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Binding of *Naja nigricollis* [^{3}H]α-Toxin to Membrane Fragments from *Electrophorus* and *Torpedo* Electric Organs

I. Binding of the Tritiated α-Neurotoxin in the Absence of Effector

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SUMMARY

WEBER, MICHEL, AND CHANGEUX, JEAN-PIERRE: Binding of *Naja nigricollis* [^{3}H]α-toxin to membrane fragments from *Electrophorus* and *Torpedo* electric organs. I. Binding of the tritiated α-neurotoxin in the absence of effector. *Mol. Pharmacol.* **10**, 1-14 (1974).

Binding of a tritiated α-neurotoxin from *Naja nigricollis* to membrane fragments purified from electric tissues of *Electrophorus electricus* and *Torpedo marmorata* was measured by ultrafiltration on Millipore filters. Isotopic dilution and pharmacological experiments showed that the tritiated α-toxin behaved exactly like the native, unlabelled compound. The number of [^{3}H]α-toxin binding sites on membrane fragments is about 10 nmoles/g of protein for *Electrophorus* and 1000 nmoles/g for *Torpedo*. The kinetics of association of [^{3}H]α-toxin with the membrane is compatible with a bimolecular mechanism of binding to a homogeneous class of sites. The second-order rate constant of association is 2.5×10^7 M^{-1} min^{-1} at 20° in Ringer's solution. It decreases with increasing ionic strength and sucrose concentration. The half-time for dissociation of the [^{3}H]α-toxin–membrane complex in the presence of an excess of unlabelled α-toxin is about 60 hr. The equilibrium dissociation constant, estimated from the kinetic data, is 20 pM.

INTRODUCTION

Venoms from a variety of highly evolved snakes possess neurotoxic properties. All these venoms consist of a complex mixture of enzymes and toxic polypeptides with vastly different pharmacological effects. As a consequence, most of the pharmacological data obtained with crude venoms were difficult to interpret until Lee and his coworkers, combining careful fractionation methods and pharmacological analysis, resolved *Bungarus multicinctus* venom into three major toxic components: a cardiotoxin with widespread effects on membrane permeability, a β-toxin which acts on the presynaptic side of the neuromuscular junction, and an α-toxin which blocks the effect of acetylcholine on its postsynaptic membrane (1). This last category of neurotoxin has become particularly useful in characterizing

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the cholinergic receptor site both *in vivo* (2, 3) and *in vitro* with excitable membrane fragments from fish electric organs (2) or with soluble preparations made from these fragments (2, 4, 5) (for a review, see ref. 6).

In this series of papers we present a quantitative analysis of the interaction of a tritiated α-neurotoxin ([^{3}H]α-toxin) from *Naja nigricollis* with a preparation of membrane fragments purified from electric organs of two different species: *Electrophorus electricus*, a freshwater fish, and *Torpedo marmorata*, a marine species. First we present kinetic and equilibrium studies in the absence of effector. Then we analyze the effect of cholinergic ligands on the interaction of the toxin with its membrane sites. Finally, in the third paper, we show that local anaesthetics behave in a manner entirely different from that of cholinergic ligands in this system. Quantitative estimates of the equilibrium and rate constants of [^{3}H]α-toxin and of several cholinergic ligands and local anaesthetics for the cholinergic receptor site are presented and compared with their "apparent" equilibrium constants given by pharmacological experiments *in vivo*. A preliminary report of this study on *Electrophorus* membrane fragments has been published (7).

METHODS

Preparation of Membrane Fragments from Electric Tissue

Electrophorus electricus excitable membrane fragments were prepared according to Changeux, Gautron, Israel, and Podleski (8) as modified by Kasai and Changeux (9). The cephalic part (90 g) of the main electric organ of a freshly killed eel was cut with scissors into fragments of approximately 1 ml and suspended in 180 ml of ice-cold 0.2 M sucrose in distilled water. The suspension was homogenized for 1.5 min with a VirTis Macro 45 apparatus at maximal speed in a 500-ml vessel maintained at 0° with crushed ice. The homogenate was then sonicated with a Branson Sonifier at energy level 6 and centrifuged at 4° for 20 min at 5000 × g (6500 rpm) in the JA20 rotor of a Beckman J21 centrifuge. The pellet was discarded, and 25 ml of the supernatant fraction were layered on top of a discontinuous gradient containing 5 ml of 1.1 M sucrose and 5 ml of 0.4 M sucrose in distilled water. The gradients were centrifuged at 4° for 5 hr at 64,000 × g (24,000 rpm) in the SW 27 rotor of a Beckman L3-50 ultracentrifuge. Seven fractions of approximately 1 ml were collected after perforation of the bottoms of the tubes. Protein was assayed in each fraction by the method of Lowry *et al.* (10), using bovine serum albumin as standard. Acetylcholinesterase was assayed by the method of Ellman *et al.* (11); ouabain-sensitive adenosine triphosphatase, by the method of Robinson (12), using the Tris salt of *p*-nitrophenyl phosphate as substrate; and sucrose, by refractometry.

Under the above experimental conditions the distribution of protein and acetylcholinesterase in the gradient was nearly the same as that found by Kasai and Changeux (9), but the final concentration of the membranes in the collected fractions was 2–3 times higher. The peaks of proteins, acetylcholinesterase, ATPase, and [^{3}H]α-toxin binding sites coincided almost exactly, although with different fractionation procedures the peaks of acetylcholinesterase and of [^{3}H]α-toxin binding could be separated (13).

