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Studies on the Cholinergic Receptor Protein of *Electrophorus Electricus*

I. An Assay *in Vitro* for the Cholinergic Receptor Site and Solubilization of the Receptor Protein from Electric Tissue

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SUMMARY

The binding of ^{14}C-decamethonium to a preparation of the electric organ of *Electrophorus electricus* has been measured by a method of rapid equilibrium dialysis. At the ionic strength of Ringer's physiological solution little nonspecific binding of decamethonium occurs. Deoxycholate extraction of membrane fragments yields a preparation which contains two classes of specific decamethonium-binding sites. From one, the ligand is displaced *reversibly* by *d*-tubocurarine, gallamine triethiodide (at concentrations lower than 10^{-5} M), carbamylcholine, and phenyltrimethylammonium, and *irreversibly* by two snake venom toxins, α-bungarotoxin and *Naja nigricollis* α-toxin. This class of site is considered to belong to the cholinergic receptor site. From the other, decamethonium is only displaced by carbamylcholine and phenyltrimethylammonium. This second class of site is identified as the catalytic site of acetylcholinesterase. The "intrinsic" binding constants of a variety of cholinergic agents for these two classes of sites are compared with their "apparent" values estimated *in vivo* on the isolated electroplax and *in vitro* on excitable membrane fragments. Some agreement exists between the two sets of data. The macromolecule possessing the cholinergic receptor site has a molecular weight larger than 50,000 daltons, is thermolabile, and is digested by Pronase. It is a protein that is easily separated from acetylcholinesterase by selective adsorption on Sepharose granules to which *N. nigricollis* α-toxin has been coupled.

INTRODUCTION

The first step in the electrogenic action of acetylcholine on a synaptic membrane is its reversible binding to a recognition site, commonly referred to as the cholinergic receptor site. The isolation of the molecule involves the difficult problem of ascertaining its specificity. In a cholinergic synapse several distinct sites are present, all of which are able to bind cholinergic ligands, and which include, in addition to the cholinergic receptor site, the catalytic and allosteric sites of the enzyme acetylcholinesterase (1, 2). Identification of the macromolecular receptor thus requires an extensive and careful comparison of the properties of the macromolecule with the physiological properties of the excitable membrane from which it has been extracted.

As emphasized by Nachmansohn (3), the

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electric organ of the gymnotid fish *Electrophorus electricus* constitutes an excellent source of biochemical material for the study of the cholinergic receptor. Indeed, one electric organ of average size contains approximately 10^5 identical electroplaxes, and each electroplax receives up to 5×10^4 nerve terminals, yielding 10^9–10^{10} identical synapses. In addition, the electric organ of *E. electricus* is presently the only source from which *single* electroplaxes can be dissected and studied electrophysiologically in a well-defined environment (4, 5). The exact correlation between biochemical and electrophysiological data which is required for a rigorous study on the cholinergic receptor protein presently can be achieved only with this fish.

In this paper, we present data which lead us to propose a binding assay characteristic of the physiological receptor site of acetylcholine. We show further that the macromolecule possessing this site can be solubilized from electric tissue in appreciable amounts, and is a protein distinct from acetylcholinesterase which retains most specific binding properties of the physiological receptor. In subsequent papers the physicochemical properties of the purified receptor protein will be presented. Preliminary reports have been published (6, 7). In the first a method of solubilization of the receptor protein was described. In the second (7) the receptor protein was shown to be distinct from acetylcholinesterase. In both cases a snake venom α-toxin was used to characterize the receptor protein. Following this lead, Miledi, Molinoff, and Potter (8) more recently have published findings on a protein from *Torpedo* which are in general agreement with ours.

MATERIALS AND METHODS

Preparation of extracts. Electric eels, *E. electricus*, were purchased live from Paramount Aquarium, Ardsley, N. Y. They were decapitated, and, after removal of the skin, the main electric organ was immediately separated from the rest of the body and freed from muscle and connective and adipose tissue. The extracts prepared from small eels were richer in receptor than those prepared from large animals, and the cephalic part of the electric organ gave better preparations than the caudal portion. Fresh electric organs were routinely used for preparation of the extracts. Occasionally extracts were made from frozen tissue, but the yield of receptor was always lower.

