II}, toxin II from the scorpion *Androctonus australis* Hector; $[^{125}I]AaH_{II}$, radiolabeled iodo derivative of AaH_{II}; MU and CU, mouse and crab unit, respectively; CM, carboxymethyl; Tris, tris(hydroxymethyl)aminomethane; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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of Merck or Prolabo. $^{22}Na^+$ and L-[^{3}H]leucine were obtained from the Commissariat â l'Energie Atomique. $Na^{125}I$ was obtained from Amersham.

Sea Anemones. *A. sulcata* were collected from the mediterranean coast near Nice, France. *Anthopleura xanthogrammica* were collected from Bodega bay, Ca, and stored at −20 °C in 95% ethanol. *Stoichactis giganteus* were collected in Sri Lanka and kept alive in the aquarium until use. *Actinodendron plumosum* were collected at Noumea, New Caledonia, and freeze-dried.

Toxicity Measurements. Toxicities were measured on both 10–20-g *Carcinus maenas* crabs and 10–20-g Swiss mice. Toxin solutions were geometrically diluted in 0.43 M NaCl (crabs) or in 0.15 M NaCl containing 1% bovine serum albumin (mice). Aliquots (0.1 mL) were injected at the junction between the body and the leg of crabs or intraperitoneally for mice. One crab unit (CU) is defined as the minimal amount of toxin which makes a 10-g crab that has been placed on its back unable to come back to its normal position 10 min after the injection. One mouse unit (MU) is the minimal amount of toxin which kills a 10-g mouse. LD_{50} (mouse) and LD_{100} (crab) values were determined as previously described (Bêress & Bêress, 1971; Rochat et al., 1977).

Purification of Sea Anemone Toxins. Sea anemone toxins were purified according to the general method described by Bêress and co-workers for the purification of toxins from *A. sulcata* (Bêress et al., 1975) with some modifications. The different steps of the purification procedure are briefly described below.

Step 1: Extraction. Sea anemones (2–8 kg) were homogenized in 1 volume of 95% ethanol, heated for 5 min at 60 °C, and centrifuged 15 min at 2000*g*. The pellets were extracted once or twice with 1 volume of 50% ethanol. The combined supernatants were stored overnight at 4 °C, centrifuged 15 min at 2000*g*, concentrated to $^1/_{10}$ of their initial volume in a rotavapor apparatus at 60 °C, and dialyzed for 3 days at 4 °C in Visking tubes (24016C75X Polylabo) against several 15-L volumes of 1 mM HCl.

Step 2: Batchwise Adsorption. The conductivity of the extracts at pH 3.0 was adjusted to 2 mS, and the toxin solutions were concentrated by batchwise adsorption on SP-Sephadex C-25 (2 g of dry SP-Sephadex C-25/kg of sea anemone). The toxic material was then eluted in three to four washes of the ion exchanger by a total volume of 200–300 mL of a 50 mM Tris-HCl buffer, pH 8.0, containing 1 M NaCl. The eluate was centrifuged 24 h at 100000*g*. This centrifugation allowed for the separation of the soluble toxin fraction from a viscous brownish pellet which was discarded.

Step 3: Gel Filtration on Sephadex G-50. The toxin solutions from step 2 were chromatographed at 4 °C on two to five Sephadex G-50 columns (Pharmacia K50/100, 5 × 100 cm) mounted in series and eluted with 0.5% acetic acid. This chromatography which, in one step, separates toxic from nontoxic fractions and eliminates the salts, was followed by measuring the toxicity of each fraction on both crabs and mice and by automatic recording of the optical density at 230 and 280 nm (Seive elugraph) or at 254 and 280 nm (Pharmacia UV2/Rec 2). Toxic fractions were pooled and lyophilized.

Step 4. Further purification of toxic fractions from step 3 was carried out by using gel filtration and ion-exchange chromatography as described under Results. After each ion-exchange chromatographic step, toxic fractions were desalted on a Sephadex G-25 column (4.4 × 44 cm) equilibrated in 1 mM HCl. All concentration steps were made by using lyophilization.

Amino Acid Analysis. Toxins were hydrolyzed in 5.7 N HCl for 24 h at 110 °C in sealed evacuated tubes. Hydrolysates were analyzed in a Biotronic or a Beckman 120 C automatic amino acid analyzer. Tryptophan was estimated spectrophotometrically (Bredderman, 1974). Molecular weights of toxins were determined from the results of the amino acid analysis with a Wang 2220 calculator according to the method of Delaage (Delaage, 1968).

Cell Cultures. Cells from the N1E 115 neuroblastoma clone were grown as previously described (Jacques et al., 1978) and allowed to differentiate for 2 days in a medium containing 1.5% dimethyl sulfoxide and 1% fetal calf serum. Cells were labeled with L-[^{3}H]leucine (0.2 μCi/mL) for 48 h before the uptake experiments to measure protein recovery from the ^{3}H counts.

$^{22}Na^+$ Uptake Measurements. The sea anemone toxin stimulation of $^{22}Na^+$ uptake by neuroblastoma cells was measured at 37 °C in the presence of 10 μM veratridine. Cells were preincubated for 20 min in a Na^+-free medium which consisted of 25 mM Hepes–Tris, pH 7.4, 140 mM choline chloride, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose, 0.1 mg/mL bovine serum albumin, 10 μM veratridine, and different concentrations of sea anemone toxins. The uptake rate of $^{22}Na^+$ was determined after a 1-min incubation of the cells in the following medium: 25 mM Hepes–Tris, pH 7.4, 130 mM choline chloride, 10 mM NaCl, 0.7 μCi/mL ^{22}NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose, 0.1 mg/mL bovine serum albumin, 0.5 mM ouabain, 10 μM veratridine, and sea anemone toxins at the concentration already used for preincubation. At the end of the uptake period, cells were washed 4 times with a medium consisting of 25 mM Tris-HCl, pH 7.4, 145.4 mM choline chloride, 1.8 mM $CaCl_2$, and 0.8 mM $MgSO_4$. Initial rates of $^{22}Na^+$ uptake were determined as previously described (Jacques et al., 1978).