In the membrane fractions which penetrated the sucrose gradient the yield of protein, acetylcholinesterase, and [^{3}H]α-toxin binding sites was 25%, 60%, and 90–100%, respectively, of the quantities added to the top of the gradient. In general only one or two fractions from the collected gradient were used for the kinetic studies; these fractions had the highest specific activity of [^{3}H]α-toxin binding sites (5–15 nmoles/g of protein) and represented only 30% of the total amount of [^{3}H]α-toxin binding sites layered on the gradient and 10% of the amount present in the total homogenate before the low-speed centrifugation.

When stored at 0° (in ice) under nitrogen, the membrane fragments are stable for weeks. Neither the rate of [^{3}H]α-toxin binding nor the affinity for decamethonium is affected by storage.

Torpedo marmorata membrane fragments particularly rich in receptor protein were prepared by the method of Cohen *et al.* (14).

Tritiated α_1-Isotoxin from Naja nigricollis Venom

α_1-Isotoxin purified from crude venom of *N. nigricollis* (15) was tritiated by the method of Menez *et al.* (16) and was a gift of Drs. Menez, Morgat, Fromageot, and Boquet. Stock solutions were made in 0.2 M sodium phosphate, pH 7.0, and stored at 4°. Before use, an aliquot of the stock solution was diluted in Ringer's physiological solution (160 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, and 2.5 mM sodium phosphate, pH 7.0). Several different batches of [^{3}H]α-toxin were used, with the following specific radioactivities and protein concentrations: 14 Ci/mmole, 450 μg/ml; 14.8 Ci/mmole, 405 μg/ml; 10.5 Ci/mmole, 395 μg/ml; 10.2 Ci/mmole, 163 μg/ml.

The radioactivity of [^{3}H]α-toxin solutions was measured in 10 ml of Bray's solution (naphthalene, 50 g; 2,5-diphenyloxazole, 4 g; *p*-bis[2-(5-phenyloxazolyl)]benzene, 0.2 g; methanol, 100 ml; ethylene glycol,20 ml; dioxane, to 1 liter) in an Intertechnique scintillation counter.

[^{3}H]α-Toxin Binding to Excitable Membrane Fragments

Electrophorus electricus. Membrane fragments were first diluted 10- or 20-fold in a medium whose final composition was that of the Ringer's solution described above except that the sucrose concentration was kept at 0.13 M. Occasionally 0.02% NaN_3 was added to prevent bacterial growth. It was verified that this concentration of azide did not change the initial rate of toxin binding or the total number of its binding sites. The suspension was incubated for 10 min at room temperature to reach thermal equilibrium. The reaction was started by adding a small aliquot of an approximately 0.1 μM solution of [^{3}H]α-toxin in Ringer's solution. At a given time, a 200-μl aliquot of the mixture was rapidly filtered on a Millipore filter (HAWP 02500) previously equilibrated with Ringer's solution, washed with 15 ml of ice-cold Ringer's solution, dried, and counted in flasks containing 10-ml of 2,5-diphenyloxazole, 3 g; *p*-bis[2-(5-phenyloxazolyl)]benzene, 0.3 g; and toluene, 1 liter.

The amount of radioactivity retained by the filter in the absence of membrane fragments was always estimated in parallel experiments in which all the components of the experimental mixture were present except the membrane fragments. This background radioactivity was found to be proportional to the total amount of free [^{3}H]α-toxin filtered and did not vary significantly within a given batch of filters. It ranged from 4 to 10% of the total radioactivity of the sample. This quantity was always subtracted from the number of counts retained on the filter in the presence of membrane fragments.

The total radioactivity of the reaction medium was estimated by placing a 100-μl drop of solution on a dry Millipore filter, which was dried again and counted as described above. Since the α-toxin binds to glass, reproducible pipetting was difficult; therefore conversion of counts per minute to moles was done by using the efficiency of the counter (28 $\pm$ 2% for several determinations made on four batches of [^{3}H]α-toxin of different specific activities).

The total number of toxin binding sites present in the membrane preparations was estimated by titration of the toxin solution by increasing concentrations of membrane fragments. After overnight exposure at room temperature with 0.02% NaN_3 always present, the samples were filtered and washed as previously indicated.

Torpedo marmorata. With *Torpedo* membrane fragments, exactly the same procedures were used, except that the dilutions (500- or 1000-fold) were made in a solution consisting of 250 mM NaCl, 5 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, and 5 mM sodium phosphate, pH 7. Filters were washed with the same solution.

RESULTS

Homogeneity of Tritiated α-Toxin Preparations

The α_1-isotoxin purified from the venom of *Naja nigricollis* by the method of Karlsson *et al.* (17) and Boquet *et al.* (15) was tritiated by the method of Menez *et al.* (16). The toxin was first iodinated, then dehalogenated in the presence of tritium gas, and finally purified on a hydroxylapatite column. The

only chemical difference between the tritiated product and the native α_1-isotoxin is the presence of a tritium atom instead of a hydrogen atom on a histidine residue.

The specific radioactivity of the preparations of [^{3}H]α-toxin used in this work ranged from 10 to 15 Ci/mmole. With 30,000 Ci/g atom as the specific radioactivity of tritium gas, 30–50% of the toxin molecules were labelled.

No significant difference was observed between tritiated and native α-toxins by polyacrylamide gel electrophoresis, by chromatography on Bio-Rex 70 columns (which separate denatured from native toxin), or by ultraviolet and optical rotatory dispersion spectroscopic analyses.[1]

Tritiated and native toxins show similar toxicities in mice and block the response of eel electroplax at similar concentrations (16, 5). Because of the low precision of these pharmacological tests, it became essential to determine the extent to which the radioactivity present in a solution of labelled toxin was indeed associated with active toxin, and whether the labelled toxin molecules showed the same reactivity towards the excitable membranes as with the native ones.

