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Some Physical Properties of the Cholinergic Receptor Protein from *Electrophorus electricus* Revealed by a Tritiated α-Toxin from *Naja nigricollis* Venom†

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ABSTRACT: The cholinergic receptor protein present in excitable membrane fragments is labeled by an α-toxin purified from the venom of *Naja nigricollis*. After tritiation *in vitro*, the α-toxin possesses a specific activity of 14 Ci/mmole and exhibits properties identical with those of the native toxin. Reversible cholinergic effectors, like carbamylcholine, decamethonium, *d*-tubocurarine, or gallamine, and two affinity labeling reagents, protect against [^{3}H]α-toxin binding. The number of [^{3}H]α-toxin binding sites in membrane fragments is found to be 10 to 20 nmoles per g of membrane protein. The receptor–[^{3}H]α-toxin complex can be separated easily from the free toxin in solution by ammonium sulfate precipitation in the presence of 1% Triton X-100; this property is used to develop an assay for the free receptor protein in solution. Extraction of labeled membrane fragments by 1% sodium deoxycholate or 1% Triton X-100 preserves the association of the toxin to a macromolecule which precipitates in the absence of detergent but migrates as a single band on gel electrophoresis in the presence of 1% sodium deoxycholate. The [^{3}H]α-toxin–receptor complex as well as the free receptor protein in the presence of either a charged detergent (deoxycholate) or a neutral one (Triton X-100) sediments in sucrose gradients with a standard sedimentation coefficient of 9.5 S and is eluted on a Sepharose 6B column with *Escherichia coli* β-galactosidase (mol wt 540,000). Sedimentation and gel filtration data cannot be reconciled assuming that the receptor protein is a classical globular protein with a normal density. Treatment of a deoxycholate extract by sodium dodecyl sulfate gives a unit of apparent mol wt 55,000 by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. This unit is the smallest one seen before dissociation of the receptor–toxin complex.

In previous papers (Changeux *et al.*, 1970a,b; Changeux *et al.*, 1971) we described and critically discussed a binding assay which led to the first unambiguous characterization *in vitro* of the physiological receptor site to which acetylcholine binds in the course of its electrogenic action (Nachmansohn, 1959, 1971). This assay was based on the joint capacity for the receptor site to bind typical cholinergic effectors (such as decamethonium or *d*-tubocurarine) or α-toxins from snake venoms (like α-bungarotoxin (Lee and Chang, 1966) or the α-toxin from *Naja nigricollis* (Boquet *et al.*, 1966)). It was further shown that the macromolecule which carries this site can be extracted by deoxycholate in a soluble form and in appreciable amounts from the electric organ of the fish *Electrophorus electricus*. The macromolecule, a protein distinct from the enzyme acetylcholinesterase, retains *in vitro*, and *in solution*, most specific binding properties of the physiological receptor (Changeux *et al.*, 1970a, 1971; Meunier *et al.*, 1971a).

More recently Miledi *et al.* (1971) published findings on *Torpedo* electric tissue labeled with radioactive [^{131}I]α-bungarotoxin. They described the irreversible binding of the radioactive toxin to membrane fragments, the protection by

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‡ R. Olsen was a postdoctoral fellow of the National Institutes of Health (1 F01 NS48648-01).

carbamylcholine and *d*-tubocurarine against this binding and the solubilization of the receptor–toxin complex by the detergent Triton X-100. These authors described centrifugation and gel filtration experiments but did not give any numerical value for the sedimentation coefficient and the apparent molecular weight of the receptor–toxin complex in the presence of Triton X-100; on the other hand they reported that, after extraction by Triton X-100, "when the sample contained sodium dodecyl sulfate (SDS), the labeled *protein* moved in two peaks as would globular proteins having molecular weights of about 88,000 and 180,000."

In this paper we present data obtained with the help of a heavily tritiated α-toxin from *Naja nigricollis* which possesses *in vivo* and *in vitro*, properties identical with those of the native α-toxin (Menez *et al.*, 1971). Estimates of the sedimentation coefficient and of the *apparent* molecular weight (Stoke's radius) on Sepharose 6B columns of the receptor–α-toxin complex in the presence of deoxycholate or Triton X-100 are reported for the first time. Thanks to an *in vitro* assay of the free cholinergic receptor site we further show that the unbound, active, receptor protein behaves in a manner similar to that of its complex with the [^{3}H]α-toxin. Finally, we propose some estimate of the molecular weight of the smallest unit to which [^{3}H]α-toxin remains associated in sodium dodecyl sulfate. This value appears to be quite different from those reported by Miledi *et al.* (1971).

Material and Methods

Tritiated α-Toxin. Pure α-toxin, prepared from venom of *Naja nigricollis* (Boquet *et al.*, 1966; Karlsson *et al.*, 1966) containing exclusively the α_1 isotoxin,[1] was tritiated following the technique described by Menez *et al.* (1971). The stock solution of tritiated toxin was made in 1 M Na phosphate buffered at pH 7.0 and contained 650 μg of toxin per ml (96 nmoles/ml assuming mol wt 6787). The specific radioactivity of the toxin was 14 Ci/mmole.

The neurotoxin from *Naja nigricollis* contains one tyrosine and two histidine residues. The tyrosine residue is buried and unavailable to the halogenation reagents. Thus only histidine has to be considered. Iodination of histidine containing peptides can be obtained by three different reagents: iodine, hypoiodide, and iodine monochloride. Iodine and hypoiodide lead to diiodohistidine since the rate of reaction of monoiodohistidine with the reagents is faster than that of the unsubstituted amino acid (Li, 1944).

By contrast, iodine monochloride leads to monoiodohistidine only (at pH 8 as well as lower pH) (Glazer and Sanger, 1964). This reagent was used for iodination of the α-toxin as a prerequisite for its tritiation. Iodination was stopped when 0.86 μmole of peptide had reacted with 0.65 μg-atom of iodine. Iodotoxin (0.14 μmole) was dehalogenated by substitution of iodine by tritium and the resulting ^{3}H peptide was diluted with 0.21 μmole of normal α-toxin. If the iodotoxin carried 1 iodine atom per mole the resulting specific radioactivity should be 12 Ci/mmole; 14 Ci/mmole were found. Taking into account the high dilutions required for such measurements, both figures are in good agreement and indicate that the original labeled material carried one ^{3}H per mole. Thus, the final product was made of 4 labeled peptides for 10 present. That most of the labeled material exhibits the relevant properties of the α-toxin is ascertained by the experiments described in the first part of the present paper. Radiolysis of the labeled toxin is unavoidable and one of its effects may well be that some peptide molecules are damaged so as to loose their full binding capacity.

The radioactivity of tritium was measured in flasks containing 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–ethyleneglycol, 20 ml–dioxane up to 1 l.). The flasks were counted in either a Packard TriCarb 574 or an Intertechnique LH 30 scintillation counter; 1 μl of the stock solution of toxin (96 pmoles) gave 4–8 × 10^6 cpm. The efficiency of counting was 15% in the Packard counter and 30% in the Intertechnique.

Acetylcholinesterase. The hydrolysis of acetylthiocholine was followed by the method of Ellman *et al.* (1961). The composition of the assay medium was 5 × 10^{-4} M acetylthiocholine chloride, 5 × 10^{-4} M dithiobisdinitrobenzoic acid, 5 × 10^{-2} M sodium phosphate buffered at pH 7.0 for a total volume of 1.0 ml. Under these conditions a change of absorbance of 1.0 unit at 412 nm corresponds to a change of concentration of acetylthiocholine of 1.36 × 10^4 moles/l.

Proteins. The method of Lowry and associates (1951) was used routinely with bovine serum albumin as a standard. In the presence of Triton X-100 the reaction was done under continuous stirring and the sample was centrifuged before reading the optical density at 650 nm.