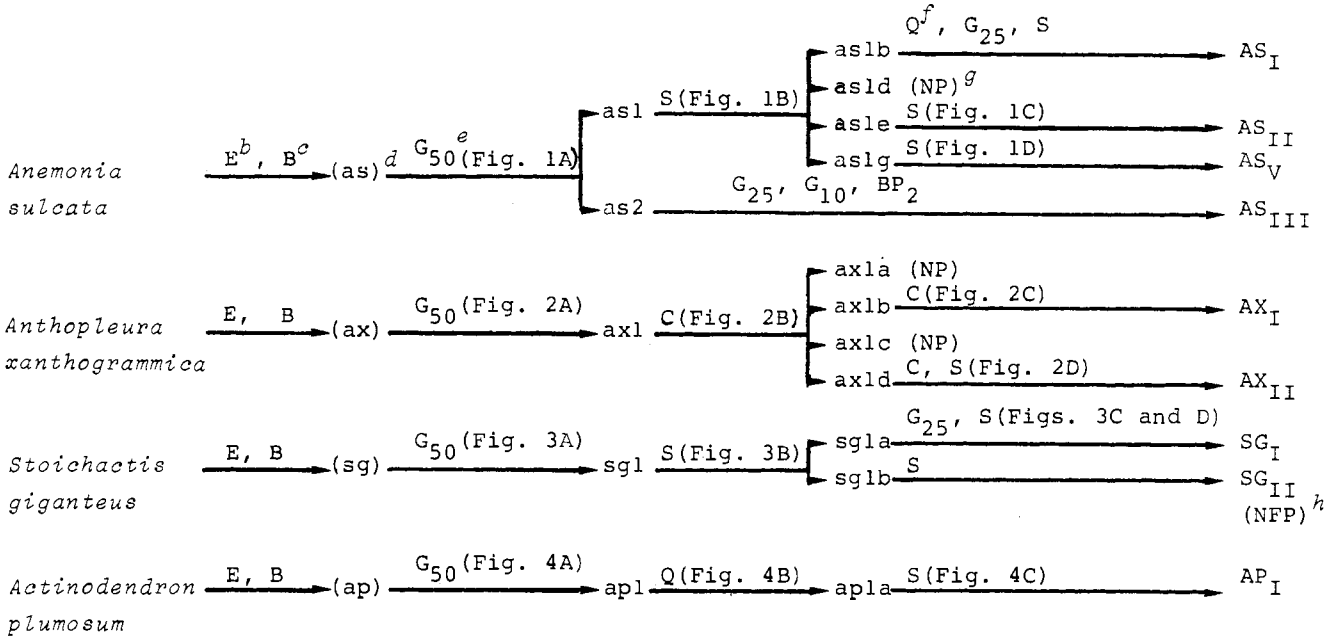
Binding Experiments. Dissociation constants of complexes formed between the different toxins and the specific sea anemone toxin receptor of rat brain synaptosomes were determined at 20 °C, pH 7.4, in competition experiments involving rat brain synaptosomes, a ^{125}I-labeled monoiodo derivative of AS_{II} ($[^{125}I]AS_{II}$), and each one of the sea anemone toxins as described elsewhere (Vincent et al., 1980). Similar competition experiments were also carried out with ^{125}I-labeled toxin II of the scorpion *Androctonus australis* Hector ($[^{125}I]AaH_{II}$) instead of $[^{125}I]AS_{II}$ (Vincent et al., 1980). Toxin II was purified from 1 g of venom of *A. australis* Hector as previously described (Miranda et al., 1970).

Miscellaneous. The titration of trypsin inhibitors in fractions containing polypeptide neurotoxins was carried out by using standard procedures (Vincent & Lazdunski, 1972).

Results

Preparation of Sea Anemone Toxins. Table I summarizes the sequence of steps used to prepare toxins from the four sea anemone species studied in this work. The main chromatographic steps are illustrated in Figures 1–4. The general technique used here is similar to that described by Bêress and co-workers for the purification of *A. sulcata* toxins (Bêress et al., 1975). However, some modifications have been introduced to obtain better yields and a better degree of purity. For example, Bêress and co-workers used three different batchwise adsorption steps on SP-Sephadex C-25 after alcoholic extraction of *A. sulcata*. The purpose of the first two steps was to remove most of the basic polypeptides that are present in this sea anemone species. Only the third step was retained in the new preparation that we describe. A comparison of our

Table I: Flow Diagram for the Purification of Sea Anemone Toxins[a]



[a] The different chromatographic steps are described in detail in Figures 1–4. [b] E, extraction. [c] B, batchwise adsorption on SP-Sephadex C-25 (see Materials and Methods). [d] (as), (ax), (sg), and (ap) refer to the crude toxin fractions obtained after extraction and batchwise adsorption. [e] G_{50}, G_{25}, and G_{10}, gel filtration on Sephadex G-50, G-25, and G-10, respectively. [f] Q, S, C, and BP_2, chromatography on QAE-Sephadex A-25, SP-Sephadex C-25, CM-Sephadex C-25, and Bio-Gel P_2 respectively. [g] (NP), no further purification attempted. [h] (NFP), toxin not fully purified.

results with those of Béress and co-workers shows that our yield in the short neurotoxin AS_{III} is ~7 times higher than that obtained by these authors. Another striking difference is that Béress and co-workers did not detect the peak designated as1g in Figure 1B which corresponds to AS_V. We believe that an important amount of AS_{III} and all of AS_V may have been lost by Béress and co-workers in the course of the first two batchwise adsorption steps. However, since these authors monitored toxicities of the different fractions on crabs only, they also may have failed to detect AS_V because its toxicity on crabs is low and because it is the least abundant of the polypeptide toxins in *A. sulcata*.

Polypeptide trypsin inhibitors are abundant in extracts of *A. sulcata*. They have molecular properties which are similar to those of polypeptide neurotoxins (Wunderer et al., 1976b). Both amino acid analysis and titration with trypsin made systematically over 13 different preparations indicated that after the equilibrium chromatography on SP-Sephadex C-25 described by Béress and co-workers, AS_{II} contained significant amounts of trypsin inhibitors (3–5% M/M). *A. sulcata* trypsin inhibitors are rich in tyrosine residues (Wunderer et al., 1976b) while this amino acid is absent in AS_{II}. The presence of a tyrosine contamination is a major problem for iodination of AS_{II} since it is well-known that tyrosine side chains are much more reactive toward iodination than histidine side chains and histidine is the sole amino acid that can be iodinated in AS_{II}. In order to fully eliminate contamination by trypsin inhibitors, we subjected AS_{II} to an ion-exchange chromatography on a SP-Sephadex C-25 column eluted with a pH gradient between 7.5 and 10.8. Figure 1C shows that this step actually eliminates, very efficiently, inhibitor contaminants. The main peak in Figure 1C corresponds to pure AS_{II}. It is devoid of any trace of tyrosine as shown by amino acid analysis and is unable to inhibit trypsin. The same type of SP-Sephadex C-25 chromatography at basic pH was used as the last purification step for AS_V (Figure 1D) and AX_{II} (Figure 2D).

Purification of toxins from *S. giganteus* on SP-Sephadex C-25 gave two toxic fractions (Figure 3B). The first one, sg1a, was purified as described in Figure 3C,D to give pure SG_I. This toxin represents 90% of the total toxicity on crabs, but it is not toxic to mice. The second fraction, sg1b, was only partially purified by a second chromatography on the SP-Sephadex C-25 column already used in Figure 3B eluted with an ionic strength gradient made of 600 mL of a 0–300 mM NaCl solution. The resulting fraction, named SG_{II}, is toxic to both crabs and mice. No further purification of SG_{II} was attempted because of the low quantity of material obtained at this stage (2.5 mg) and also because of the unstability of the toxic activity with time.