In a first series of experiments, solutions of [^{3}H]α-toxin were titrated with increasing amounts of excitable membrane fragments. The preparation of tritiated toxin was first diluted in Ringer's solution at a final concentration which, in general, ranged between 0.1 and 3 nM. Then various amounts of membrane fragments were added to the medium. After overnight incubation at room temperature,[2] the total radioactivity of the suspension and the fraction of radioactivity bound to the membrane fragments were measured by Millipore ultrafiltration (see METHODS). Figure 1 shows the results of two experiments of this kind performed with different preparations of [^{3}H]α-toxin. For the fresh preparation, almost 100% of the counts were removed from the solution by an excess of membrane fragments. All the radioactivity was thus associated with a molecular species which bound strongly to the membrane fragments. For the preparation of toxin stored a few months at 4°, approximately 40% of the counts were no longer bound by an excess of membrane fragments. These counts moved simultaneously with $^{22}Na^+$ on a Sephadex G-50 column and slightly after $^{22}Na^+$ on Bio-Gel P-2 [see Fig. 13 of the following paper (18)]. They were presumably associated with either tritiated amino acids, or small peptides, and they probably represented degradation products of tritiated toxin molecules. This loss in binding capacity occurred with all our preparations, approximately 7%/month of storage at 4°. Preparations which had lost more than 50% of their binding capacity were always discarded.

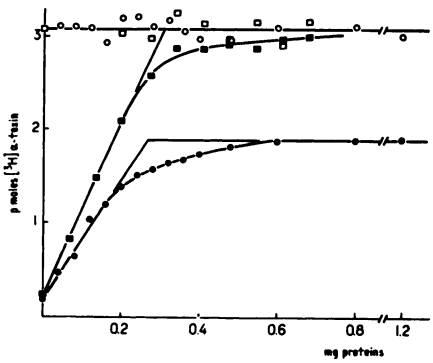

FIG. 1. *Titration of stock solution of [^{3}H]α-toxin by increasing amounts of excitable membrane fragments*

From 0 to 150 μl of two different membrane suspensions from *Electrophorus* were diluted in Ringer's solution containing 0.13 M sucrose. The reaction was started by adding [^{3}H]α-toxin. ○ and □, total radioactivity of the mixture; ● and ■, radioactivity remaining on filter after overnight incubation; ■ and □, freshly prepared [^{3}H]α-toxin 10.5 Ci/mmole; concentrations of *active* [^{3}H]α-toxin in the reactive medium = 3.5 nM, and of α-toxin binding sites, 9.2 nmoles/g of protein; ● and ○, [^{3}H]α-toxin (14.8 Ci/mmole) used after 4 months of storage at 4°; concentration of *active* [^{3}H]α-toxin in the reaction medium = 1.3 nM, and of α-toxin binding sites, 6.5 nmoles/g of protein.

[1] A. Menez unpublished observations.

[2] The amount of counts retained on the filter did not change after 1–2 days of incubation at room temperature (see, for example, Fig. 1 of the following paper (18)). In some experiments this value started to decline when the incubation time was extended beyond 2 days. This decay might have resulted from enzymatic degradation of either membrane or toxin. Such experiments were generally discarded.

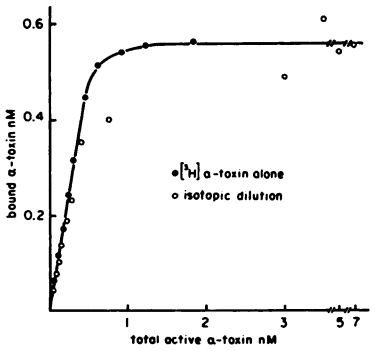

FIG. 2. *Isotopic dilution of* [³H]α*-toxin by unlabelled α-toxin*

For binding of [³H]α-toxin, membrane fragments of *Electrophorus* (7.0 g of protein per liter) were diluted 200-fold in Ringer's solution supplemented with 0.02% NaN_3. Varied amounts of [³H]α-toxin (10.2 Ci/mmole) were then added. After 24 hr of incubation at room temperature, the amount of bound [³H]α-toxin was determined as described under METHODS. The percentage of counts bound to an excess of membrane fragments was 63% of the total. Correction was made to express the bound toxin as a function of active [³H]α-toxin, assuming 63% of toxin molecules (labelled as well as unlabelled) to be active. For isotopic dilution, 75 pM [³H]α-toxin in Ringer's solution was supplemented with different amounts of a freshly prepared solution of unlabelled α-toxin (0.035–7.0 nM). Membrane fragments were then diluted 200-fold in these media, and radioactivity bound was measured as above. The background of the filters was estimated by first incubating membrane fragments with 7.0 nM unlabelled toxin for 10 min, then adding 75 pM [³H]α-toxin. The total concentration of bound toxin was calculated by assuming [³H]α-toxin and unlabelled α-toxin to be 63% and 100% active, respectively.

Monitoring of the toxicity on mice showed that an increase in the lethal dose accompanied the loss of binding capacity. Although the precision of such an experiment is rather poor, it appeared that the loss of toxicity was in the same range as the loss of binding capacity. In other words, the radioactivity remaining in solution in the presence of an excess of membrane fragments seemed to be due to a general degradation of the solution of toxin rather than to selective radiolysis of the labelled molecules.

This point was further studied with a stock solution of [³H]α-toxin (10.2 Ci/mmole) in which only 63% of the counts were bound to an excess of membrane fragments. Two series of experiments were performed, in which the concentration of membrane fragments was kept constant while that of α-toxin was varied within a large range. In the first series the concentration of toxin was changed by adding increasing amounts of labelled toxin. In the second increasing quantities of unlabelled α-toxin from a freshly prepared solution were added to a fixed concentration of tritiated α-toxin (isotopic dilution). In the latter experiment the actual amount of toxin bound should be given by multiplying the counts per minute bound by the ratio of the "total" toxin concentration to that of the "labelled" toxin molecules. Superimposition of the two sets of data was obtained, assuming that only 63% of the toxin molecules (both labelled and unlabelled) present in the stock of tritiated toxin were active while all toxin molecules were active in the fresh solution of unlabelled toxin (Fig. 2). If selective radiolysis of the labelled molecules had occurred, 88% (33 × 0.63 + 67) of the toxin molecules present in the stock solution should have been "active." Superimposition of the two sets of data was not obtained with this assumption. Therefore, after storage, the fraction of the total "active" population of toxin molecules (labelled plus unlabelled) is identical with the fraction of the total radioactivity bound at equilibrium by an excess of membrane fragments.