Preparation of Excitable Membrane Fragments. Membrane fragments were prepared following the technique of Changeux *et al.* (1969) as modified by Kasai and Changeux (1971). Routinely, homogenization was carried out from cephalic parts of fresh electric organs taken from small eels. To 20 g of electric organ minced with scissors was added 50 ml of ice-cold 0.2 M sucrose in H_2O. Homogenization was carried out at 0° in a Virtis apparatus, in a 100-ml glass vessel at 95% of maximal speed. The homogenate was submitted to ultrasound for 60 sec at 0° in a Mullard apparatus L277 and centrifuged at 4° in the *S*S34 rotor of a Sorvall centrifuge at 6500 rpm (5000*g*) for 20 min; 19 ml of supernatant was then layered on top of a discontinuous sucrose gradient containing 5 ml of 1.1 M and 5 ml of 0.4 M sucrose. Centrifugation was carried out in a SW 25.1 rotor in a Beckman ultracentrifuge Model L at 25,000 rpm (90,000*g*) for 270 min at 4°. Fractions of approximately 1 ml were collected after perforation of the bottom of the tube with a needle. The tubes containing the fractions with the highest activity of acetylcholinesterase were used in the experiments. The specific activity of acetylcholinesterase usually ranged between 0.5 and 3 moles of acetylthiocholine hydrolyzed per hr and per g of protein. The concentration of proteins was 5 to 10 mg per ml.

Sedimentation in Sucrose Gradients. The sample (0.15 ml) supplemented with 400 μg of horse heart cytochrome *c*, 500 μg of beef liver catalase, and 20 μg of *Escherichia coli* β-galactosidase (provided by Dr. A. Ullmann) to a final volume of 0.20 ml was added on top of a 5-ml linear sucrose gradient containing 5 × 10^{-3} M Tris (pH 8.0)–5 × 10^{-3} M sodium citrate–1% deoxycholate or 1% Triton X-100. The concentration of sucrose was 20% at the bottom of the tube and 5% at the top. The tubes were centrifuged in a SW 65 K Beckman rotor of a Spinco Model L ultracentrifuge at 60,000 rpm (390,000*g* average) for 150 min at either 20° or 4°. Fractions of approximately 0.11 ml were collected after perforation of the bottom of the tube.

After centrifugation, β-galactosidase was assayed by following the hydrolysis of orthonitrophenyl β-galactoside according to the method of Horiuchi *et al.* (1962); catalase was estimated by following the decrease of absorption of a solution contain-

[1] P. Boquet, to be published.

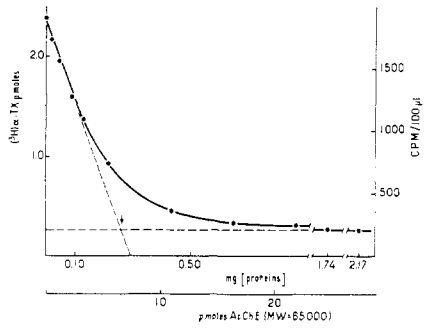


FIGURE 1: Titration of a solution of [^{3}H]α-toxin by excitable membrane fragments. Excitable membrane fragments purified by the technique described in Methods were added as a suspension in 0.7 M sucrose and at the indicated concentrations to the solution of toxin. The final solution in a volume of 0.5 ml contained: 2.3 pmoles of [^{3}H]α-toxin, 1.6×10^{-1} M NaCl, 5×10^{-3} M KCl, 2×10^{-3} M $MgCl_2$, 2×10^{-3} M $CaCl_2$, 3×10^{-4} M sodium phosphate, pH 7.0, and 3.5×10^{-1} M sucrose. The mixture was incubated 45 min at room temperature and immediately centrifuged in a Beckman ultracentrifuge Model L with a rotor 30 or 40 at 30,000 rpm (approximately 100,000 *g*) for 45 min at 4°. Aliquots of the supernatant were counted. The number of [^{3}H]α-toxin binding sites found with this particular preparation was 8.4 nmole/g of protein (see Menez *et al.*, 1971).

ing enough H_2O_2 to give an absorbance of 1.5–1.7 at 240 nm in 5×10^{-2} M Na phosphate (pH 7.0). The sedimentation coefficients were estimated by the method of Martin and Ames (1961) using β-galactosidase ($s_{20,w} = 16.0$) and catalase ($s_{20,w} = 11.4$) as references.

Labeling of Membrane Fragments by [^{3}H]α-Toxin. The suspension of excitable membrane fragments in 7×10^{-1} M sucrose was mixed with an equal volume of [^{3}H]α-toxin solution in twice concentrated Ringer's saline medium (3.2×10^{-1} M NaCl, 10^{-2} M KCl, 4×10^{-3} M $CaCl_2$, 4×10^{-3} M $MgCl_2$, 5×10^{-3} M Na phosphate, pH 7.0). The concentration of [^{3}H]α-toxin in the concentrated Ringer's solution was, except when noted, 65 ng/ml (9.6 pmole/ml). The membrane fragments were treated with toxin at room temperature for 90 min, centrifuged 30 min at 30,000 rpm (100,000*g*) in a rotor 40 of a Beckman preparative ultarcentrifuge, and resuspended in the appropriate buffer. The relative concentrations of [^{3}H]α-toxin and membrane fragments were such that approximately 1% of the cholinergic receptor sites were occupied by the toxin.

Preparation of Soluble Extracts. In several experiments, soluble deoxycholate extracts of the active receptor protein were made following the method described by Changeux *et al.* (1971). The extracts contained from 1 to 10 mg of protein per ml and hydrolyzed up to 7 moles of acetylthiocholine per hour and per g of proteins.

Preparations of the soluble receptor–[^{3}H]α-toxin complex were often made from purified membrane fragments previously labeled with the tritiated toxin. A pellet of about 20 mg of protein of labeled membrane fragments was resuspended in 1.6 ml of 0.5 M Tris (pH 8.0) containing 3% sodium deoxycholate and gently stirred for 60 min at room temperature. The solution was then centrifuged at 100,000*g* (30,000 rpm) in a rotor 30 of a Beckman ultracentrifuge for 30 min. Under these conditions 70–90% of the radioactivity originally bound to the membrane fragments was recovered in the supernatant.

Gel Filtration on Sepharose 6B (Pharmacia). The Sepharose beads were decanted several times and extensively washed in 5×10^{-3} M Tris (pH 8.0), 5×10^{-3} M sodium citrate, and 1% deoxycholate (or Triton X-100) at 4°. The column was then packed, in general, with a bed volume of 200 ml and a height to diameter ratio of approximately 18. The column was washed with 500 ml of the equilibration buffer; 1–2 ml of the soluble preparation containing approximately 10 mg of protein per ml and approximately 40,000 cpm per ml, supplemented with 200 μg of β-galactosidase, 5 mg of catalase, and 2 mg of cytochrome *c* was then applied to the column.

Elution was carried out at 4° with the equilibration buffer at a flow rate of about 20 ml/hour. Fractions of 3.2–3.3 ml were collected by a Gilson fraction collector model "Miniescargot" MTDC. The radioactivity of 0.5 ml of each sample was then counted in 10 ml of Bray's solution. β-Galactosidase and catalase were assayed following the methods previously described. The presence of cytochrome *c* was revealed by measuring the optical density at 550 nm. Stoke's radii were estimated by linear interpolation using β-galactosidase (mol wt = 540,000), catalase (mol wt = 240,000), and cytochrome *c* (mol wt = 12,384) as references.

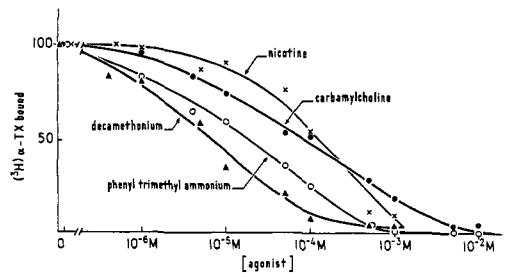
Assay for the Free Receptor Protein. To 1.0-ml samples containing increasing amounts of soluble extract in 5×10^{-3} M Tris (pH 8.0), 5×10^{-3} M trisodium citrate, and 1% Triton X-100 was added 1.0 μl of α-toxin solution. The ratio of cold to tritiated toxin was adjusted in such a manner that the absolute concentration of α-toxin in the final dilution was of the same order of magnitude as the concentration of receptor assayed. The mixture was then incubated for 60 min at room temperature. Then, 371 mg of solid ammonium sulfate or 1.5 ml of a solution saturated with ammonium sulfate was added to each tube yielding a final solution 60% saturated in ammonium sulfate. After complete dissolution the samples were centrifuged 30 min at 100,000*g* (30,000 rpm) in a rotor 40 of a Spinco ultracentrifuge at 4°. The radioactivity of 0.1 ml of the supernatant was counted.