In the course of the purification of AP_I (Figure 4) it has been observed that crude extracts of *A. plumosum* not only contain AP_I but also a nonpolypeptidic toxin which is dialyzable and therefore cannot be purified by our standard procedure. This toxin was only partially purified by chromatography of the crude extract on Sephadex G-50 followed by a QAE-Sephadex A-25 column eluted with a linear ionic strength gradient from 100 to 500 mM NaCl (not shown). The nonpolypeptidic toxic component from *A. plumosum* has low toxicity on crabs and mice (0.02 CU/μg and 0.02 MU/μg).

The procedures described in Table I and Figures 1–4 have been used to prepare eight pure toxins from the four different sea anemone species studied in this paper. The amino acid composition of these toxins is presented in Table II together with their molecular weight, their absorbance at 280 nm, and the yield of their purification. The eight toxins are pure according to the following criteria. (i) In the last chromatographic step, the ratios CU/OD and MU/OD (when toxicity on mice is present) are constant along the peak corresponding to the toxin. (ii) Each toxin is devoid of at least 1 of the 18 amino acids. The missing amino acids are undetectable by amino acid analysis.

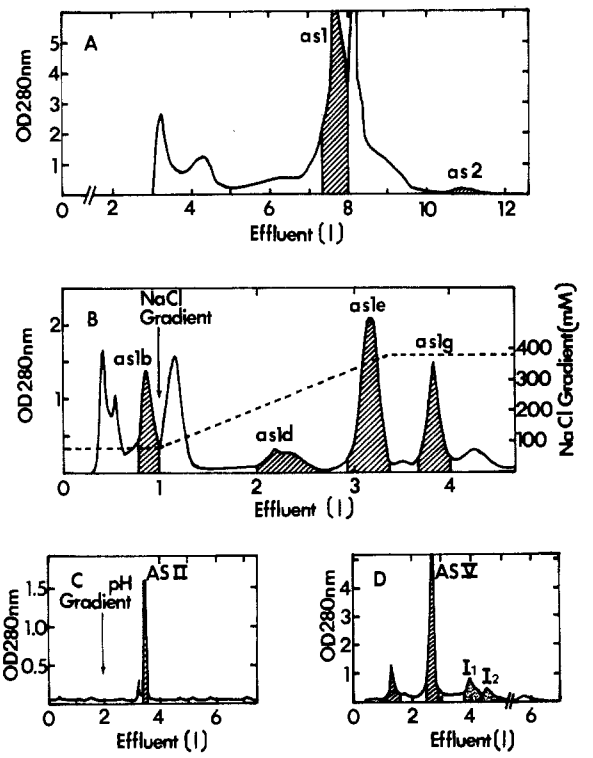


FIGURE 1: (A) Gel filtration on Sephadex G-50 of the batchwise extract (step 2 of the purification procedure) from 5 kg of wet drained *A. sulcata*. Columns (5 × 93 cm) were five in series. Eluting solvent, 0.5% acetic acid; flow rate, 140 mL/h. The fraction toxic to crabs is shaded. (B) SP-Sephadex C-25 chromatography of 2.7 g of fraction as1 from the above gel filtration. Column, 3 × 117 cm; eluting solvent, 40 mM sodium phosphate buffer, pH 6, and a NaCl gradient between 80 and 380 mM (dotted line); flow rate, 100 mL/h. Fractions as1b, as1e, and as1g contain toxins I, II, and V, respectively. Shaded fractions are toxic to crabs. (C) SP-Sephadex C-25 chromatography of 230 mg of desalted toxic material obtained from fraction as1e. Column, 3 × 77 cm; eluting solvent, first 100 mM ammonium carbonate buffer, pH 7.5, and then a linear gradient made of 2.8 L of 100 mM ammonium carbonate at pH 7.5 and 2.8 L of the same buffer adjusted to pH 10.5 with concentrated ammonia; flow rate, 100 mL/h. The fraction corresponding to pure AS_{II} is shaded. (D) SP-Sephadex C-25 chromatography of 1.6 g of desalted fraction as1g obtained by the procedure described for the previous steps from 33 kg of wet drained *A. sulcata*. Column, 4.4 × 70 cm; eluting solvent, a linear gradient made with 5 L of 100 mM ammonium acetate, pH 7.5, and 5 L of the same buffer adjusted to pH 10.5 with concentrated ammonia; flow rate, 200 mL/h. Recovery: the first peak (shaded) is AS_{II}, 75 mg; AS_V (shaded), 250 mg, I_1, 290 mg, and I_2, 150 mg, are trypsin inhibitors. Toxins I and III from *A. sulcata* were purified from fractions as1b and as2, respectively, according to Béress et al. (1975).