The concentrations of [³H]α-toxin molecules given in this paper are always expressed in terms of "active" molecules, not in amounts of stock solution of radioactive toxin. Each time a new preparation of membrane fragments was made, i.e., every 1–2 weeks, the fraction of "active" toxin molecules present in the stock solution of [³H]α-toxin used was routinely measured.

Number of [³*H*]α*-Toxin Binding Sites*

The number of [³H]α-toxin binding sites present on the membrane fragments was estimated by the two methods given in the previous paragraph, using the filtration assay. For a constant concentration of [³H]α-toxin, the slope of the linear part of

the titration curve gives the number of [^{3}H]α-toxin binding sites per mass of membrane protein. When the concentration of [^{3}H]α-toxin was varied, by adding either labelled or unlabelled α-toxin, the same information was obtained from the plateau at high α-toxin concentrations. Finally, ultracentrifugation (16) was used instead of filtration, to separate free and bound toxin. All these methods gave almost the same number of sites (Table 1).

With *Electrophorus* membrane fragments, the number of toxin sites ranged from 5 to 20 nmoles/g of protein, and their concentration in most of our preparations ranged from 50 to 150 nM. These preparations contained acetylcholinesterase. The number of acetylcholinesterase molecules can be estimated from enzymatic assays at 25°, using acetylthiocholine as substrate (11) and assuming a molecular weight of 260,000 (19, 20) and a specific activity of 610 moles of acetylthiocholine per hour per gram of protein (21). Since these values are still uncertain for the membrane-bound enzyme, the numbers proposed should be considered tentative. Within this set of assumptions, we found for most preparations a ratio of [^{3}H]α-toxin *sites* to acetylcholinesterase *molecules* of about 2. This ratio varies with the method of extraction and purification of the membrane fragments.

TABLE 1

Number of α-toxin binding sites obtained by three different methods with the same preparation of membrane fragments from Electrophorus

For each determination of the number of α-toxin binding sites a complete titration curve was constructed (see Figs. 1 and 2). The error given is that which results from the measurement of the slope or plateau value of the relevant titration curve.

Method	Toxin binding sites
	nmoles/g protein
Millipore assay	
1. Active toxin fixed at 1 nM, binding site concentration varied	18 ± 2
2. Binding site concentration fixed	
a. [^{3}H]α-Toxin	16 ± 1
b. Unlabelled α-toxin[a]	16 ± 2
Ultracentrifugation	14 ± 2

[a] Measured by the isotope dilution method.

Binding studies performed with crude homogenates before purification permit an estimate of the total number of toxin sites per kilogram of fresh organ. In the cephalic part of the main electric organ from an *Electrophorus* 1 m long, we found 110 ± 20 nmoles/kg of fresh tissue. The central part of the organ contained less sites than the front part. Organs from small eels contained more sites than those of large ones. On the average we extracted only 20–30 nmoles/kg of fresh tissue.

According to Miledi *et al.* (4), the electric tissue from *Torpedo* is much richer in α-toxin sites than that from *Electrophorus*. By the method of Cohen *et al.* (14) we prepared membrane suspensions with a specific activity of 1100 ± 600 nmoles of [^{3}H]ϕ-toxin sites per gram of protein. In this preparation the number of [^{3}H]α-toxin sites was generally more than 100 times the number of acetylcholinesterase molecules, and the protein concentration was 0.5 ± 0.2 g/liter. The total number of toxin sites per kilogram of fresh organ was 1000 ± 200 nmoles, a value very close to that found by Miledi *et al.* (4) (Table 2).

Equilibrium Constant of [^{3}H]α-Toxin Reaction with Membrane Sites

Because of the particularly high affinity of *N. nigricollis* α-toxin for its membrane sites, precise determination of the equilibrium constant is difficult. For example, at the concentrations used in Figs. 1 and 2, the equilibrium titration curves do not differ significantly from linearity until about 80% of the sites are occupied. It thus becomes difficult to estimate the concentration of free [^{3}H]α-toxin when the concentration of membrane fragments is fixed, as in Fig. 2, or of the free binding sites when the concentration of [^{3}H]α-toxin is fixed, as in Fig. 1. Measurement was improved by working at concentrations of the fixed component lower than 0.1 nM, but under these conditions the limiting factor becomes the small quantities of radioactivity to be measured and the

TABLE 2

Biochemical properties of membrane fragments from Electrophorus and Torpedo

Values are means ± standard error for eight preparations (*Torpedo*) or 10 preparations (*Electrophorus*). To calculate acetylcholinesterase concentrations, we assumed for the pure enzyme a molecular weight of 260,000 (19, 20) and a specific activity of 610 moles of acetylthiocholine hydrolyzed per hour per gram of protein (21). Ouabain-sensitive ATPase activity was measured by following hydrolysis of *p*-nitrophenyl phosphate (12).