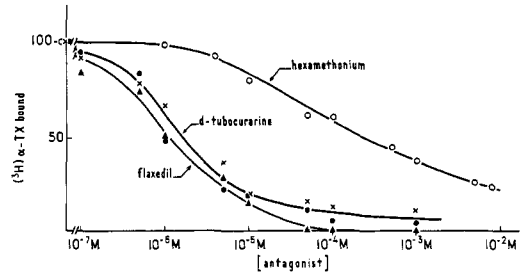
Polyacrylamide Gel Electrophoresis. Gels were made following the procedure described in Weber and Osborn (1969). In one series of experiments, the gels contained 1% deoxycholate, 5×10^{-3} M Tris-HCl (pH 8.0), 5×10^{-3} M trisodium citrate, and 5% polyacrylamide. In another series the gels were made in 0.1% sodium dodecyl sulfate, 10^{-1} M sodium phosphate (pH 7.1), with 7.5% polyacrylamide. The proteins used as references were β-galactosidase from *E. coli* (mol wt 135,000), beef liver catalase (mol wt 60,000), yeast alcohol dehydrogenase (Sigma) (mol wt 37,000), and horse heart cytochrome *c* (mol wt 12,384). These proteins were visualized by staining 6 hr in 0.2% Coomassie Brilliant Blue in 7.5% acetic acid and destaining 2 days in acetic acid. In most of the experiments dansylated lysozyme and bovine serum albumin, prepared by the technique described by Inouye (1971), were used as standards.

Electrophoresis was run in a Beckman duostat. The gels were cut into 1-mm slices with a set of Gillette razor blades and incubated overnight in 0.5 ml of 15% H_2O_2 at 60–70°. Then 0.1 ml of Hyamine hydroxide was added. After 3 min at 60°, the sample was finally mixed with 10 ml of Bray's solution and counted. Quenching of 70% was observed with this system. Of the remaining 30%, 50–60% were recovered in the peaks of all gels described in the text.

Results

1. Specific Binding of the Tritiated α-Toxin from Naja nigricollis to the Cholinergic Receptor Protein. As already mentioned (Menez *et al.*, 1971), the tritiated toxin kills 20-g mice with a lethal dose LD_{50} of 2.4 μg and an LD_{100} of 3.35 μg which is close to the LD_{50} of 1.7 μg and the LD_{100} of 2.0





FIGURES 2 (LEFT) and 3 (RIGHT): Protection against [^{3}H]α-toxin binding to excitable membrane fragments by reversible cholinergic agonists and antagonists. Exposure to the tritiated toxin was carried out under the conditions described in Methods under the paragraph labeling of membrane fragments except that (1) the membrane fragments were incubated with the indicated concentration of cholinergic agent before the addition of [^{3}H]α-toxin, (2) the time which elapsed between the addition of membrane fragments to [^{3}H] α-toxin and the start of the centrifugation was only 15 min. The curves relative to flaxedil, decamethonium, phenyltrimethylammonium, and carbamylcholine were made with the same membrane preparation; those relative to hexamethonium and *d*-tubocurarine with a second preparation, and that for nicotine, with a third. With *d*-tubocurarine, one experiment was done at 4° (▲), the other at 22° (x). The concentration of protein in the incubation medium was between 1.5 and 2.5 mg/ml. The conditions were such that 50–70% of the total amount of [^{3}H]α-toxin added was bound after 15-min exposure.

μg found with the native toxin. On the isolated electroplax a 1-μg/ml solution of the tritiated toxin blocks the depolarization caused by a cholinergic agonist carbamylcholine to the same extent (75–85%) as a 1-μg/ml solution of the native compound. Thus no significant difference can be demonstrated in the gross toxicity and in the physiological action of the native and tritiated toxins.

The specific radioactivity of the [^{3}H]α-toxin used in these experiments (14 Ci/mmole) is approximately 40% of the theoretical value calculated if in each toxin molecule, one hydrogen atom is exchanged by one tritium atom (30 Ci/mmole). Only four out of ten toxin molecules are labeled and one might argue that the activity assays are not sensitive enough to tell a loss of 40% activity. We therefore studied the interaction of the tritiated toxin with excitable membrane fragments prepared from homogenates of electric organ by a simple centrifugal assay (see Methods and captions of Figures 1 and 2). We first confirmed that the tritiated toxin binds to the membrane fragments. The reaction proceeded with a second-order rate constant of approximately 10^7 mole^{-1} min^{-1} at 22°.

The toxin–membrane complex was not dissociated by subsequent centrifugation and resuspension of the membrane fragments. Under the present experimental conditions the binding of the toxin appeared to be irreversible.

Figure 1 shows the titration of a solution of [^{3}H]α-toxin by increasing amounts of excitable membrane fragments. After extensive exposure of the membrane fragments to the toxin, the fragments were centrifuged and the supernatant counted. In the experiment reported in Figure 1 approximately 10% of the counts remained in solution, showing that at least 90% of the radioactivity was borne by physiologically active α-toxin. This quantity was found to decrease slowly with the time of storage of the toxin solution. After storage of the stock solution of tritiated toxin for 3 months at 4° only 50% of the radioactivity was found to bind to membrane fragments.

By extrapolation of the titration curve a direct count of the total number of toxin binding sites present in our membrane preparation was obtained. This number always depended on the purity of the membrane fragments but was usually close to 10–20 nmoles per g of membrane protein.

We then checked that the binding of the toxin to bacterial membranes was negligible. In addition autoradiographic studies (to be published) showed that [^{3}H]α-toxin binds exclusively to the innervated membrane of the electroplax.

In order to gain further information on the nature of the site on the innervated membrane to which [^{3}H]α-toxin binds, the effect of a variety of *reversible* cholinergic agents was tested. The suspension of membrane fragments was first exposed to a given concentration of effector under the conditions given in "Methods" for a few minutes, then the radioactive toxin was added, and the mixture incubated at room temperature for 20 min within the centrifuge. Figures 2 and 3 indicate that all the cholinergic agents tested markedly decrease the amount of toxin associated with the membrane fragments after 15-min incubation. At high levels of effector this fraction even becomes negligible. We then tried to estimate the potency with which a given cholinergic agent protects against toxin binding. In the first series of experiments using the ultracentrifugal assay we determined the concentration (I_{50}) of cholinergic effector which reduces by 50% the amount of [^{3}H]α-toxin bound by the control (see caption of Figure 2) after the same length of time. This empirical value, I_{50}, was found to be of the same order of magnitude as the apparent dissociation constant measured with the isolated electroplax or the excitable microsacs.[2] In addition, when the agonists and antagonists were ranged by increasing affinities, the I_{50}'s ranged in the same order (Figure 4).

Figure 5 shows the effect of the preincubation of membrane fragments with compounds known to act *in vivo* as *irreversible* cholinergic antagonists. Two of them were tried: *p*-(trimethylammonium)benzenediazonium fluoroborate (Changeux *et al.*, 1967) or the dinaphthyldecamethonium mustard of Rang and Ritter, $DNC_{10}M$ (Rang and Ritter, 1969). Both prevent [^{3}H]α-toxin binding. Here again the concentrations giving protection *in vitro* fall into the range of those which block *in vivo* the response of the electroplax to cholinergic agonists.