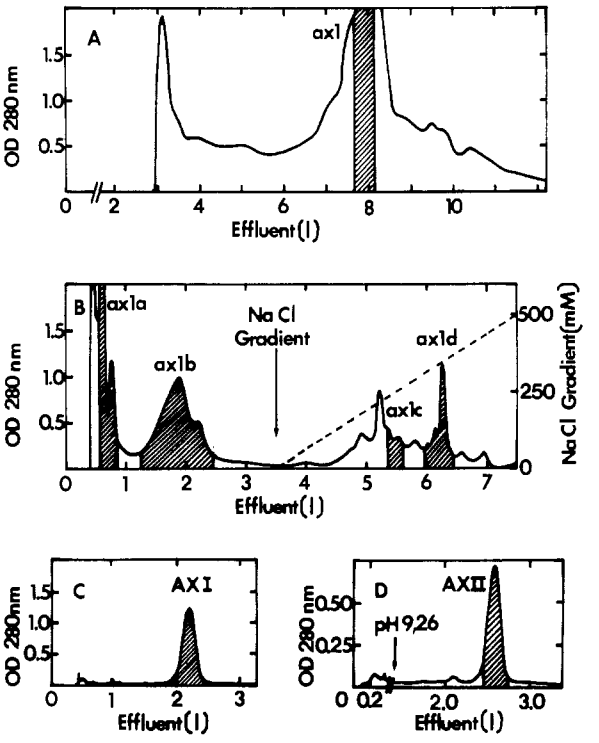


FIGURE 2: (A) Sephadex G-50 gel filtration of the batchwise extract (step 2 of the purification procedure) from 3.5 kg of of *A. xanthogrammica* on five successive columns (5 × 93 cm) in series. Eluting solvent, 0.5% acetic acid; flow rate, 140 mL/h. The fraction toxic to both crabs and mice is shaded. (B) CM-Sephadex C-25 chromatography of 2.6 g of fraction ax1 obtained from the above gel filtration. Column, 4.4 × 88 cm; eluting solvent, 30 mM sodium phosphate buffer, pH 7.5, and a NaCl gradient from 0 to 500 mM (dotted line); flow rate, 100 mL/h. Shaded fractions are toxic to crabs and mice. Peaks ax1b and ax1d contain AX_I and AX_{II}, respectively. The minor fractions ax1a and ax1c contain together 3% of the MU and 7% of the CU. (C) CM-Sephadex C-25 equilibrium chromatography of 140 mg of fraction ax1b. Column, same as in (B); eluting solvent, 30 mM sodium phosphate buffer, pH 7.5; recovery, 82 mg of pure AX_I (shaded). This toxin corresponds to Anthopleurin A (ApA) of Norton et al. (1976). (D) The toxic fractions ax1d obtained from 6.7 kg of *A. xanthogrammica* were pooled and chromatographed on a CM-Sephadex C-25 column (4.4 × 82 cm) eluted with a 30 mM sodium phosphate buffer, pH 7.5, and a NaCl gradient from 100 to 350 mM (4 L) (not shown). 105 mg of toxic material was obtained after desalting on Sephadex G-25 and lyophilization. 82 mg of this material was applied on a SP-Sephadex C-25 column (3 × 59 cm). Eluting solvent, 100 mM ammonium acetate, pH 9.0 and then pH 9.26, as shown by the arrow; flow rate, 50 mL/h; recovery, 38.5 mg of pure AX_{II} (shaded). This toxin corresponds to Anthopleurin B (ApB) of Norton et al. (1976).

Pharmacological and Biochemical Properties of Sea Anemone Toxins. The toxic properties of the eight sea anemone toxins were estimated by measurement of LD_{50} on mice and of LD_{100} on crabs. Results presented in Table III show that although all the toxins are highly toxic to crabs the toxicity to mice of AS_I, AS_{III}, SG_I, and AP_I is so low that no precise value of LD_{50} can be given. Moreover, toxicities to crabs and mice are not directly correlated. AX_{II} is the most toxic to mice (LD_{50} = 8 μg/kg) and also the least toxic to crabs (LD_{100} = 78 μg/kg). AS_I and AS_{II} exhibit similar toxicities on crabs (LD_{100} = 4.4 and 3.7 μg/kg, respectively), but the toxicity of AS_{II} on mice is more than 40 times higher than that of AS_I.

Sea anemone toxins bind specifically and reversibly to Na^+ channels in rat brain synaptosomes (Vincent et al., 1980). Competition experiments involving rat brain synaptosomes, a radioactive monoiodo derivative of AS_{II} ($[^{125}I]AS_{II}$), and each one of the unlabeled toxins were used to determine the dissociation constants (K_d) of complexes formed between sea anemone toxins and the synaptic receptor (Vincent et al., 1980). Results are shown in Figure 5A. They demonstrate that AS_{III}, SG_I, and AP_I which are almost completely devoid of toxicity on mice are also unable to compete efficiently with $[^{125}I]AS_{II}$ for binding to its receptor. Conversely, AS_{II}, AS_V, AX_I, and AX_{II} that possess high toxicities on mice are very good competitors for the binding of $[^{125}I]AS_{II}$ to the specific sea anemone toxin receptor. Experiments in Figure 5A were carried out with a $[^{125}I]AS_{II}$ concentration (10 nM) much smaller than the K_d value (240 nM) of the $[^{125}I]AS_{II}$–receptor complex (Vincent et al., 1980). Under these experimental conditions, $K_{0.5}$ values, i.e., the concentrations of unlabeled toxins that induce half-displacement of bound $[^{125}I]AS_{II}$, represent the dissociation constants of the unlabeled sea anemone toxin–receptor complexes. K_d values calculated from Figure 5A are presented in Table III. A good correlation is observed for the different sea anemone toxins between K_d values obtained with rat brain synaptosomes and LD_{50} values

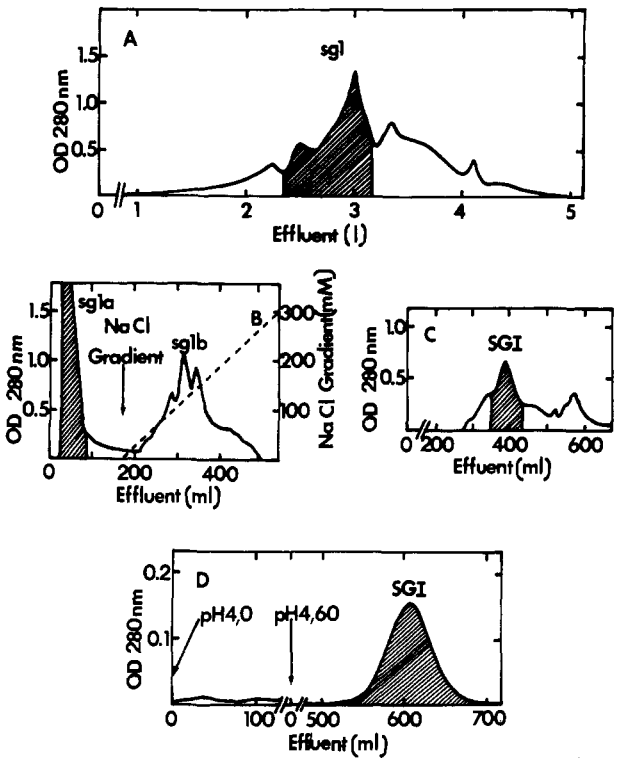


FIGURE 3: (A) Gel filtration on Sephadex G-50 of the batchwise extract (step 2 of the purification procedure) from 1.5 kg of wet drained *S. giganteus*. Columns (5 × 93 cm) were two in series. Eluting solvent, water; flow rate, 140 mL/h. The fractions toxic to crabs is shaded. It was lyophilized and rechromatographed on the same columns. (B) SP-Sephadex C-25 chromatography of the desalted toxin fraction sgl (330 mg). Column, 1.24 × 51 cm; eluting solvent, 40 mM sodium phosphate buffer at pH 6.0 and 200 mL of a 0–300 mM NaCl gradient (arrow); flow rate, 15 mL/h. Peak sgla (shaded) is toxic to crabs; peak sglb is toxic to mice. (C) Gel filtration on Sephadex G-25 of fraction sgla. Column, 4.4 × 45 cm; eluting solvent, 1 mM HCl; flow rate, 160 mL/h. The toxic fraction is shaded. (D) SP-Sephadex C-25 chromatography of 11 mg of desalted toxic fractions obtained at the previous step (SG_I). Column, 2 × 37 cm; eluting solvent, 100 mM ammonium acetate. The column was washed at pH 4.0, and then the toxin (shaded) was eluted at pH 4.60 as shown by the arrows. Flow rate, 24 mL/h; recovery, 6.0 mg of pure SG_I.