Species	Protein	Acetylcholinesterase (*A*)		[^{3}H]α-Toxin binding sites (*B*)		*B/A*	ATPase
		Specific activity	Concentration	Specific activity	Concentration		
	g/liter	*moles acetylthiocholine/hr/g protein*	*nM*	*nmoles/g protein*	*nM*		ΔA_{420}/*min/g protein*
Electrophorus	11 ± 2.5	1.8 ± 0.5	38 ± 14	7.3 ± 2.5	77 ± 22	2.2 ± 0.6	0.53
Torpedo	0.60 ± 0.15	0.4 ± 0.2	1.6 ± 0.4	1100 ± 600	560 ± 250	330 ± 120	0.08

reproducibility of the pipetting of [^{3}H]α-toxin solutions.

A rough estimate of the equilibrium constant was given by the titration curve established in the presence of a fixed concentration, A_0, of active [^{3}H]α-toxin. If we suppose that the α-toxin, T, binds reversibly to a homogeneous class of sites R, the dissociation constant is given by $K_\alpha = (R)(T)/(RT)$. When the total concentrations of both α-toxin and binding sites are equal to A_0, we have $(R) = (T) = A_0x$ and $(RT) = A_0(1 - x)$, where x is the fraction of the total toxin molecules which are not bound to the membrane fragments. The dissociation constant is then given by the formula $K_\alpha = A_0\,x^2/(1 - x)$. x was determined from a saturation curve similar to that shown in Fig. 1. The linear part of the curve near the origin was first extrapolated until its intersection (equivalence point) with a horizontal line drawn from the plateau. Then, at this concentration of protein, $(1 - x)$ was determined on the graph as the ratio of bound to total toxin concentration.

With membrane fragments from *Electrophorus*, when $A_0 = 200$ pM, we measured $K_\alpha = 50$ pM.

The best estimates for K_α were made in the presence of either decamethonium or *d*-tubocurarine (18). Under these conditions

$$K_\alpha = (2 \pm 1) \times 10^{-11}\ \text{M}$$

Preliminary experiments have shown that with *Torpedo* membrane fragments and with the same *N. nigricollis* α-toxin, the equilibrium dissociation constant is of the same order of magnitude.

Kinetics of Dissociation of [^{3}H]α-Toxin–Electrophorus Membrane Site Complex

The kinetics of dissociation of the toxin-membrane complex was measured by adding an excess of unlabelled α-toxin (3×10^4 times the concentration of tritiated toxin) to previously labelled membrane fragments from *Electrophorus*. The reaction was followed for 70 hr at room temperature. During the first 8 hr of incubation the radioactivity retained on the filters decayed exponentially but reached a plateau value close to 50% of that initially bound. However, some precipitation of the membrane fragments frequently occurred after an equally long incubation at room temperature in Ringer's solution. This partial release thus could have been due to an alteration of our preparation rather than to heterogeneity of the binding sites. From the exponential part of the curve, we estimated a dissociation rate constant:

$$k_{-1} = (2.1 \pm 0.2) \times 10^{-4}\ \text{min}^{-1}$$

and a time for half-dissociation:

$$\tau_{1/2} = 55 \pm 5\ \text{hr}$$

Similar values were obtained with *Torpedo* membrane fragments and after solubilisation of *Electrophorus* membrane fragments by Triton X-100 (18).

Kinetics of Association of [^{3}H]α-Toxin with Electrophorus Membrane Fragments

Since the rate of dissociation of the toxin from its membrane site is rather low, the association kinetics can be reliably measured by our Millipore filtration method. Figure 3 shows the time course of [^{3}H]α-toxin binding to membrane fragments. In this experiment the concentration of [^{3}H]α-toxin was 2.8 nM and that of toxin binding sites was 4.5 nM. Half-completion of the reaction was reached

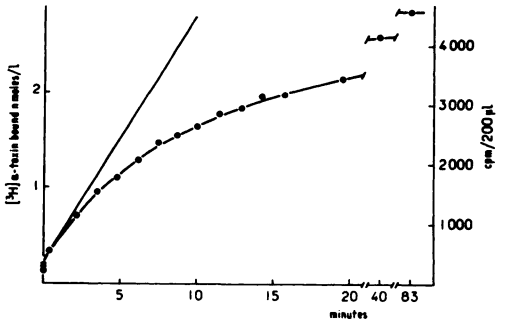

FIG. 3. *Time course of reaction of [^{3}H]α-toxin with excitable membrane fragments*

Membrane fragments (300 µl) from eel electric organ containing 8 g of protein per liter were diluted 10-fold in standard medium, and the reaction was started by adding 150 µl of [^{3}H]α-toxin (14.8 Ci/mmole). At the times shown, 200 µl of the incubation mixture were filtered as described under METHODS. The final concentration of toxin binding sites was 4.5 nM; active [^{3}H]α-toxin, 2.8 nM; protein, 0.8 mg/ml.

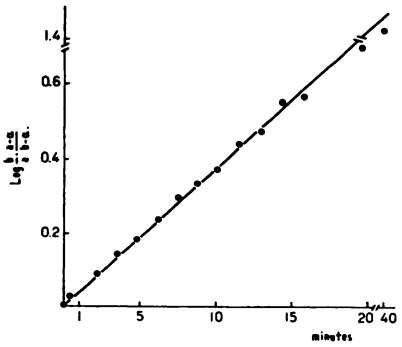

FIG. 4. *Analysis of time course of [³H]α-toxin binding in terms of an irreversible bimolecular reaction (Eq. 1)*

a is the concentration of toxin binding sites, 4.5 nM; *b*, the concentration of active [³H]α-toxin, 2.8 nM; α, the concentration of bound toxin at a given time. The data are those of Fig. 3. The slope of the straight line is 0.037 min^{-1}, and the second-order rate constant is 2.18×10^7 M^{-1} min^{-1}.