The qualitative agreement observed between the potency with which a given compound protects against [^{3}H]α-toxin binding and acts as a cholinergic agent on the excitable membrane confirms our earlier conclusion that the α-toxin does bind to the cholinergic receptor protein. The fact that

[2] More quantitative studies have been recently developed with the help of a rapid filtration assay for [^{3}H]α-toxin binding. From the protection experiments, "real" dissociation constants of the cholinergic effectors from the cholinergic receptor site are measured and found to coincide almost exactly with the "apparent" dissociation constants obtained by following the permeability response *in vivo* or *in vitro*. The difference of shape seen on Figure 2 between the inhibition curves by nicotine and carbamylcholine is not significant (Weber *et al.*, 1972).

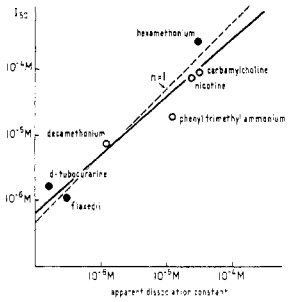


FIGURE 4: Comparison of the apparent dissociation constants of a series of agonists and antagonists with their ability to protect against [^{3}H]α-toxin binding. The apparent dissociation constants, measured *in vivo* with the isolated electroplax, are taken from the work of Higman *et al.* (1963), Bartels and Podleski (1964), Changeux *et al.* (1967). I_{50} is the concentration of cholinergic agent which reduces by 50% the total amount of toxin bound after 15-min exposure at room temperature. The experimental data are those of Figures 2 and 3. The solid line was drawn following the least-squares method; its slope is $n = 0.88$.

the protection is almost complete at concentrations of cholinergic agent that are still physiological further shows that, under our experimental conditions, the [^{3}H]α-toxin binds *exclusively* to the cholinergic receptor protein present in the excitable membrane fragments.

It was demonstrated in previous works by ourselves and others (Changeux *et al.*, 1970a,b; Miledi *et al.*, 1971) that exposure of membrane fragments to detergents like sodium deoxycholate or Triton X-100 gives a soluble preparation of receptor protein which still binds cholinergic agonists and antagonists and the native α-toxin. The [^{3}H]α-toxin binds as well to the receptor protein in solution; Figure 6 illustrates how this property can be used to assay the free receptor protein. In the presence of a 60% saturated solution of ammonium sulfate the receptor protein and its complex with the toxin precipitate while the free toxin remains in solution. Titration of the active receptor protein can thus be obtained after extensive reaction of a limited quantity of soluble receptor with stoichiometric amounts of [^{3}H]α-toxin. The difference between the total quantity of [^{3}H]α-toxin added and the free toxin remaining in solution after precipitation of the protein–toxin complex by ammonium sulfate gives the concentration of free receptor protein. The extract used in the

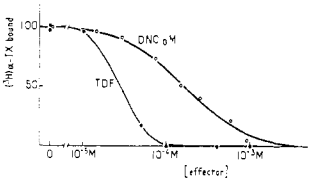


FIGURE 5: Protection against [^{3}H]α-toxin binding by affinity labeling reagents of the cholinergic receptor site. Membrane fragments were preincubated for 20 min at room temperature with the indicated concentration of dinaphthyldecamethonium mustard ($DNC_{10}M$) of Rang and Ritter (1969) and of trimethylammoniumbenzenediazonium difluoroborate (TDF, Fenton and Singer, 1965; Changeux *et al.*, 1967). The composition of the preincubation medium was the same as the one used consistently in labeling experiments (see Methods). The reaction was stopped by adding enough L-histidine to make its final concentration 10^{-2} M. After 15 min the tritiated [^{3}H]α-toxin was added and the mixture incubated 50 min at room temperature and then centrifuged under the same condition as for Figures 2 and 3.

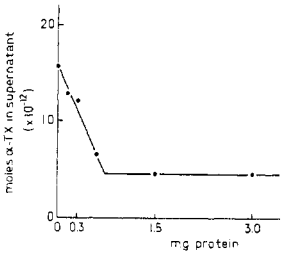


FIGURE 6: Titration of a solution of [^{3}H]α-toxin by increasing amounts of free receptor protein extract. Experimental procedure is described in Methods.

experiment shown in Figure 6 contained 13.9 nmoles of free toxin receptor sites per g of protein.

2. Gel Electrophoresis in the Presence of 1% Deoxycholate. Sodium deoxycholate treatment of membrane fragments labeled with [^{3}H]α-toxin yields, under the conditions specified in Methods, solubilization of 70–90% of the counts. The question one then asks is, to what kind of molecule are these counts associated? A first answer to this question was given by the gel electrophoresis of the soluble deoxycholate extract in the *presence of 1% deoxycholate*. Figure 7 shows that most of the radioactivity migrated toward the anode as a single peak but that this peak was clearly distinct from the peak of free toxin obtained on a separate gel.

Deoxycholate extraction thus does not release the free toxin in solution but preserves its association with a negatively charged membrane component which migrates in the gel as a homogeneous species. For the reasons of specificity mentioned above this component carries the cholinergic receptor site.

3. Sedimentation of the Receptor Protein Solubilized by Deoxycholate and Triton X-100. Figure 8 shows the result of the centrifugation at 20° of a deoxycholate extract of labeled membrane fragments in a sucrose gradient performed in 1% deoxycholate. This pattern presents several interesting features. First of all, the radioactivity sedimented as a single symmetrical peak which was clearly distinct from the peak of free [^{3}H]α-toxin. Assuming that the standard sedimentation coefficients of catalase and β-galactosidase at 20°, for a solvent with the viscosity and density of water are, respectively, 11.4 S and 16.0 S in 1% deoxycholate, one then finds that the standard sedimentation coefficient of the bound toxin is, by linear interpolation, 10.0 S. Interestingly under these conditions the acetylcholinesterase extracted from the membrane fragments forms aggregates with sediment much

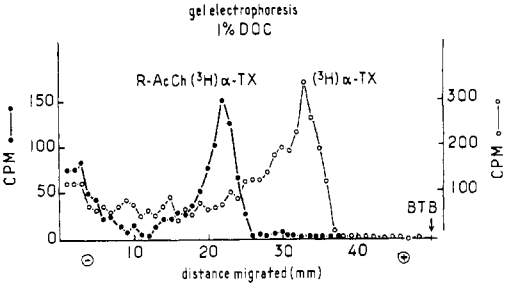


FIGURE 7: Polyacrylamide gel electrophoresis in 1% deoxycholate of a soluble deoxycholate extract of membrane fragments labeled by [^{3}H]α-toxin. An electrophoresis of the free [^{3}H]α-toxin was run simultaneously, in parallel, in a separate gel.

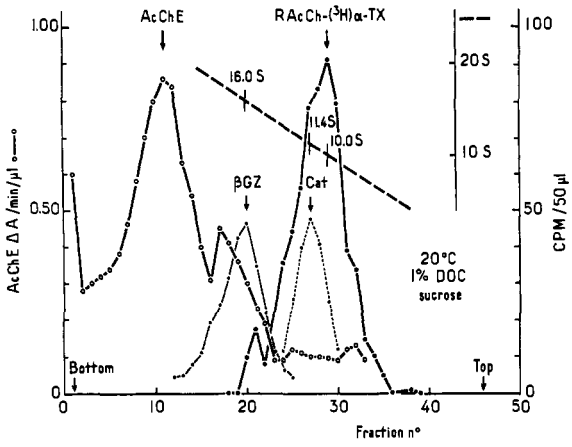


FIGURE 8: Sedimentation in a sucrose gradient containing an ionic detergent, 1% deoxycholate, of membrane fragments labeled by [³H]α-toxin and dissolved in 1% deoxycholate. The conditions are given in Methods. The temperature was 20°. Cat is catalase, βGZ is β-galactosidase, AcChE is acetylcholinesterase.