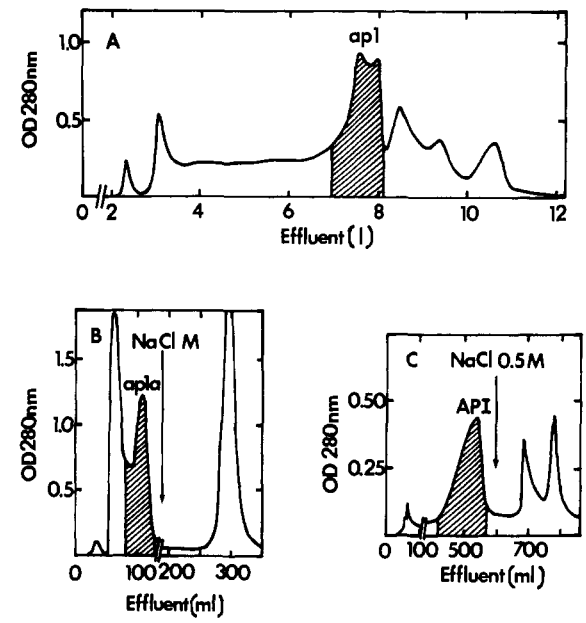


FIGURE 4: (A) Gel filtration on Sephadex G-50 of the batchwise extract (step 2 of the purification procedure) from 230 g of lyophilized *A. plumosum*. There were five columns (5 × 93 cm) in series. Eluting solvent, 0.5% acetic acid; flow rate, 200 mL/h. The fraction toxic to crabs is shaded (apl). (B) QAE-Sephadex A-25 chromatography of 430 mg of desalted material toxic to crabs (apl) obtained as described above. Column, 1.5 × 78 cm; eluting solvent, 50 mM Tris, pH 8; flow rate, 15 mL/h. The toxic fraction is shaded (apla). A nontoxic material was retained on the column and could be eluted by 1 M NaCl (arrow). (C) SP-Sephadex C-25 equilibrium chromatography of fraction apla. Column, 2 × 55 cm; eluting solvent, 20 mM sodium phosphate buffer, pH 5.5; flow rate, 20 mL/h. The toxic fraction is shaded; it is pure AP_I. Nontoxic fractions were retained on the column and could be eluted with 0.5 M NaCl (arrow). Recovery of AP_I after desalting on Sephadex G-25 and lyophilization was 19 mg.

on mice, but not between K_d values and LD_{100} values on crabs. AX_{II} is the toxin that has the best affinity for the sea anemone toxin receptor (K_d = 35 nM) and the highest toxicity on mice (LD_{50} = 8 μg/kg) but the lowest toxicity on crabs (LD_{100} = 78 μg/kg).

AS_{II} and scorpion toxins have been reported to bind to a common receptor site in rat brain synaptosomes (Ray et al., 1978). K_d values found for unlabeled AS_{II} in competition experiments involving either [^{125}I]AS_{II} or a radioactive monoiodo derivative of one of the toxins from the scorpion *A. australis* Hector ([^{125}I]AaH_{II}) as the labeled ligand were indeed identical (Vincent et al., 1980). This result is confirmed in Figure 5B and extended to the three other sea anemone toxins prepared from *A. sulcata*. K_d values determined for each unlabeled *Anemonia* toxin in competition experiments with either [^{125}I]AS_{II} (Figure 5A) or [^{125}I]AaH_{II} (Figure 5B) are identical within the experimental error. This is also true for the four other sea anemone toxins described in this paper (not shown).

Table III shows that AS_V, AX_I, and AX_{II} bind to the specific sea anemone toxin receptor with better affinities than AS_{II}. Radioactive monoiodo derivatives of AS_V, AX_I, and AX_{II} were prepared and purified by using techniques that have been described for the preparation of [^{125}I]AS_{II} (Vincent et al., 1980). Iodination of AX_{II} results in a significant loss of toxicity on mice (~90%) which prevents the use of [^{125}I]AX_{II} to study the interaction between sea anemone toxins and the Na^+ channel. Conversely, iodination of both AS_V and AX_I preserves the toxic activity as previously demonstrated for AS_{II} (Vincent et al., 1980). The dissociation constant of the [^{125}I]AS_V–receptor complex is 84 nM, ~3 times better than those obtained with [^{125}I]AS_{II} and [^{125}I]AX_I (240 nM). Similar values for the maximal binding capacity of rat brain synaptosomes (3.2 ± 0.5 pmol/mg of protein) were obtained with the three different iodotoxins.

Sea anemone toxins and veratridine have a synergistic action on the action potential Na^+ ionophore of neuroblastoma and other excitable cells (Jacques et al., 1978; Romey et al., 1980). The mixture of toxins selectively prolongs the time of opening of the Na^+ channel, a property that can be monitored by ion transport methods (Catterall, 1976; Jacques et al., 1978). Figure 6 shows that AS_{II}, AS_V, AX_I, and AX_{II} produce a marked increase in the rate of $^{22}Na^+$ uptake by neuroblastoma cells measured in the presence of 10 μM veratridine. This increase in the rate of $^{22}Na^+$ uptake through the Na^+ channel is completely abolished by 1 μM tetrodotoxin. The stimulation produced by AS_I, AS_{III}, SG_I, and AP_I is hardly measurable. Values of ED_{50}, i.e., the concentration of sea anemone toxin that induces a half-maximal stimulating effect on the $^{22}Na^+$ uptake rate of neuroblastoma cells, are given in Table III. The most active toxin is AX_{II}. Its ED_{50} value is 7 nM, ~2 times lower than that of AS_V and 30 times lower than that of AS_{II}, the most commonly used sea anemone toxin. The stimulating effect on the $^{22}Na^+$ uptake rate is clearly correlated to the affinity of toxins for the rat brain receptor and to their toxicity on mice but not to their toxicity on crabs (Table III).