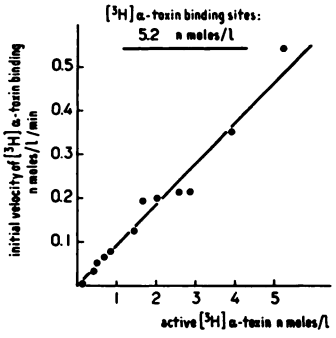

FIG. 5. *Effect of total concentration of [³H]α-toxin on initial rate of [³H]α-toxin binding*

The concentration of toxin binding sites was 5.2 nM, and that of protein was 1.25 mg/ml. The slope of the straight line gives a second-order rate constant of 1.77×10^7 M^{-1} min^{-1}.

in 8 min. Extrapolation of the curve to zero time gives a number of counts equal to or slightly higher than the background of the filters measured in the absence of membrane fragments. Since the rate of dissociation of the toxin-receptor complex is considerably slower than the rate of association, and since the concentration of the reactants is more than 100 times larger than the equilibrium dissociation constant, the reverse reaction can legitimately be neglected. Then, assuming that [³H]α-toxin binds in an irreversible manner to a homogeneous class of independent binding sites, the kinetics of association of [³H]α-toxin with its receptor site should be described by the equation

$$k_1 t = \frac{1}{a - b} \ln \frac{b}{a} \times \frac{a - \alpha}{b - \alpha} \qquad (1)$$

where *a* is the total concentration of sites, *b* the total concentration of active toxin, α the concentration of sites occupied by the toxin at a given time, and k_1 the second-order association rate constant.

Figure 4 shows that the kinetic equation fits the data for up to 40 min, the time at which approximately 90% of the active [³H]α-toxin is bound to the membrane fragments.

At 20°, in the presence of 0.13 M sucrose and at ionic strength Γ/2 = 0.18 OSM,

$$k_1 = (1.7 \pm 0.5) \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$$

As expected for a bimolecular reaction with a homogeneous population of sites, the initial rate of [³H]α-toxin binding varies linearly with the total concentration of both [³H]α-toxin (up to 5 nM) (Fig. 5) and receptor sites (up to 8 nM) (Fig. 6).

The ratio of k_{-1} to k_1 gives an estimate of

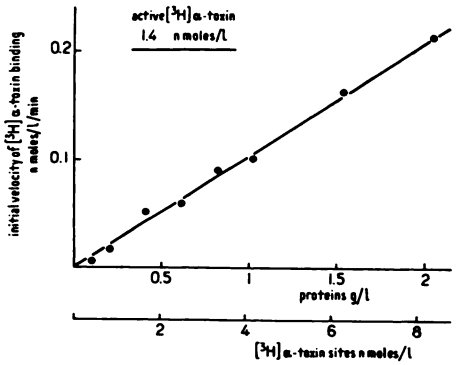

FIG. 6. *Effect of total concentration of toxin binding sites on initial rate of [³H]α-toxin binding*

Between 10 and 200 μl of a suspension of membrane fragments from *Electrophorus* (12.5 g/liter of protein) were diluted in a final volume of 1.2 ml of Ringer's solution. The sucrose concentration was kept at 0.15 M. The concentration of active [³H]α-toxin was 1.4 nM. The slope of the straight line gives a second-order rate constant of 1.75×10^7 M^{-1} min^{-1}.

TABLE 3

Number of [³H]α-toxin binding sites on excitable membrane fragments as a function of ionic strength

Membrane fragments from *Electrophorus* (10 μl) were diluted 12-fold in a medium similar to Ringer's solution, except that the concentrations of all components were multiplied by the factor indicated in the first column. The reaction was started by adding [³H]α-toxin. After overnight incubation at room temperature, the suspensions were centrifuged at 100,000 × *g* for 1.5 hr in the No. 40 rotor of a Beckman L3-50 ultracentrifuge. The amount of toxin bound was estimated by difference in the radioactivity of the solutions before and after centrifugation.

Relative concentration of Ringer's solution	Γ/2	No. of [³H]α-toxin binding sites
	osM	*nmoles/g*
1.7	0.31	14.2
1	0.18	13.5
0.2	0.036	15.7
0.1	0.018	21.5
0.05	0.009	54
0	0	300

K_α, the equilibrium dissociation constant:

$$\frac{k_{-1}}{k_1} = 14 \text{ pM}$$

This value is close to that obtained in the equilibrium studies.

With *Torpedo* membrane fragments the association rate constant is

$k_1 = (3 \pm 1) \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$ (in the absence of sucrose)

Effect of Physical Parameters on [³H]α-Toxin Binding

In order to obtain reproducible results, several properties of the reaction medium have to be carefully controlled.

Ionic strength. Table 3 shows that the total number of [³H]α-toxin molecules bound remains constant when the ionic strength is varied, as long as it is maintained higher than Γ/2 = 0.04 osM. Below Γ/2 = 0.04 osM the number of toxin molecules bound increases dramatically, up to 300 nmoles/g of protein at zero ionic strength. At low ionic strength a nonspecific binding of α-toxin occurs. A similar phenomenon was observed with a cholinergic agonist, decamethonium (22), and probably resulted from electrostatic effects.

Figure 7 illustrates that in the region of ionic strength where specific binding occurs, k_1 decreases markedly when the ionic strength increases.

Sucrose concentration. Since the membrane fragments were initially prepared in the presence of sucrose, significant quantities of sucrose were often present in the reaction medium. In the presence of sucrose the total amount of toxin bound at equilibrium does not change, but the initial rate of [³H]α-toxin binding decreases markedly (Fig. 8). However, k_1 does not decrease linearly with the reciprocal of the viscosity of the medium, as would be expected if sucrose were affecting the kinetics simply by changing the viscosity of the medium and, hence, the diffusion rate of the ligand.