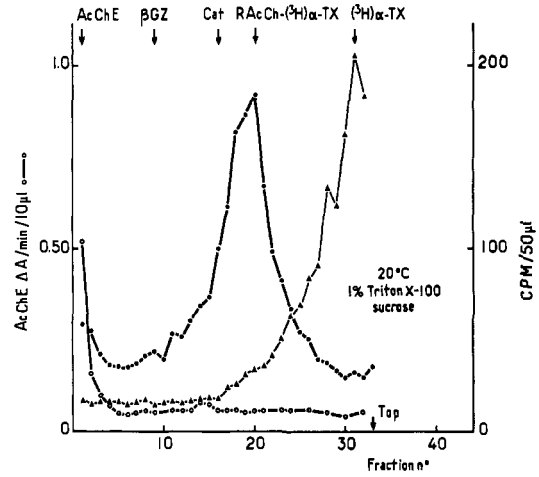


FIGURE 9: Sedimentation in a sucrose gradient containing a nonionic detergent, 1% Triton X-100, of membrane fragments labeled by [³H]α-toxin and dissolved in 1% Triton X-100. Conditions are the same as for Figure 8. The free [³H]α-toxin was centrifuged in the same rotor but in a separate tube. Abbreviations are the same as for Figure 8.

faster than the bound toxin (approximate standard sedimentation coefficient 20 S). This experiment illustrates particularly well the previously established fact (Meunier *et al.*, 1971a; Miledi *et al.*, 1971) that the cholinergic receptor site and the catalytic site of acetylcholinesterase are carried by different polypeptide chains.

When the experiment was performed at 20° with membrane fragments solubilized by 1% Triton X-100 and in the presence of 1% Triton X-100, similar results were obtained. The standard sedimentation coefficient of the bound toxin was 9.5 S in the experiment represented in Figure 9 and again the peak of radioactivity was distinct from the peak of acetylcholinesterase (in this particular experiment most of the enzyme was packed at the bottom of the tube).

When the run was made at 4° instead of 20° in the presence of either 1% Triton X-100 or 1% deoxycholate and with the same markers, the same standard sedimentation coefficients were measured.

The observation of identical sedimentation patterns of the labeled protein relative to β-galactosidase and catalase, and the absence of unusual behavior of the standards in the two detergents strongly suggest that the standard sedimentation coefficient measured is an intrinsic physical property of the [³H]α-toxin–receptor complex.

It was then of interest to see if binding of the [³H]α-toxin to the solubilized receptor had some effect on its sedimentation velocity. We therefore centrifuged an unlabeled Triton X-100 extract of excitable membrane fragments and subsequently titrated with the tritiated toxin the free cholinergic receptor sites in the collected fractions following the technique described in Methods. Figure 10 shows that the standard sedimentation coefficient of the macromolecular structure which carries the free cholinergic receptor site(s) is again 9.5 S. Binding of the [³H]α-toxin thus has no significant effect on the sedimentation properties of the cholinergic receptor protein.

4. Gel Filtration on Sepharose 6B Columns. As already reported in previous work (Changeux *et al.*, 1971; Miledi *et al.*, 1971), the receptor protein is not soluble in aqueous solvents in the absence of detergents. Gel filtration of receptor extracts thus was always performed *in the presence* of detergents. Figures 11 and 12 show the filtration profile of Sepharose 6B columns of soluble extracts of purified membrane fragments labeled with [³H]α-toxin. One filtration was done in the presence of 1% deoxycholate, the other in the presence of 1% Triton X-100. The distribution of radioactivity appeared to be exactly the same in the effluent of the two columns. Under the present experimental conditions, where only a small fraction (1%) of the cholinergic receptor site was labeled by the [³H]α-toxin, two main peaks were present.

These two peaks were clearly distinct from the peak of free toxin which shortly followed cytochrome *c*, both on separate columns, and on columns made with extracts loaded with a large excess of toxin.

The first minor peak of radioactivity corresponds to proteins excluded from the gel: *i.e.*, to particles larger than spherical particles of molecular weight 4×10^6. As expected from the centrifuge experiments acetylcholinesterase is recovered among the excluded proteins. The relative amount of radioactivity present in this peak appears to vary from experiment to experiment: 10% in some cases, 30% in others. It increases upon incomplete dissolution of the membrane fragments or prolonged storage of the extracts.

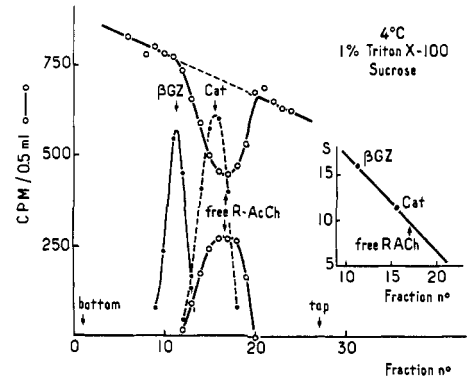
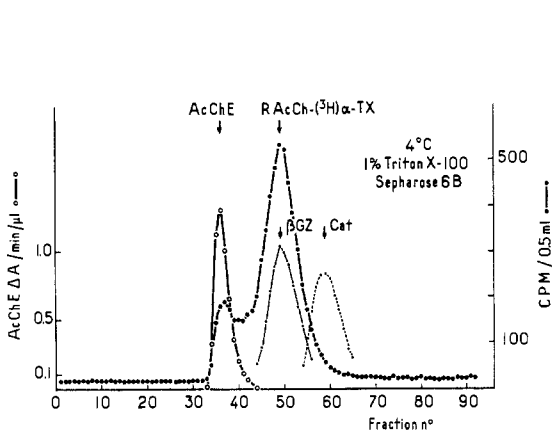
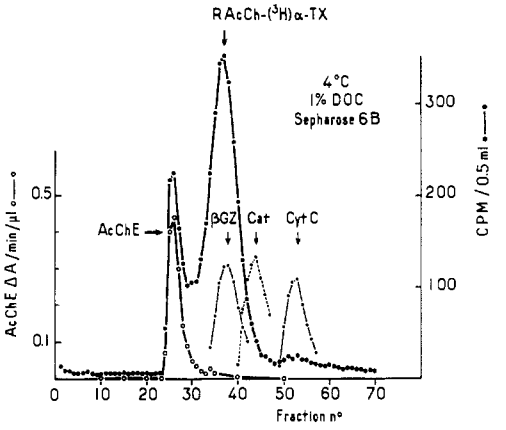


FIGURE 10: Sedimentation in a sucrose gradient containing 1% Triton X-100 of a soluble extract of unlabeled membrane fragments. The free receptor protein is assayed in fractions collected after centrifugation as described in Methods. Other conditions are the same as for Figure 9.





FIGURES 11 (RIGHT) and 12 (LEFT): Filtration on a Sepharose 6B, column in the presence of an ionic detergent (1% deoxycholate, Figure 11) or a nonionic detergent (1% Triton X-100, Figure 12) of membrane fragments labeled by [³H]α-toxin and solubilized by the same detergent. Conditions are given in Methods. Elution was always carried out at 4°.

The second peak of radioactivity, in which the majority of the counts were recovered, repeatedly coincided or slightly preceded that of *E. coli* β-galactosidase used as a marker. It was as sharp and symmetrical as that shown by β-galactosidase. Moreover, the radioactive peak and β-galactosidase had the same position in the presence of either 1% deoxycholate or 1% Triton X-100. In addition the position of the peak of β-galactosidase was also the same with the same column, in the presence and in the absence of detergents. Figures 11 and 12 show that no counts were recovered between this peak and that of free toxin. The peak of radioactivity thus reveals a unit of well-defined size: it is the smallest one which can be extracted, up to now, from membrane fragments by deoxycholate or Triton X-100; as shown later this unit is still able to bind cholinergic ligands in the absence of radioactive toxin.

Keeping in mind that gel filtration gives indications on the Stoke's radius of a macromolecule rather than on its *true* molecular weight, an *apparent* molecular weight of the component present in the major peak of radioactivity was determined by using β-galactosidase, catalase, and cytochrome *c* as standards. We checked that with columns run in the presence of 1% Triton X-100, the log of the known molecular weight of the three markers plotted as a function of the fraction number in which these markers appear gave a straight line. The same line with the same slope was obtained as when the same markers were used with this column in the absence of Triton X-100. We thus feel that apparent molecular weights obtained by gel filtration in Triton X-100 are reliable. In the presence of 1% deoxycholate we noticed, however, some deviations in elution behavior of standard proteins. For example, cytochrome *c* and commercial acetylcholinesterase (from Sigma Inc.) appeared to possess apparent molecular weights larger than the ones expected from their real molecular weights. In any case the apparent Stoke's radius of the receptor–toxin complex in Triton X-100 or deoxycholate was always in the same range as that of a globular protein of mol wt 550,000 ± 50,000.