Routinely we cut 300 g of electric tissue in pieces of approximately 20 g. Each piece was then minced into fragments of less than 1 cm with scissors. To 20 g of minced tissue were added 40 ml of the following ice-cold buffer: 160 mM NaCl, 5 mM KCl, 10 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, and 10 mM $CaCl_2$. Homogenization was carried at 0°, in a VirTis apparatus, in a 100-ml glass vessel at 75% of maximal speed. The homogenates were then pooled and stored at 0° for sufficient time (1–10 hr) to give a bubble-free suspension. The homogenate was then centrifuged at 4° in the SS 34 rotor of a Sorvall centrifuge at 15,000 rpm ($28,000 \times g$) for 15 min. The supernatant fluids were discarded. The pellets were resuspended in the same volume of 50 mM Tris (pH 8.0), and 100 mM NaCl at room temperature. To the continuously stirred suspension was then added a 10% solution of sodium deoxycholate to make the final solution 1% in deoxycholate. The yield of receptor strongly depends on the commercial origin of deoxycholate. Initial experiments gave excellent results with a batch of deoxycholate from Light & Company. A second batch from the same company gave preparations with a very poor yield. Finally, we selected deoxycholate from Schuchardt & Company, which regularly gave a good yield of receptor protein. The activity of acetylcholinesterase in the solution was followed during the extraction and usually reached a plateau after 50 min. The homogenate was then cooled to 0° and centrifuged in the cold at $28,000 \times g$ for 15 min. The supernatant fluid could be centrifuged at $100,000 \times g$ for 1 hr without loss of its binding capacity. In order to improve the stability of the preparation of receptor, deoxycholate was routinely precipitated from the extract by addition, under continuous stirring, of 1 M $MgCl_2$ to give a final concentration of 70–100 mM $MgCl_2$. The deoxycholate precipitate was then removed by low-speed centrifugation in the cold at $28,000 \times g$ for 15 min. The extract retained its binding

capacity for several days at 0°. Recent improvements in the preparation of the extract and a procedure for purification of the receptor protein will be described in a subsequent paper.

Approximately 350 ± 50 g of membrane fragments in the bulk pellet (33 ± 5 g, dry weight, or 27 ± 3 g of protein) can be obtained from 1 kg of tissue (80 ± 5 g, dry weight). Extraction of the pellet with 1% deoxycholate yields, after precipitation by $MgCl_2$, 4 ± 1 g of protein.

Extracts contained 5–10 mg of protein per milliliter, and the specific activity of acetylcholinesterase in the extracts ranged from 5 to 10 moles of acetylthiocholine hydrolyzed per hour per gram of protein. In order to effect significant displacement in our equilibrium dialysis experiments, the extract was usually concentrated 3–4-fold by pressure filtration with a Diaflo XM 50 membrane (Amicon).

Equilibrium dialysis. Either decamethonium[*methyl*-^{14}C] bromide (20.9 mCi/mmole) or decamethonium[*methyl*-^{3}H] bromide (100–500 mCi/mmole) (Radiochemical Centre, Amersham) was used as the radioactive ligand. We routinely followed the method of equilibrium dialysis described by Gilbert and Muller-Hill (9). In order to achieve a high surface to volume ratio, 0.2 ml of extract was employed in dialysis bags 1 cm wide and 13 cm long. The bag was knotted at both ends to minimize the volume of entrapped air. The solution was then dialyzed at 4° against a large excess of a solution containing 160 mM NaCl, 5 mM KCl, 10 mM $MgCl_2$, 2 mM $CaCl_2$, and 2 mM Tris, pH 8.0.[1] Except when mentioned, the concentration of radioactive decamethonium in the dialysis medium was 0.5 μM.

The flask was shaken on a Eberbach (Ann Arbor, Mich.) horizontal rotator in the cold for 4–12 hr. Except when *d*-tubocurarine was used, equilibrium was reached after about 3½ hr; however, incubation was routinely carried out overnight. After equilibration the bag was opened carefully and 0.1 ml of its contents was mixed with 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). The flasks were subsequently counted in a Packard liquid scintillation counter. Self-quenching was found to be less than 1% with ^{14}C-decamethonium. The concentration of protein in the bag was estimated by the method of Lowry *et al.* (10). Only extracts in which the number of counts displaced exceeded 500 were used. The counting error was generally less than 1% of the total.

Snake venom toxins. Pure α-bungarotoxin was a gift from Dr. C. Y. Lee, Taiwan University. It was prepared by cation exchange chromatography from the venom of *Bungarus multicinctus* by the method of Lee *et al.* (11).

Pure α-toxin from the venom of *Naja nigricollis* was a gift from Dr. P. Boquet of the Institut Pasteur. It was prepared according to the methods of Boquet, Izard, Jouannet, and Meaume (12) and Karlsson, Eaker, and Porath (13).

Covalent coupling of N. nigricollis α-toxin to Sepharose 4B. We used the method described by Axen, Porath, and Ernback (14). Sepharose 4B (Pharmacia) was extensively washed with distilled water. To 10 ml of the washed suspension, containing approximately 5 ml of Sepharose beads, were added 5 ml of cyanogen bromide in water (100 mg/ml). During the reaction the pH was kept at 11.0 with concentrated NaOH. After 8 min the reaction was almost complete, and the granules of Sepharose were then washed with 150 ml of 0.1 M $NaHCO_3$, pH 9.0. Several batches of activated Sepharose were prepared consecutively and mixed. The final suspension of activated Sepharose was then decanted, and the supernatant solution was discarded. To a total volume of 36 ml of decanted Sepharose were then added 36 ml of 0.1 M $NaHCO_3$, pH 9.0, and 3 mg of α-toxin in 1 ml of H_2O. The mixture was gently agitated overnight at 4° in an Erlenmeyer flask. The suspension was decanted, and the supernatant fluid was assayed for the presence of α-toxin (the toxin was not detected in this solution). The gran-