Temperature. The concentration of sucrose was varied to maintain constant viscosity at all the temperatures considered. Under these conditions (Table 4) the initial rate of [³H]α-toxin binding varied only

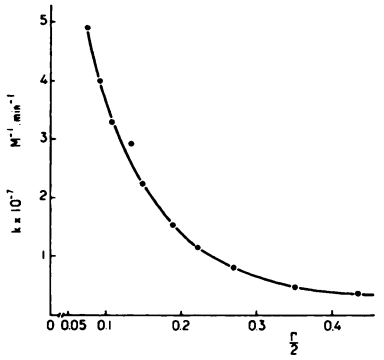

FIG. 7. *Effect of ionic strength on initial rate of [³H]α-toxin binding*

Membrane fragments (60 μl) from *Electrophorus* were diluted 20-fold in a medium containing all the components of Ringer's solution except NaCl. The NaCl concentration was varied from 0.05 to 0.41 M. The reaction was started by adding 30 μl of 89 nM [³H]α-toxin. In the final solution the concentration of toxin binding sites was 4.0 nM, that of active [³H]α-toxin was 2.5 nM, and that of protein was 0.65 mg/ml. The ionic strength of the Ringer's solution was 0.18 osM.

slightly with temperature. Between 20 and 30° $Q_{10} = 1.3 \pm 0.1$.

DISCUSSION

Since the early experiments *in vivo* by Lee and Chang (1) and *in vitro* by Changeux *et al.* (2) and Miledi *et al.* (4), several research groups have been challenged by the exceptional properties of snake venom α-toxins and have made them radioactive without loss of their pharmacological properties (24–30). Table 5 summarizes the main results and compares them with ours. All groups except ours used chemical derivatives of the native α-toxins. The method of Menez *et al.* (16) gives a labelled toxin which possesses exactly the same structure as the native toxin, except for a single hydrogen atom replaced by tritium. This preparation possesses the same toxic properties as the native toxin and is stable for 3–6 months even with its high specific activity (10–14 Ci/mmole).

In agreement with the early work of Menez *et al.* (16) and Meunier *et al.* (5), the [³H]α-toxin binds to excitable membrane fragments prepared from both *Electrophorus* and *Torpedo*. Isotopic dilution of the labelled toxin with freshly prepared, native α-toxin shows that tritiation of the toxin is not accompanied by significant change of its binding properties with respect to its membrane sites.

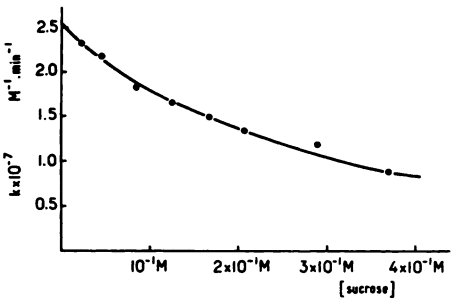

FIG. 8. *Effect of sucrose on initial rate of [³H]α-toxin binding*

A suspension (60 μl) of membrane fragments from *Electrophorus* was diluted 20-fold in the standard medium, except that the concentration of sucrose was varied from 0.022 to 0.37 M. The reaction was started by adding 30 μl of 89 nM [³H]α-toxin. The concentration of toxin binding sites was 4.0 nM, that of active [³H]α-toxin was 2.5 nM, and that of protein was 0.65 mg/ml.

TABLE 4

Variation of second-order rate constant k_1 with temperature at constant viscosity

Initial rates of toxin binding were measured as described under METHODS except that the sucrose concentration was varied to give a constant viscosity at each temperature. The amount of sucrose to be added was calculated from data in the *Handbook of Biochemistry* (23).

Temperature	1.72 cp	1.37 cp
°C	$M^{-1}\ min^{-1}$	$M^{-1}\ min^{-1}$
10	0.33×10^7	0.71×10^7
15	0.40×10^7	0.71×10^7
20	0.45×10^7	0.81×10^7
25	0.50×10^7	0.83×10^7
30	0.62×10^7	0.96×10^7

The number of [³H]α-toxin sites present in our preparations of membrane fragments has been estimated by three different methods and found to be close to 5–20 nmoles/g of protein with *Electrophorus* and 1100 ± 600 nmoles/g of protein with *Torpedo*. There exists general agreement between these results and those of other workers (Table 5) with the exception of results reported by Fiszer de Plazas and de Robertis (29). With eel membrane fragments prepared by our method, they found 50 times more sites for [¹³¹I]α-bungarotoxin than we observed for [³H]α-toxin. The reason for this discrepancy is not known.

For both *Electrophorus* and *Torpedo* membrane fragments, all our results at this time are consistent with the hypothesis that [³H]α-toxin binds to a homogeneous class of membrane sites. This result contrasts with the recent finding of Raftery *et al.* (31), who presented evidence for the heterogeneity of [¹²⁵I]α-bungarotoxin binding sites in a total homogenate of *Torpedo californica* electric tissue. A similar heterogeneity of the binding sites of [³H]α-bungarotoxin on rat diaphragm was recently reported by Chiu *et al.* (32). This discrepancy may have arisen from the fact that our work was done with purified membrane fragments, not with total homogenates of electric tissue.