Surprisingly, this value did not agree with the molecular weight value expected from a sedimentation coefficient of 9.5 S assuming the receptor–toxin complex to be globular and to have a partial molal volume ($\bar{v}$) equal to that of the standard proteins. We therefore checked if some aggregation had occurred within the column during the filtration by centrifuging in a sucrose gradient a sample from the peak of radioactivity which came out of the column with β-galactosidase. The sedimentation coefficient of the radioactive species was still 9.5 S. Irreversible aggregation of the receptor–toxin complex thus did not account for the high value of the apparent size.

We then investigated to discover if the actual association of the toxin with the receptor protein had some influence on its extractability and on its filtration properties. In the experiment represented in Figure 13, the extracts added to the column were labeled by traces of [³H]α-toxin. The concentration of free cholinergic receptors was then much larger (1 : 100) than that of bound receptor. In the effluent we assayed both the complex [³H]α-toxin by counting directly the radioactivity of the sample and the free cholinergic receptors sites using the ammonium sulfate precipitation assay. Figure 13 shows that the profiles of elution of the free and bound receptors superimpose exactly. Thus, there is no significant effect of toxin binding on the apparent molecular weight measured by gel filtration in the presence of detergents.

5. Gel Electrophoresis in Sodium Dodecyl Sulfate. Gel filtration in the presence of 0.5% sodium dodecyl sulfate of a Triton X-100 extract of *Torpedo* electric tissue labeled by [¹³¹I]α-bungarotoxin gives, according to Miledi *et al.* (1971), peaks of radioactivity which correspond to *apparent* molecular weights of 88,000 and 180,000. We therefore tried to see if this was true as well with *Electrophorus* extracts but we

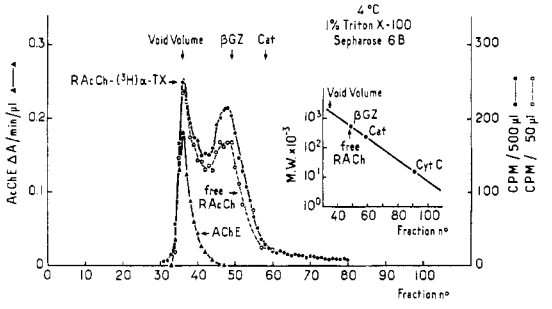


FIGURE 13: Filtration on a Sepharose 6B column equilibrated with 1% Triton X-100 of membrane fragments labeled by a trace of [³H]-α-toxin and dissolved in 1% Triton X-100. After elution, the radioactivity of a sample of each fraction was counted (●), and in the same fraction the *free receptor protein* (○) was measured with the standard assay.

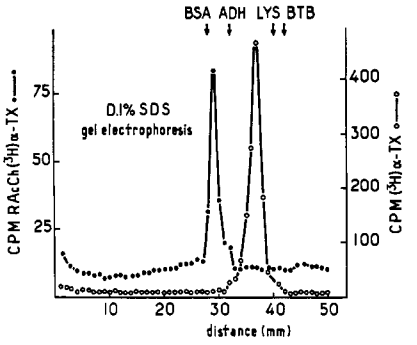


FIGURE 14: Polyacrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate of a soluble deoxycholate extract of [^{3}H]α-toxin-labeled membrane fragments. (●) [^{3}H]α-Toxin-labeled membranes dissolved in 0.3 M NaCl–0.3 M Tris (pH 8.0) and 2% deoxycholate and dialyzed against 1% sodium dodecyl sulfate 16 hr at room temperature; 3000 cpm was applied to the gel. (○) Parallel gel of [^{3}H]α-toxin alone in twofold Ringer's solution incubated with 1% sodium dodecyl sulfate 4 hr at room temperature; 5000 cpm was applied. Standards, in parallel gel, are compared to the tracking dye, Bromothymol Blue.

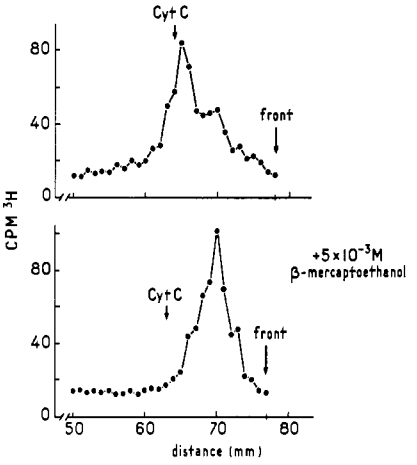


FIGURE 15: Polyacrylamide gel electrophoresis of pure [^{3}H]α-toxin in 0.1% sodium dodecyl sulfate. Upper figure: Sample had been incubated overnight at 37° in twofold Ringer's solution containing 1% sodium dodecyl sulfate. Lower figure: Sample had been incubated overnight at 37° in twofold Ringer's solution containing 1% sodium dodecyl sulfate and 5 mM β-mercaptoethanol. For both, standards were included in the same gel and about 10^4 cpm was applied.

used polyacrylamide gel electrophoresis instead of gel filtration and ran, in parallel, controls with the [^{3}H]α-toxin alone.

(a) ELECTROPHORESIS OF THE PURE [^{3}H]α-TOXIN. Figure 14 (open circles) shows the electrophoresis in 0.1% sodium dodecyl sulfate of a sample of pure toxin preincubated for 3 hr at room temperature (or 1 hr at 37°) with 1% sodium dodecyl sulfate. Despite the good evidence that the α-toxin has a molecular weight of 6787, the peak of radioactivity was repeatedly found between lysozyme (mol wt 14,300) and yeast alcohol dehydrogenase (mol wt 37,000). Under these conditions the apparent molecular weight of the free toxin was 23,000 ± 5000. Overnight incubation of the toxin in the presence of 5×10^{-3} M β-mercaptoethanol and 1% sodium dodecyl sulfate, followed or not by overnight alkylation at room temperature with 3×10^{-2} M iodoacetamide, showed a marked decrease in the apparent size of the radioactive material. Figure 15 shows it to have become smaller than that of cytochrome *c* and to approximate the value expected from its actual molecular weight.

(b) ELECTROPHORESIS OF [^{3}H]α-TOXIN-LABELED MEMBRANES SOLUBILIZED BY 1% SODIUM DODECYL SULFATE. The labeled membrane fragments were dissolved directly at 37° for 1 hr in 1% sodium dodecyl sulfate buffered at pH 7.0 with 10^{-1} M Na phosphate (incubation at room temperature failed to give adequate solubilization of the proteins). Electrophoresis in 0.1% sodium dodecyl sulfate of the soluble extract (not shown) yielded only one peak of radioactivity between the monomer (mol wt 16,500) and the dimer (mol wt 33,000) of dansylated lysozyme incorporated in the same gel. If the extract was supplemented by sodium dodecyl sulfate incubated toxin, again only one peak was obtained with an apparent molecular weight of 25,000 ± 3000, *i.e.*, the same as that of the free toxin. Either the receptor–toxin complex splits into small molecular weight units of 25,000 or, more probably, the receptor–toxin complex dissociates. The peak of radioactivity observed would then correspond, in the latter case, to the free toxin.

(c) ELECTROPHORESIS OF [^{3}H]α-TOXIN-LABELED MEMBRANES SOLUBILIZED BY 1% TRITON X-100. In order to preserve the association of the toxin with its receptor site during the solubilization we applied to the gel-labeled membranes dissolved by Triton X-100 following the procedure described in Methods (3% Triton X-100, 0.5 M Tris-HCl, pH 8.0). After solubilization, the extract was made 1% in sodium dodecyl sulfate and submitted immediately to electrophoresis. The upper trace of Figure 16 shows the patterns observed. (The same pattern was observed with incubation at 37° for 1–3 hr in the presence of 1% sodium dodecyl sulfate.) There is a broad peak of radioactivity peaking about mol wt 90,000 between the internal markers dansylated bovine serum albumin (mol wt 66,500) and its dimer (mol wt 133,000).

