# PURIFICATION FROM *TORPEDO MARMORATA* ELECTRIC TISSUE OF MEMBRANE FRAGMENTS PARTICULARLY RICH IN CHOLINERGIC RECEPTOR PROTEIN

Jonathan B. COHEN, Michel WEBER, Monique HUCHET and Jean-Pierre CHANGEUX

*Unité de Neurobiologie, Département de Biologie Moléculaire, Institut Pasteur, Paris, France*

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## 1. Introduction

The study of membrane excitation *in vitro* has been made possible by the development of a fractionation procedure of the electric tissue of *Electrophorus electricus* [1] yielding closed membrane fragments, or microsacs, which respond to cholinergic agonists by a change of selective permeability to cations and which bind nicotinic agents [2] and snake venom $\alpha$-toxins [2–5]. However, the resulting preparations had a rather low content of cholinergic receptor site (up to 50 nmoles per gram of protein). In order to permit an experimental study of the receptor protein in its membrane environment by the most common physical techniques, a preparation with a higher content and specific activity in receptor protein was needed. We report in this letter a method of fractionation of membrane fragments from *Torpedo marmorata* electric tissue which satisfies this requirement. The purified membrane fragments obtained contain up to 10% w/w of their membrane protein as cholinergic receptor protein and less than 0.02% as acetylcholinesterase.

## 2. Materials and methods

### 2.1. *Preparation of membrane fragments*

Live *Torpedo marmorata* were furnished by the Marine Biological Station of Arcachon and kept for a few days in Paris before use in an aquarium containing continuously filtered and oxygenated artificial sea water at 14°. The electric organs of an average size *Torpedo* were dissected from the live animal without anesthesia and immediately freed from skin and connective tissue. The total weight of tissue was in general close to 150 g per animal.

60 g of fresh electric tissue were then minced with scissors, suspended in 120 ml of distilled water in a 500 ml vessel, and homogenized at 4° in a Virtis apapparatus for 2 min at 90% of its maximal speed. The homogenate (HT) was sonicated at 4° for three periods of 10 sec with a Branson B-12 Sonifier at force 6. Between each period a 10 sec rest was observed to minimize heating. The sonicated homogenate (SH) was centrifuged at 5000 g for 10 min and the pellet discarded. The supernatant (S) was then centrifuged at high speed in the SW 27 rotor of a Beckman L3-50 ultracentrifuge on a sucrose density gradient formed either by layering from bottom to top 2.0 ml each of 1.5, 1.4, 1.3, 1.2, 1.1, 1.0 and 0.8 M sucrose or by two cycles of freezing and thawing 14 ml of a 1.2 M sucrose solution [6]. In the last case the thawing was performed at 4°, the freezing at −28°. 23 ml of S were layered on the gradients. The tubes were centrifuged for 4.5 hr at 4° at 80,000 *g* (24,000 rpm). Fractions of 0.7 ml were collected after perforating the bottom of the tube with a needle, and proteins, acetylcholinesterase and toxin binding were assayed in each fraction.

### 2.2. *Assays*

Proteins were estimated by the method of Lowry [7] using bovine serum albumin as the standard. Acetylcholinesterase activity was assayed with acetylthiocholine as substrate by the method of Ellman [8].

In order to convert from the observed change in optical density per time per volume of membrane suspension to concentration units, it was assumed that acetylcholinesterase had a molecular weight of 260,000 and that the acetylthiocholine turnover number for the pure enzyme was 750 moles per hr per g protein [9].

The cholinergic receptor protein present in the membrane fragments was labelled by the $\alpha_1$-isotoxin from the venom of *Naja nigricollis* tritiated by the method of Menez et al. [4]. 1 $\mu$l of a 58 $\mu$M, 10.5 Ci/mmole [$^3$H]$\alpha$-toxin stock solution was added to 30 ml of sonicated crude homogenate (SH), and the mixture incubated for 30 min at 22° prior to the low speed centrifugation. Under these conditions less than 1% of the receptor sites were labelled. The radioactivity of 0.1 ml samples of labelled SH, S, and the sucrose gradient fractions were counted in flasks containing 10 ml of Bray's solution in an Intertechnique scintillation counter.