The toxin binding sites present in our

TABLE 5

Reported binding parameters for association in vitro of labeled α-neurotoxins with membrane preparations from Electrophorus and Torpedo electric organs

Species	Authors	α-Toxin	Dissociation constant	No. of sites		Rate constants
				Per gram of protein of membrane fragments	Per kilogram of fresh organ	
			M	*nmoles*		
Electrophorus electricus	Menez *et al.* (16)	[^{3}H]α-toxin (native), *Naja nigricollis*		8.4		
	Raftery *et al.* (24)	[^{125}I]α-Bungarotoxin	7×10^{-8}		45	
	Fiszer de Plazas and de Robertis (29)	[^{131}I]α-Bungarotoxin	(8.8×10^{-7})	(490)	150–450	
	Fulpius *et al.* (25)	[^{3}H]α-toxin (modified), *Naja naja siamensis*	9.2×10^{-10}			$k_1 = 1.0 \times 10^7$ M^{-1} min^{-1} $k_{-1} = 4.35 \times 10^{-3}$ min^{-1}[a]
	Lindstrom and Patrick (27)	[^{125}I]α-toxin, *N. naja*	$0.97\text{–}2.3 \times 10^{-9}$	6–16		$k_1 = 3.1 \times 10^6$ M^{-1} min^{-1} $k_{-1} = 4.1 \times 10^{-3}$ min^{-1}
	Present study	[^{3}H]α-toxin (native), *N. nigricollis*	2×10^{-11}	5–20	100	$k_1 = 2.5 \times 10^7$ M^{-1} min^{-1} $k_{-1} = 2 \times 10^{-4}$ min^{-1}
Torpedo marmorata	Miledi *et al.* (4)	[^{131}I]α-Bungarotoxin		1100	1000	
	Franklin and Potter (28)				1000	$k_1 = 2 \times 10^7$ M^{-1} min^{-1}
	Present study	[^{3}H]α-toxin (native), *N. nigricollis*		1100 ± 600	1000 ± 100	$k_1 = 3 \times 10^7$ M^{-1} min^{-1}

[a] Fast component.

membrane suspensions constitute only a small fraction of those present in fresh electric tissue (10–30%). In particular, the yield of our low-speed centrifugation is rather poor (25–50%). Our fractionation methods may select certain *classes* of toxin binding sites, although we do not have direct evidence, at present, for or against this eventuality.

With *Electrophorus* electroplax, using high-resolution radioautography, Bourgeois *et al.* (13) have shown that the density of toxin sites per surface area is approximately 100 times larger under the synapses [$(30 \pm 10) \times 10^3$ sites/μ^2] than between the synapses. If we assume that our preparation of membrane fragments is homogeneous (which in fact appears unlikely), then we calculate from the observed specific activities that there should be approximately 200 toxin sites/μ^2 of microsac membrane. This value is close to that expected for extrasynaptic membrane fragments.

The difference observed between *Electrophorus* and *Torpedo* might come from the fact that subsynaptic areas constitute at least 50% of the innervated surface of the electroplax in *Torpedo* and only 2% in *Electrophorus*. It is therefore probable that our *Torpedo* microsacs derive mostly from subsynaptic areas.

In agreement with earlier results (2), we find a number of toxin binding sites in the same range as that of acetylcholinesterase molecules in *Electrophorus* membrane fragments. With *Torpedo* membrane fragments, in agreement with Cohen *et al.* (14), the two values are very different. In fact, the exact stoichiometry which exists *in situ* on the cytoplasmic membrane cannot be inferred from these data, since we know that the ratio of esterase to toxin sites varies *in vitro* according to the conditions of homogenization and fractionation.

Early studies of Tazieff-Depierre and Pierre (33) have shown with cat sciatic-gastrocnemius preparation that the curare-like effect of *N. nigricollis* α-toxin is reversible. We have confirmed this result with [^{3}H]α-toxin and *Electrophorus* membrane fragments, and for the range of toxin concentration explored (up to 10 nM free [^{3}H]α-toxin) we found the equilibrium dissociation of the [^{3}H]α-toxin–membrane fragment complex to be close to 20 pM. This value is the smallest one measured *in vitro* to date with a labelled α-toxin.

For chemically modified α-toxins from different species of snakes, much lower affinities have been found by others (see Table 5). Assuming that the chemical modifications do not alter the properties of the toxins, the discrepancy might come from the zoological origin of the α-toxin. Lee *et al.* (34) reported that the effect on the rat phrenic nerve–diaphragm preparation of α-toxin from the Formosan cobra (*Naja naja atra*) was reversible, while that of the Indian cobra (*N. naja*) was irreversible. Also, it should be noted that the reversibility of a given α-toxin could vary with the zoological origin of the receptor sites (34).

The kinetics of association of [^{3}H]α-toxin with *Electrophorus* and *Torpedo* microsacs is consistent with a bimolecular reaction between homogeneous populations of toxin molecules and binding sites. In both cases the association rate constant is $2\text{–}3 \times 10^7$ M^{-1} min^{-1}. Similar values have been found by others (see Table 5), using α-toxins having rather different affinities (some of them had affinities two orders of magnitude lower than our [^{3}H]α-toxin).

In agreement with Lee *et al.* (34), the difference in affinities observed between the various toxins would arise mainly from the difference in the dissociation rate constants.

The time course of the dissociation of the [^{3}H]α-toxin–membrane complex in the presence of an excess of unlabelled toxin follows an exponential decay for at least 8 hr, with a dissociation rate constant $k_{-1} = 2 \times 10^{-4}$ min^{-1}, but only approximately 50% of the counts are released. Although this measure is rather imprecise, the dissociation rate constant k_{-1} seems more than 40 times smaller than that found by Fulpius *et al.* (25) with *N. naja siamensis* α-neurotoxin and purified receptor protein.

The particularly high affinities observed within this group of α-toxins could be explained by the establishment of an especially stable bond between α-toxin and its receptor site. We have no evidence for such a mechanism. On the other hand, affinities of this order of magnitude are also observed with

polypeptide hormones like insulin and their membrane receptor sites (35). The most likely interpretation of these low dissociation constants is that *several* amino acid residues contribute to the establishment of the toxin–membrane site complex, as in the case of the well-known polypeptide inhibitors of trypsin (36). That the rate of association of *N. nigricollis* α-toxin is sensitive to ionic strength suggests that charged groups contribute to the binding energy. The precise nature of these groups on the α-toxin and on its receptor site has still to be determined, although the study appears feasible with the present system.

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