However, when free toxin was treated under the same conditions, a very similar pattern was obtained (lower trace of Figure 16). Unaccountably the toxin alone gave a broad peak

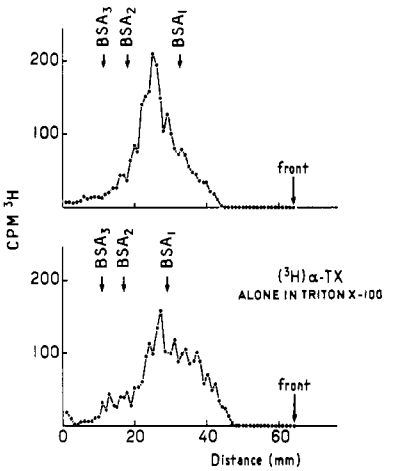


FIGURE 16: Comparison of the patterns of radioactivity obtained after gel electrophoresis in 0.1% sodium dodecyl sulfate [^{3}H]α-toxin-labeled membrane fragments dissolved in Triton X-100 and free [^{3}H]α-toxin submitted to the same treatment. Upper figure: [^{3}H]α-toxin-labeled membranes dissolved in 3% Triton X-100, in 0.5 M Tris, pH 8.0, and made 1% in sodium dodecyl sulfate at the time of electrophoresis. Lower figure: [^{3}H]α-toxin alone in 3% Triton X-100 in 0.5 M Tris, pH 8.0, made 1% in sodium dodecyl sulfate at the time of electrophoresis. For both, standards were included in the same gels and about 8×10^3 cpm was applied.

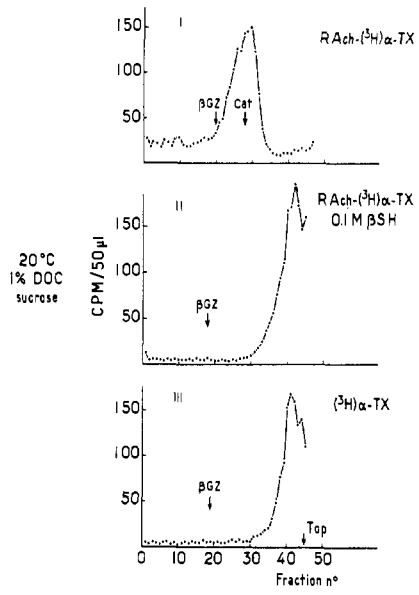


FIGURE 17: Dissociation of the [^{3}H]α-toxin–cholinergic receptor complex by prolonged exposure to 0.1 M β-mercaptoethanol. The sample containing 0.15 mg of labeled membrane proteins dissolved in 0.5 M Tris (pH 8.0), 3% Na-deoxycholate, and approximately 5400 cpm was supplemented with enough β-mercaptoethanol to make 0.1 M. After 24 hr at room temperature, 7 μg of β-galactosidase was added to the sample which was then layered on top of a 5-ml linear sucrose gradient (5 to 20% w:v in Tris 5×10^{-3} M (pH 8.0), sodium citrate 5×10^{-3} M, sodium–deoxycholate 1% and 0.1 M β-mercaptoethanol) and centrifuged for 160 min at 60,000 rpm (390,000g) in the rotor SW 65 K of a Beckman centrifuge (Spinco Model L) thermostated at 20°. Controls were made during the same run, using an aliquot of the starting sample (nonreduced) and free tritiated α-toxin. Fraction collection, β-galactosidase and radioactivity assays have been described in Methods.

of radioactivity which resembled that of the dissolved labeled membrane. Conclusions regarding the latter are thus made impossible, except for the warning that certain combinations of detergents can cause artifacts in the physical behavior of the α-toxin.

(d) ELECTROPHORESIS OF [^{3}H]α-TOXIN-LABELED MEMBRANES SOLUBILIZED BY 1% DEOXYCHOLATE. Since in the presence of Triton X-100 an artefactual behavior of the free [^{3}H]α-toxin was noticed, we studied the effect of sodium dodecyl sulfate on the receptor protein after solubilization by deoxycholate.

In a first series of experiments we used extracts made from labeled crude membrane fragments following the method of Meunier *et al.* (1971b) (0.3 M NaCl, 0.3 M Tris-HCl (pH 8.0), and 2% deoxycholate for 1.5 hr at room temperature under agitation). The extract was dialyzed overnight at 22° against 1% sodium dodecyl sulfate before electrophoresis in 0.1% sodium dodecyl sulfate following the standard procedure. In Figure 14 are shown the patterns of radioactivity obtained with either the crude extract (closed circles) or the free α-toxin previously incubated 4 hr at room temperature in twofold Ringer's solution supplemented with 1% sodium dodecyl sulfate (open circles). The extract gave a single peak of radioactivity of apparent molecular weight 52,000 ± 7000 (estimated by comparison with standards run at the same time on separate gels) while the [^{3}H]α-toxin alone gave 25,000 as described above in section a.

This finding was confirmed by a second series of experiments where the conditions of solubilization by deoxycholate and sodium dodecyl sulfate treatment were varied. In particular, *incubation* for 0–4 hr at 37° in the presence of 1% sodium dodecyl sulfate replaced *dialysis* against sodium dodecyl sulfate. Internal standard dansylated bovine serum albumin and lysozyme were also included in the gels.

However, a prolonged exposure (15 hr at 37°) to 1% sodium dodecyl sulfate resulted in a single peak of radioactivity on sodium dodecyl sulfate gel electrophoresis with an apparent molecular weight of 25,000, the molecular weight of the free toxin. It is likely that under these conditions, the receptor–toxin complex dissociated.

Because of the unusual behavior of the free toxin in the presence of detergents these studies were extended to a variety of incubation conditions. In particular, the free toxin was put into 1% sodium dodecyl sulfate or 0.1% sodium dodecyl sulfate plus 2% deoxycholate *before* electrophoresis. Again a peak with an apparent size of 25,000 was observed. In contrast with what was noticed with Triton X-100, deoxycholate treatment did not change the behavior of the free toxin.

6. Dissociation of the Receptor–Toxin Complex. Extensive treatment by sodium dodecyl sulfate leads to a dissociation of the receptor–toxin complex. As already mentioned, the α-toxin from *Naja nigricollis* possesses 4 disulfide bridges and was shown to be inactivated when treated by disulfide reducing agents. Figure 17 shows that free [^{3}H]α-toxin is released after overnight exposure of the complex at 22° to 0.1 M β-mercaptoethanol. Interestingly, the free receptor protein was not inactivated and preserved its ability to bind the toxin. When the experiment was done under conditions where all the receptor sites present were labeled by the toxin, an exposure to β-mercaptoethanol had to be prolonged for a complete dissociation to occur. In that case, a significant inactivation of the receptor protein was seen.

Discussion

Tritiation of the α-toxin from *N. nigricollis* following the procedure described by Menez *et al.* (1971) yields a protein with a specific activity of 14 Ci/mmole which has properties *in vivo* and *in vitro*, identical with those of the native toxin. In the preparation used throughout this work, 90% of the tritiated toxin was able to bind irreversibly to excitable membrane fragments and thus was active material.

As expected, reversible and irreversible cholinergic agents prevented [^{3}H]α-toxin association to the membrane fragments. Concentrations which prevent toxin from binding lie in the same range as those active *in vivo* on the isolated electroplax (reference in Changeux and Podleski, 1968, and Kasai and Changeux, 1971) or *in vitro* on excitable microsacs (Kasai and Changeux, 1971). Qualitative agreement was observed between the ability of a given compound to protect and its apparent affinity as determined from electrophysiology of the electroplax. No *major* difference was even seen in this respect between agonists and antagonist. This result, which is in agreement with earlier findings of Changeux *et al.* (1970a,b), contrasts with Miledi *et al.* (1971) who claim that the effect of carbamylcholine in reducing the rate of α-bungarotoxin binding to *Torpedo* membrane fragments was "more pronounced" than that of *d*-tubocurarine. Nevertheless quantitative studies are in progress to make an objective comparison between the actual dissociation constant of a given cholinergic agent from the cholinergic receptor site and its efficiency in slowing down the rate of toxin binding (Weber and Changeux).[3]

[3] M. Weber and J.-P. Changeux, unpublished results.