Estimation of the concentration of toxin binding sites was made by titration of a known quantity of [$^3$H]$\alpha$-toxin with increasing volumes of membrane suspension. Samples of the fraction to be tested were diluted from 150 to 5000-fold in a modified Elasmobranch Ringer's medium (250 mM NaCl, 5 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM pH 7 Na phosphate buffer) to a final volume of 500 $\mu$l. The medium was then supplemented with 10 $\mu$l of a $5.8 \times 10^{-8}$ M [$^3$H]$\alpha$-toxin solution. The reaction was complete after 4 hr at 22°. 400 $\mu$l of the reaction medium were then filtered on a Millipore filter, HAWP 02500, and washed with 20 ml of 4° Ringer's solution. The dried filter was counted in 10 ml of toluene POPOP-PPO counting solution. To determine the radioactivity corresponding to the total quantity of [$^3$H]$\alpha$-toxin added per tube, 0.08 ml of the reaction medium was placed on a filter, dried, and counted. With fresh toxin solutions it was observed that in the absence of membrane fragments less than 10% of the total counts were retained on the filter but that 100% of the counts were retained in the presence of excess membrane fragments. The receptor concentration was found by using the initial slope of the titration curve to determine the quantity of membrane fragments necessary to react with all the toxin added in the tube.

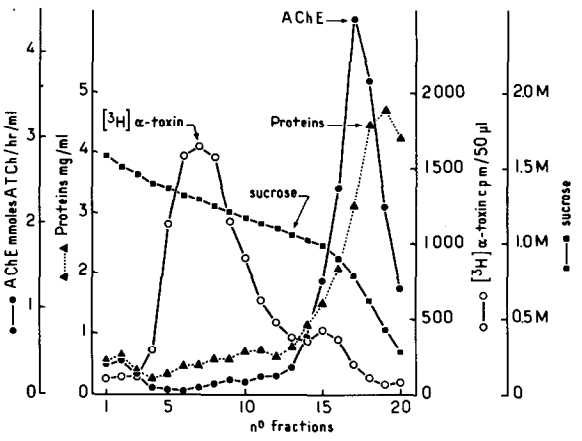


Fig. 1. Distribution of [$^3$H]$\alpha$-toxin, acetylcholinesterase and total protein in a sucrose gradient after ultracentrifugation of a homogenate of *Torpedo marmorata* electric organ. 20% of the protein, 80% of the receptor and 53% of the esterase enter the gradient. The recovery of material placed on the gradient is 86% for the protein and esterase and 92% for the receptor (The content of a small observed pellet is not included).

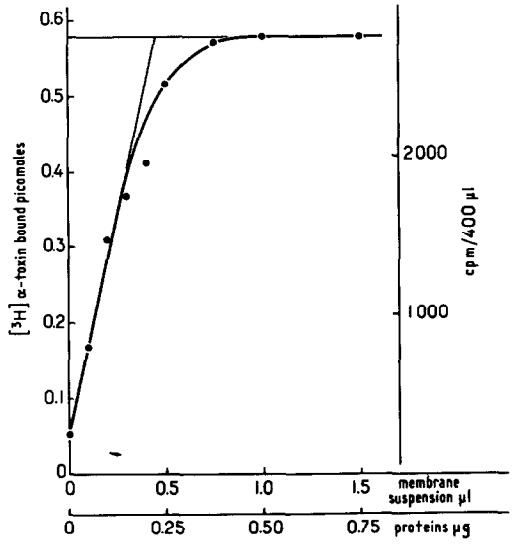


Fig. 2. Titration of a solution of [$^3$H]$\alpha$-toxin by a purified preparation of receptor rich membrane fragments.

### 3. Results and discussion

In table 1 are given the results of a typical purification of receptor rich membrane fragments following the procedure given in Methods. The profiles of the distribution of total protein, acetylcholinesterase and [$^3$H]α-toxin binding sites in the final sucrose gradient are found in fig. 1. Both low and high speed ultracentrifugations give a clear separation of membrane fragments rich in toxin binding material from membrane fragments rich in acetylcholinesterase*. After high speed centrifugation, the density of sucrose at which they band is higher in the former (1.3 ± 0.1 M sucrose) than in the latter case (0.8 M sucrose). The separation is accompanied by a marked increase of the specific activity of toxin binding sites (approx. 80-fold in fraction no. 7) with a reasonable yield for this step (approx. 40% in fractions 5–8). The maximum specific activity of acetylcholinesterase found in the final gradient does not significantly differ from that of the crude homogenate.

A titration of the toxin binding sites in a receptor rich membrane fraction is presented in fig. 2. It gives 2300 nmoles of sites per g protein. If one takes 45,000 for the molecular weight of the smallest protein subunit to which the toxin binds [5], then 12% of the protein present in these membrane fragments would consist of receptor protein and only 2 parts per 10,000 of acetylcholinesterase. Membrane fragments of this sort containing 8 mg of total protein can be obtained from 60 g of fresh electric organ.

That the toxin binding protein present in these membrane fragments does correspond to the cholinergic receptor protein was confirmed by the fact that decamethonium, a typical nicotinic agonist, decreases the rate of [$^3$H]α-toxin binding. The concentration of decamethonium which decreases by half the initial rate of binding and which, under certain hypotheses, is a measure of the dissociation constant of the effector for the receptor site [10], was found to be similar to that found with excitable membrane fragments of *E. electricus* (table 2). In addition, the second order rate constants of [$^3$H]α-toxin binding in the absence of effector were similar in the two cases (table 2).