Table II: Amino Acid Composition of Sea Anemone Toxins

amino acid	*A. sulcata*				*A. xanthogrammica*		*S. giganteus*	*A. plumosum*
	AS_I	AS_{II}	AS_{III}	AS_V	AX_I	AX_{II}	SG_I	AP_I
Asp	4.95 (1)[a]	3.75 (2)	0.99 (0)	3.66 (4)	4.11 (2)	4.92 (5)	8.06 (8)	4.57 (5)
Asn	(4)	(2)	(1)		(2)			
Thr	1.93 (2)	1.79 (2)	0.00 (0)	1.98 (2)	2.96 (3)	0.85 (1)	3.89 (4)	4.20 (4)
Ser	3.70 (4)	3.74 (4)	1.84 (2)	3.95 (4)	5.71 (6)	2.90 (3)	2.63 (3)	3.94 (4)
Glu	2.13 (1)	0.93 (0)	1.97 (1)	0.00 (0)	1.19 (1)	0.00 (0)	2.16 (2)	2.15 (2)
Gln	(1)	(1)	(1)		(0)			
Pro	2.76 (2-3)	3.96 (4)	3.64 (4)	4.30 (4)	4.31 (4)	6.99 (7)	2.14 (2)	3.94 (4)
Gly	7.91 (8)	7.68 (8)	4.78 (5)	7.26 (7)	8.03 (8)	7.43 (7)	4.30 (4)	8.44 (8)
Ala	2.53 (2-3)	1.10 (1)	0.00 (0)	1.16 (1)	1.26 (1)	1.12 (1)	5.42 (5)	2.29 (2)
Cys	5.58 (6)	5.51 (6)	5.63 (6)	5.53 (6)	5.62 (6)	6.06 (6)	5.72 (6)	4.36 (4)
Val	1.03 (1)	1.93 (2)	1.03 (1)	1.92 (2)	1.98 (2)	1.15 (1)	1.67 (2)	3.74 (4)
Met	1.09 (1)	0.00 (0)	0.00 (0)	0.00 (0)	0.00 (0)	0.00 (0)	0.00 (0)	0.00 (0)
Ile	2.68 (3)	2.61 (3)	0.00 (0)	1.93 (2)	1.11 (1)	1.88 (2)	1.94 (2)	0.00 (0)
Leu	1.09 (1)	3.31 (3)	0.00 (0)	3.87 (4)	3.85 (4)	2.92 (3)	2.86 (3)	2.88 (3)
Tyr	1.10 (1)	0.00 (0)	1.75 (2)	0.00 (0)	1.24 (1)	1.02 (1)	0.94 (1)	0.00 (0)
Phe	1.19 (1)	0.00 (0)	0.00 (0)	0.00 (0)	0.00 (0)	1.02 (1)	1.04 (1)	1.97 (2)
Lys	2.04 (2)	3.16 (3)	0.81 (1)	3.87 (4)	2.10 (2)	2.96 (3)	2.24 (2)	2.02 (2)
His	0.00 (0)	1.97 (2)	0.00 (0)	1.73 (2)	2.09 (2)	1.93 (2)	0.00 (0)	0.86 (1)
Arg	1.80 (2)	1.06 (1)	0.98 (1)	1.04 (1)	1.14 (1)	3.61 (4)	4.08 (4)	1.85 (2)
Trp	1.97 (2)	2.94 (3)	2.00 (2)	2.93 (3)	2.87 (3)	3.68 (4)	1.19 (1)	2.15 (2)
total[b]	46	47	27	46	49	51	50	49
M_r[c]	4821	4948	2939	4883	5138	5725	5395	5109
$A^{1\%}_{1cm}$ at 280 nm	26.4	33.9	48.4	34.2	34.5	38.8	15.6	23.9
yield[d]	15	116	10	7.6	60	7.8	4.0	11

[a] Numbers in parentheses are calculated from sequence data for AS_I, AS_{II}, AS_{III}, and AX_I or represent the nearest integer for AS_V, AX_{II}, SG_I, and AP_I. [b] Total number of amino acids calculated from the numbers in parentheses. [c] Molecular weight calculated from the numbers in parentheses. [d] The yield in pure toxin is given in milligrams of toxin per kilogram of wet sea anemone.

Table III: Pharmacological Properties of Sea Anemone Toxins

toxin	toxicity: LD_{50} on mice (μg/kg)	toxicity: LD_{100} on crabs[a] (μg/kg)	binding to synaptosomes, K_d[b] (nM)	$^{22}Na^+$ uptake of neuroblastoma cells, ED_{50}[c] (nM)
AS_I	>4 000	4.4	7 000	>10 000
AS_{II}	100	3.7	150	200
AS_{III}	>18 000	6.7	>10 000	>10 000
AS_V	19	10.4	50	15
AX_I	66	22	120	47
AX_{II}	8	78	35	7
SG_I	>2 000	14	>10 000	>10 000
AP_I	>6 000	32	>10 000	>10 000

[a] Values of LD_{100} on crabs were calculated from the relationship LD_{100} = 100/number of CU per microgram of toxin. [b] K_d values were calculated from results shown in Figure 5. [c] ED_{50} values were calculated from results shown in Figure 6.

Discussion

The general purification procedures described here led us to isolate three new sea anemone toxins, AS_V, SG_I, and AP_I. The purification of the five other toxins studied in this paper had been previously described by other groups (Bêress et al., 1975; Norton et al., 1976). However, these toxins can now be obtained in a simpler way, in a better state of purity, or in higher yields.

All these toxins but one are polypeptides consisting of ~50 amino acids, cross-linked by two or three disulfide bridges, with a molecular weight of ~5000 (Table II). Other sea anemone toxins recently isolated from *Condylactis aurantica* (Bêress et al., 1976), *Condylactis gigantea* (Yost & O'Brien, 1978), *Bolocera tuediae* (Bêress & Zwick, 1980), *Anthopleura elegantissima* (Quinn et al., 1974; Norton et al., 1978), and *Stoichactis* species (Turlapaty et al., 1973; Devlin, 1974; Mebs & Gebauer, 1980) also possess the same chemical characteristics. One sea anemone toxin, AS_{III} (M_r 2939), is shorter, and its sequence (Martinez et al., 1977; Bêress et al., 1977) has no evident homology with those of longer toxins. Another toxin of M_r ~2000 has been recently isolated from *Parasicyonis actinostoloides* (Ishikawa et al., 1979).

Of all the toxins described in Table III, there are some which are more active on crustaceans (AS_I, AS_{III}, SG_I, and AP_I) and others on mammals (AX_{II}). Within the eight toxins that have been studied, toxicities on crabs vary by a maximum factor of 20 whereas differences varying by a factor of more than 2000 were observed for LD_{50} values on mice. Results in Table III for the different sea anemone toxins demonstrate that there exists a striking correlation between their binding affinity to rat brain synaptosomes (K_d), their efficiency (ED_{50}) in increasing the rate of $^{22}Na^+$ uptake through the Na^+ channel of mouse neuroblastoma cells, and their toxicity on mice (LD_{50}). Conversely, results in Table III show that values of K_d, ED_{50}, and LD_{50} on mice of the eight toxins are not correlated to their toxicity on crabs and even vary in the opposite direction. For example, AX_{II} is the toxin that has the highest activity (K_d, ED_{50}, LD_{50}) on mammals, mammalian tissue, or mammalian membranes and the lowest toxicity on crabs. The only exception to this general observation in the series of the sea anemone toxins studied in this work is AS_{II} which is highly active on both mammals and crustaceans.