In any case, complete protection against [^{3}H]α-toxin binding was observed at a still physiological concentration of the pharmacologically active compound, which indicates that, under the present experimental conditions, the [^{3}H]α-toxin binds *exclusively* to the cholinergic receptor site. In agreement with this conclusion is the finding that the number of [^{3}H]α-toxin receptor sites is very close to the number of decamethonium receptor sites blocked by α-bungarotoxin found by the same authors using cold toxin. However Kasai and Changeux found that, in their preparation, there were approximately twice as many α-bungarotoxin binding sites as decamethonium binding sites. This difference might be due to the fact that during storage a nonnegligible fraction (up to 50%) of the cold toxin they used was not in its active or "binding" conformation. Thanks to the tritiated toxin this ambiguity can now be overcome.

The highly selective and irreversible binding of the [^{3}H]α-toxin allows the detection of quantities of cholinergic receptor sites as low as 0.1 pmole. This assay is applicable to receptor free in solution since the receptor–toxin complex can easily be separated from the free toxin by ammonium sulfate precipitation.

Of the [^{3}H]α-toxin bound to excitable membrane fragments 70–100% is released in solution upon treatment by sodium deoxycholate or Triton X-100. The solubilized radioactivity no longer sediments in sucrose gradients or passes through Sepharose 6B columns at the place expected for the free [^{3}H]α-toxin in solution. Deoxycholate and Triton X-100 extraction preserves the association of the toxin to a macromolecule, which, for the reasons mentioned before, carries the cholinergic receptor site and thus, is, or contains, the cholinergic receptor protein. This membrane component, as previously mentioned (Changeux *et al.*, 1971), and in agreement with Miledi *et al.* (1971), precipitates in the absence of detergent, but it migrates on gel electrophoresis in the presence of deoxycholate as a single band. Our conditions of labeling and of extraction are such that the toxin–receptor complex obtained fulfill several criteria for homogeneity.

Centrifugation in sucrose gradient of the labeled receptor protein gave consistently a standard sedimentation coefficient of 9.5 S in the presence of either 1% deoxycholate or Triton X-100, at 4° or at 22°. The same value was obtained with the free receptor protein. Under exactly the same conditions the toxin–receptor complex (or the free receptor protein) was largely recovered after filtration on a Sepharose 6B column in a major symmetrical and sharp peak with an Stoke's radius close to that of *E. coli* β-galactosidase suggesting an *apparent* molecular weight of 540,000 ± 50,000. There exists a large discrepancy between the two sets of results since the sedimentation velocity expected from a 540,000 mol wt globular protein should be close to 16 S. The sedimentation coefficient might be underestimated as compared to the apparent molecular weight but the reverse might be true as well.

A nonglobular, rod-like shape of the receptor complex, similar to that postulated by Massoulié *et al.* (1971) for another membrane protein of the same origin: acetylcholinesterase, might account for the first situation considered. A deviation in the same direction would happen if our conditions of extraction were such that the membrane unit which carries the cholinergic receptor site still contains lipids in quantities large enough to decrease its density below that of typical globular proteins. The receptor protein might as well associate with detergent molecules and make some kind of micromicelle which would present in gel filtration experiments an apparent molecular weight larger than the one expected from the protein it contains. This last hypothesis is in agreement with the recent studies by A. Helenius and K. Simons on a low-density lipoprotein from plasma and several membrane proteins. In all these cases, considerable binding of sodium deoxycholate and Triton X-100 was noticed whereas a number of standard hydrophilic proteins bind little or no detergent.[4] Experiments are presently being carried out to test these various alternatives and to estimate the exact molecular weight.

At this point, it is of interest to compare our results with those published by Miledi *et al.* (1971) with the α-bungarotoxin-tagged protein from *Torpedo* tissue. On Sephadex G200 their complex was eluted in the void volume *before* acetylcholinesterase. In glycerol density gradients the same authors report that the toxin-labeled protein sedimented between acetylcholinesterase and β-galactosidase. These results contrast with those obtained with *Electrophorus* tissue since in all our experiments acetylcholinesterase makes, in the presence of either Triton X-100 or deoxycholate, large aggregates which move faster than the main peak of receptor protein both on Sepharose 6B columns and in sucrose gradients. The reasons for this discrepancy is not yet clear and might possibly be relevant to either a zoological difference between the two species of fish or to a difference of ionic strength in the centrifugation medium. Using exclusively Sephadex G-200 for gel filtration, Miledi *et al.* (1971) were unable to report an estimate of the molecular weight of the complex in Triton X-100 except that it was larger than 200,000. This last observation is however consistent with our quantitative estimate of a Stoke's radius equal to that of β-galactosidase.

From experiments carried out in the presence of sodium dodecyl sulfate after Triton X-100 extraction, Miledi *et al.* (1971) claimed that the labeled protein splits into units of apparent molecular weight of about 88,000 and 180,000. However, they do not give any information on the behavior of the toxin alone placed in the same condition. We have not attempted to repeat these experiments but have run gel electrophoresis of Triton X-100 extracts in the presence of sodium dodecyl sulfate. Controls with the free toxin were made in parallel. Both samples gave a distribution of counts reaching a peak around 80,000 but covering molecular weight regions from 300,000 to 10,000. We thus abandoned Triton X-100 extraction in favor of deoxycholate extraction for study in sodium dodecyl sulfate gel electrophoresis. In deoxycholate and/or sodium dodecyl sulfate, the [^{3}H]α-toxin alone gave a sharp peak with an apparent molecular weight around 20,000 (suggesting either a drastic change of conformation or a discrete but finite aggregation of the toxin in the presence of sodium dodecyl sulfate). Conditions were defined for gel electrophoresis of the labeled protein which gave a peak of radioactivity corresponding to an apparent molecular weight smaller than that measured in the *absence* of sodium dodecyl sulfate in deoxycholate or Triton X-100 but larger than that of the free toxin. This unit was the smallest one seen before dissociation of the toxin. It had an apparent molecular weight of 55,000 ± 5,000. The nature of this component is not yet completely understood. First of all, it should be recalled that the apparent molecular weight of this unit was not measured under conditions of complete reduction and saturation of sodium dodecyl sulfate, which as shown by Reynolds and Tanford (1970), are those required for reproducible determination of molecular weights. Moreover, we ignore the number of toxin molecules included in the 55,000 unit.

[4] Manuscript in preparation.

If we assume, *without proof*, that only one α-toxin chain of 6787 molecular weight associates with this unit, then one might consider that the receptor protein contains a subunit of approximately 48,000 molecular weight which tightly binds the α-toxin. Interestingly this value seems in agreement with the one proposed independently by Karlin and associates (personnal communication) for the polypeptide chain labeled by an affinity reagent.

It thus appears plausible that the form of the cholinergic receptor protein which is solubilized by deoxycholate and Triton X-100 is a well-defined polymer made up of a finite number of smaller subunits. It is not possible at present to ascertain whether this polymer is made exclusively of such units and that it corresponds to the "native state" of the receptor protein within the membrane. It can only be said that in this state, the protein is able to bind cholinergic ligands and snake venom α-toxins.

Finally, we would like to mention the analogy of the solubility properties of the receptor protein with those of another receptor protein: the opsin from visual cells of the retina (Heller, 1968). In both cases, the molecule is not soluble in aqueous solvents in the absence of detergents. This property, which is shared by numerous membrane proteins, might be associated with the presence of a hydrophobic "tail." Such a differentiation of the surface of the molecule, which is well documented in the cases of cytochrome b_5 (Spatz and Strittmatter, 1971) might serve to anchor the receptor protein to the membrane.

Acknowledgment

We thank Michel Weber for comments and suggestions relative to the kinetics of α-toxin binding and Monique Huchet and Simone Mougeon for their technical assistance.

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