* Note added in proofs: A similar observation has been made independently by P. Molinoff (personal communication).

The present results contrast with those previously reported with *Torpedo* and *Electrophorus*. Miledi et al. [11] prepared membrane fragments from *Torpedo* which contained 180 nmoles of [$^{131}$I]α-bungarotoxin binding sites per g of protein and the same amount of acetylcholinesterase. The method used to purify excitable membrane fragments from *Electrophorus* gave preparations with a specific activity of receptor sites in the order of 50 nmoles/g protein and equivalent amounts of acetylcholinesterase [1, 2].

Autoradiographic studies of receptor distribution in eel electroplax [12] and mouse skeletal neuromuscular junction [14] show that the density of receptor lies between 10,000 and 30,000 sites per $\mu^2$ of subsynaptic membrane. Such high densities leave little room for other proteins if one considers the receptor to be distributed subsynaptically in a monolayer. In the eel electroplax receptor density is 100 times lower in the extrasynaptic regions than in the subsynaptic ones. This is presumably true as well with *Torpedo*, although in *Torpedo* up to 50% of the total surface of the innervated membrane consists of subsynaptic areas while approx. only 5% belong to this category in *Electrophorus*. These observations lead us to conclude that the receptor rich membrane fragments we isolate presumably originate in subsynaptic areas of the *Torpedo* innervated membrane.

A puzzling question is raised by our observations that *in vitro* acetylcholinesterase and receptor appear to be bound to different membrane fragments. Histochemical studies of the *Torpedo* electroplax [13] show a uniform distribution of esterase along sub- and extrasynaptic regions of the innervated face, but the method used does not permit the determination of absolute enzyme concentration. In mouse diaphragm equal quantities of esterase and receptor have been found in the end plate region [14]. However, in the frog neuromuscular junction it has been shown that the esterase can be solubilized by mild enzymatic treatment without seriously affecting receptor function [15], an observation consistent with the view that esterase and receptor might be integrated into the membrane in a different manner. The differential distribution of receptor and esterase observed *in vitro* might thus be the consequence of the homogenization procedure.

In order to examine this point the tissue was homogenized in a medium [16] containing 0.3 M

Table 1
Purification of receptor rich membrane fragments from *Torpedo* electric tissue.

| | Proteins | | [$^3$H]α-toxin binding sites | | Acetylcholinesterase molecules | |
|---|---|---|---|---|---|---|
| | (g/l) | Yield (%) | (nmoles/ g protein) | Yield (%) | (nmoles/ g protein) | Yield (%) |
| Sonicated fragments (SH) | 9.0 | 100 | 30 | 100 | 4.3 | 100 |
| Low speed supernatant (S) | 4.25 | 47 | 34 | 63 | 1.35 | 15 |
| High speed centrifugation gradient fraction | | | | | | |
| 7 | 0.5 | 0.2 | 2300 | 7.1 | 0.9 | 0.03 |
| 5–9 | – | 0.9 | – | 28 | – | 0.2 |
| 17 | 3.1 | 1.0 | < 1 | 0.8 | 7.2 | 1.8 |
| 16–18 | – | 3.3 | – | 2.7 | – | 4.2 |

Table 2
Rates of [$^3$H]α-toxin binding to receptor rich membrane fragments.

| | *Torpedo* | *Electrophorus* [10] |
|---|---|---|
| Bimolecular rate constant | $(3 \pm 1) \times 10^7$ $M^{-1} \times min^{-1}$ | $1.7 \times 10^7$ $M^{-1} \times min^{-1}$ |
| Concentration of decathonium which reduces initial rate by 50% | $(0.8 \pm 0.5) \times 10^{-6}$ M | $0.8 \times 10^{-6}$ M |

NaCl and 0.2 M sucrose instead of distilled water. The yield and distribution of receptor in the final gradient did not change, but the distribution of acetylcholinesterase did change. Although the percent of esterase found in the low speed supernatant was the same, a second esterase peak was found coincident with the receptor peak (the esterase content in this peak was still about 100 times smaller than that of receptor). A redistribution of the esterase might thus occur during the fractionation procedure, and it is not yet clear whether or not the stoichiometry of receptor to esterase varies from subsynaptic areas to extra synaptic ones.

In any case, the preparation of membrane fragments from *Torpedo* electric tissue very rich in cholinergic receptor protein is of considerable utility as a first step for the purification of the receptor protein and as a basic material for the study of the physical properties of the receptor protein integrated in a biological membrane.

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