A comparison of results presented in Table III and Figures 1–3 shows that there also exists a correlation between the order of elution of toxins belonging to a given sea anemone species on a cation-exchanger column eluted with an ionic strength gradient and their toxicity on mammals. Figure 1B shows that *A. sulcata* toxins are eluted from the SP-Sephadex C-25 column in the following order: (i) AS_I (peak as1b); (ii) AS_{II} (peak as1e), and (iii) AS_V (peak as1g). On the other hand, the increasing order of activity of these toxins on mammals is $AS_I < AS_{II} < AS_V$ (Table III). This correlation is also observed for AX_I and AX_{II} (Figure 2B and Table III) and for SG_I and SG_{II} (Figure 3B, Table III, and results not shown for the partially purified SG_{II}). Within a given sea anemone species, the most cationic toxin is also the most active on

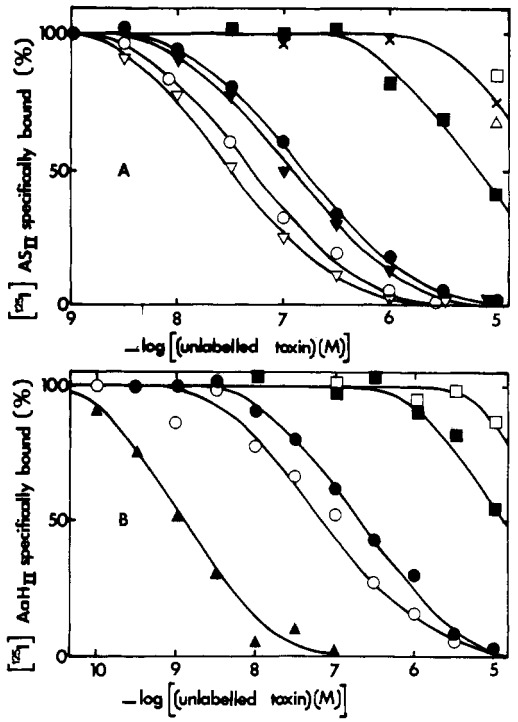


FIGURE 5: Competition between [^{125}I]AS_{II} or [^{125}I]AaH_{II} and unlabeled toxins for binding to synaptosomes. (A) Competition between [^{125}I]As_{II} and unlabeled sea anemone toxins. [^{125}I]AS_{II} (10 nM) was incubated with synaptosomes (1 mg of protein/mL) and increasing concentrations of AS_I (■), AS_{II} (●), AS_{III} (□), AS_V (○), AX_I (▼), AX_{II} (▽), SG_I (×), or AP_I (△). The incubation medium consisted of 140 mM choline chloride, 5.4 mM KCl, 2.8 mM $CaCl_2$, 1.3 mM $MgSO_4$, 0.1% bovine serum albumin, and 20 mM Tris-HCl, pH 7.4. (B) Competition between [^{125}I]AaH_{II} and unlabeled AaH_{II} or sea anemone toxins. [^{125}I]AaH_{II} (0.4 nM) was incubated with synaptosomes (1 mg of protein/mL) and increasing concentrations of AaH_{II} (▲), AS_I (■), AS_{II} (●), AS_{III} (□), or AS_V (○) in the same incubation medium as in (A). Radioactivity specifically bound to synaptosomes was determined after incubation for 30 min at 20 °C.

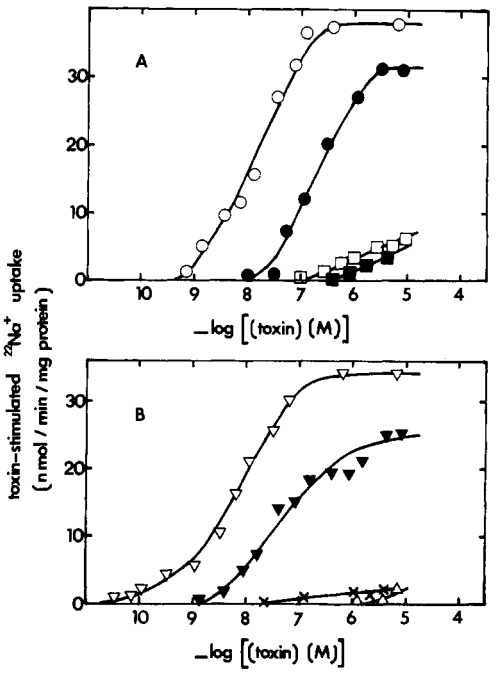


FIGURE 6: Sea anemone toxin-induced increase in the rate of $^{22}Na^+$ uptake by N1E 115 neuroblastoma cells. Uptake experiments were performed as described under Materials and Methods at 37 °C in the presence of 10 μM veratridine, 10 mM Na^+, and increasing concentrations of sea anemone toxins. (A) Dose–response curves for AS_I (■), AS_{II} (●), AS_{III} (□), and AS_V (○). (B) Dose–response curves for AX_I (▼), AX_{II} (▽), SG_I (×), and AP_I (△). The toxin-stimulated $^{22}Na^+$ uptakes were fully inhibitable by 1 μM tetrodotoxin (not shown). The tetrodotoxin-insensitive basal rate of $^{22}Na^+$ uptake has been subtracted.

mammals and the least active on crustaceans. The only toxin which is an exception to this rule is AS_{II}.

Scorpion toxins, similarly to sea anemone toxins, prolong action potentials by specifically slowing down the inactivation of the Na^+ channel (Romey et al., 1975; Catterall, 1976; Bernard et al., 1977). Among all the scorpion toxins which have been purified (Miranda et al., 1970; Zlotkin, 1973; Babin et al., 1975; Possani et al., 1977, 1978), the most toxic are those extracted from the scorpion *A. australis* Hector, and the most active on mice is toxin II from this venom which has a LD_{50} of 9 μg/kg. LD_{50}'s of toxins from *A. australis* Hector, *Buthus occitanus tunetanus*, and *Leiurus quinquestriatus quinquestriatus* are between 9 and 225 μg/kg (Miranda et al., 1970). Table III indicates that sea anemone toxins which are the most toxic to mice have LD_{50} values comparable to those of AaH_{II}. Sea anemone toxins compete with [^{125}I]AaH_{II} for binding to the scorpion toxin receptor. However, in spite of the similarities of the LD_{50}'s, the most toxic sea anemone toxins and the most toxic scorpion toxins remain far apart in their affinities for the Na^+ channel. The best scorpion toxin has an affinity for the channel which is ~60 times higher than that of the best sea anemone toxin. Other differences between the two different types of polypeptide toxins have already been discussed elsewhere (Vincent et al., 1980).

AS_{II} is one of the most abundant sea anemone toxins and for that reason the most widely used. This paper demonstrates that although AS_{II} is our most active toxin on crustaceans, there exist three other sea anemone toxins, namely, AS_V, AX_I, and AX_{II}, that are more active than AS_{II} on the mammalian Na^+ channel. These toxins are potentially better tools than AS_{II} to study the molecular mechanism of functioning of this channel in excitable membranes of mammalian origin. It has been shown in this work that both AS_V and AX_I but not AX_{II} can be radiolabeled with ^{125}I and used for biochemical studies of the specific sea anemone toxin receptor in rat brain synaptosomes.

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References

Abita, J. P., Chicheportiche, R., Schweitz, H., & Lazdunski, M. (1977) *Biochemistry 16*, 1838–1844.

Babin, D. R., Watt, D. D., Goos, S. M., & Mlejnek, R. V. (1975) *Arch. Biochem. Biophys. 166*, 125–134.

Barhanin, J., Hugues, M., Schweitz, H., Vincent, J. P., & Lazdunski, M. (1981) *J. Biol. Chem. 256*, 5764–5769.

Bêress, L., & Bêress, R. (1971) *Kiel. Meeresforsch. 27*, 117–127.

Bêress, L., & Zwick, J. (1980) *Mar. Chem. 8*, 333–338.

Bêress, L., Bêress, R., & Wunderer, G. (1975) *FEBS Lett. 50*, 311–314.

Bêress, L., Wunderer, G., & Wachter, E. (1977) *Hoppe-Seyler's Z. Physiol. Chem. 358*, 985–988.

Bêress, R., Bêress, L., & Wunderer, G. (1976) *Hoppe-Seyler's Z. Physiol. Chem. 357*, 409–414.

Bergman, C., DuBois, J. M., Rojas, E., & Rathmayer, W. (1976) *Biochim. Biophys. Acta 455*, 173–184.
Bernard, P., Couraud, F., & Lissitzky, S. (1977) *Biochem. Biophys. Res. Commun. 77*, 782–788.
Bredderman, P. J. (1974) *Anal. Biochem. 61*, 298–301.
Catterall, W. A. (1976) *J. Biol. Chem. 251*, 5528–5536.
De Barry, J., Fosset, M., & Lazdunski, M. (1977) *Biochemistry 16*, 3850–3855.
Delaage, M. (1968) *Biochim. Biophys. Acta 168*, 573–575.
Devlin, J. P. (1974) *J. Pharm. Sci. 63*, 1478–1480.
Ishikawa, Y., Onodera, K., & Takeuchi, A. (1979) *J. Neurochem. 33*, 69–73.
Jacques, Y., Fosset, M., & Lazdunski, M. (1978) *J. Biol. Chem. 253*, 7383–7392.
Martinez, G., Kopeyan, C., Schweitz, H., & Lazdunski, M. (1977) *FEBS Lett. 84*, 247–252.
Mebs, D., & Gebauer, E. (1980) *Toxicon 18*, 97–106.
Miranda, F., Kopeyan, C., Rochat, H., Rochat, C., & Lissitzky, S. (1970) *Eur. J. Biochem. 16*, 514–523.
Norton, T. R., Shibata, S., Kashiwagi, M., & Bentley, J. (1976) *J. Pharm. Sci. 65*, 1368–1374.
Norton, T. R., Kashiwagi, M., & Shibata, S. (1978) in *Drugs and Food from the Sea* (Kaul, P. N., & Sinderman, C. J., Eds.) pp 37–50, University of Oklahoma Press, Norman, OK.
Possani, L. D., Alagon, A. C., Fletcher, P. L., Jr., & Erickson, B. W. (1977) *Arch. Biochem. Biophys. 180*, 394–403.
Possani, L. D., Ramirez, G. A., Fletcher, P. L., Jr., & Gurrola, A. H. (1978) *FEBS Lett. 91*, 261–264.
Quinn, R. J., Kashiwagi, M., Norton, T. R., Shibata, S., Kuchii, M., & Moore, R. E. (1974) *J. Pharm. Sci. 63*, 1798–1800.
Rathmayer, W., & Bêress, L. (1976) *J. Comp. Physiol. 109*, 373–382.
Ray, R., Morrow, C. S., & Catterall, W. A. (1978) *J. Biol. Chem. 253*, 7307–7313.
Rochat, H., Tessier, M., Miranda, F., & Lissitzky, S. (1977) *Anal. Biochem. 82*, 532–548.
Romey, G., Chicheportiche, R., Lazdunski, M., Rochat, M., Miranda, F., & Lissitzky, S. (1975) *Biochem. Biophys. Res. Commun. 64*, 115–121.
Romey, G., Abita, J. P., Schweitz, H., Wunderer, G., & Lazdunski, M. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4055–4059.
Romey, G., Renaud, J. F., Fosset, M., & Lazdunski, M. (1980) *J. Pharmacol. Exp. Ther. 213*, 607–615.
Tanaka, M., Haniu, M., Yasunobu, K. T., & Norton, T. R. (1977) *Biochemistry 16*, 204–208.
Turlapaty, P., Shibata, S., Norton, T. R., & Kashiwagi, M. (1973) *Eur. J. Pharmacol. 24*, 310–316.
Vincent, J. P., & Lazdunski, M. (1972) *Biochemistry 11*, 2967–2977.
Vincent, J. P., Balerna, M., Barhanin, J., Fosset, M., & Lazdunski, M. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1646–1650.
Wunderer, G., & Eulitz, M. (1978) *Eur. J. Biochem. 89*, 11–17.
Wunderer, G., Fritz, H., Wachter, E., & Machleidt, W. (1976a) *Eur. J. Biochem. 68*, 193–198.
Wunderer, G., Bêress, L., Machleidt, W., & Fritz, H. (1976b) *Methods Enzymol. 45*, 881–888.
Yost, G. A., & O'Brien, R. D. (1978) *Arch. Biochem. Biophys. 185*, 483–487.
Zlotkin, E. (1973) *Experentia 29*, 1319–1327.

Reliability of Nitroxide Spin Probes in Reporting Membrane Properties: A Comparison of Nitroxide- and Deuterium-Labeled Steroids†

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ABSTRACT: The reliability for the study of membrane properties of the steroid nitroxide spin probe, 3-doxylcholestane, was tested by comparison of analogous data for the deuterated steroid, cholesterol-3α-*d*. Good agreement between the two probes was found for the dependence of their order parameters on variation of temperature or cholesterol concentration in egg phosphatidylcholine bilayers. This finding is contrasted with the results of a previous study of fatty acid probes where poor agreement was found for the spectral responses of nitroxide- and deuterium-labeled species. The angular dependence of the ESR spectra of nitroxide-labeled probes in oriented multibilayer films was examined to determine if the probes were oriented in a tilted fashion in the bilayer. The 3-doxylcholestane probe and a doxylstearic acid labeled at position 14 orient with their long molecular axes perpendicular to the bilayer plane. In contrast, the stearic acid probe nitroxide labeled at position 5 does not appear to orient in such a fashion. We suggest that the behavior of the latter probe reflects the difficulty of inserting a bulky nitroxide group into a highly ordered region of the bilayer rather than an inherent tilting of the phospholipid acyl chains. On the basis of the comparisons between various types of probes, some suggestions are made concerning the choice of ESR spin probe to obtain reliable information in membrane studies.

Nitroxide spin probes are commonly used in the study of phospholipid membrane organization (Berliner, 1976; Schreier et al., 1978). The reliability of data derived from ESR[1] studies of spin probes may be questioned since the bulky, polar nitroxides may not accurately report on the natural properties of the membrane. We feel that it is important to assess the nature and magnitude of the perturbation induced in the membrane by introduction of the nitroxide. Previously, we

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[1] Abbreviations used: NMR, nuclear magnetic resonance; ESR, electron spin resonance; doxyl, the 4′,4′-dimethyloxazolidinyl-*N*-oxy derivatives of ketones; CSL, the doxyl derivative of cholestan-3-one; 5-SASL, the doxyl derivative of 5-ketostearic acid; 14-SASL, the doxyl derivative of 14-ketostearic acid; PC, phosphatidylcholine.

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