[1] This medium has the same composition as physiological Ringer's solution, except that sodium phosphate is replaced by Tris. The ionic strength is 0.18.

ules were then washed extensively with 0.03 M sodium citrate at pH 8.0. The suspension was stored at 4° in the presence of 0.02% NaN_3. The α-toxin coupled to the Sepharose beads maintains its antigenic activity toward horse antiserum to *N. nigricollis* venom.[2]

RESULTS

Selection of decamethonium as cholinergic ligand for binding studies. Our initial attempts to isolate the cholinergic receptor employed the affinity label *p*-(trimethylammonium)benzenediazonium fluoroborate. This compound blocked irreversibly the response of the electroplax to cholinergic agonists *in vivo* (15) but was far too reactive for specific radioactive labeling of the cholinergic receptor site. It also became covalently attached to membrane proteins, and the complex could not be dissociated easily. To circumvent these difficulties, we used the method of equilibrium dialysis which Gilbert and Müller-Hill have employed to assay and isolate the genetic repressor of the *Lac* operon (9).

In the first experiments carried out with this technique (16) acetylcholine was selected as the cholinergic ligand, since its "apparent" affinity for the isolated electroplax *in vivo* was about two orders of magnitude higher than that for the catalytic site of acetylcholinesterase (17, 18). However, even in the presence of potent enzyme inhibitors such as eserine, paraoxon, and phospholine, significant hydrolysis of acetylcholine occurred in the equilibrium medium. We therefore decided to monitor binding of decamethonium, a cholinergic agonist which is not hydrolyzed by acetylcholinesterase and which possesses a low "apparent" dissociation constant for the cholinergic receptor of the isolated electroplax (1.2 μM) (19).

Figure 1 shows the effect of ionic strength on the binding of ^{14}C-decamethonium to a suspension of membrane fragments prepared by low-speed centrifugation of a crude homogenate of electric organ. Physiological Ringer's solution ($\Gamma/2 = 0.180$) was sequentially diluted with water. Below $\Gamma/2 = 0.018$ the increase in radioactive bound decamethonium was nearly inversely proportional to the ionic strength, but, above $\Gamma/2 = 0.018$ the quantity bound did not vary appreciably with ionic strength and represented 10–20 nmoles of decamethonium per gram of protein in the presence of 0.56 μM free decamethonium. At low ionic strength, the amount of ligand bound increased almost linearly with the concentration of free decamethonium and was completely displaced by any quaternary ligand. We thus concluded that under these conditions decamethonium was bound to nonspecific sites. At high ionic strength, in contrast, saturation occurred at high concentrations of free decamethonium, and the compound was displaced from its binding sites by characteristic cholinergic ligands.

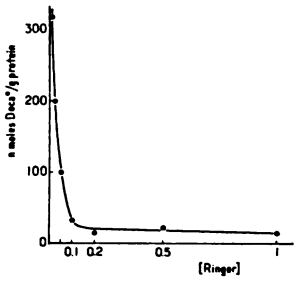

FIG. 1. *Effect of ionic strength on binding of ^{14}C-decamethonium (Deca*) to a suspension of membrane fragments*

The membrane fragments were obtained by low-speed centrifugation and resuspended in physiological Ringer's solution (see MATERIALS AND METHODS). The solution was diluted with water in the proportions given on the abscissa [*1* represents undiluted Ringer's solution ($\Gamma/2 = 0.180$); *0.1* represents 10-fold dilution]. The concentration of protein inside the dialysis bag was approximately 7 mg/ml. The concentration of free decamethonium outside the bag was 1 μM.

Extraction of decamethonium-binding macromolecules from electric tissue. Since the cholinergic receptor macromolecule was expected to be membrane-bound, our first step was the preparation of a concentrated suspension of membrane fragments. This was achieved by homogenization of electric tissue in a VirTis apparatus, which gives large membrane fragments that sediment on low-speed centrifugation. Since decamethonium binds to these membrane fragments (Fig. 1), and since a large part of the bound decame-

[2] P. Boquet, unpublished observations.

thonium was displaced by 10 μM gallamine, 10 μM *d*-tubocurarine, or 10 μg/ml of α-toxin when the concentration of free decamethonium was 0.5 μM, the membrane fragments contain the receptor protein.

Considerable effort was made to solubilize quantitatively the membrane components which specifically bind decamethonium, without loss of the total binding capacity. Neither 1 M Li_2SO_4 nor 1 M $(NH_4)_2SO_4$ caused the release of material binding both decamethonium and gallamine, although 1 M KI had a slight effect. Various enzymes were tested at 200 μg/ml: collagenase, lipase, and trypsin. Only trypsin resulted in a slight solubilization. Several detergents (1% Nonidet, 1% Tween 20, and 1% Triton X-100) had a smaller solubilizing effect than 1% sodium deoxycholate.

The best solubilization of membrane-bound proteins was achieved by continuous stirring of the membrane fragments at room temperature in the presence of 1% deoxycholate, 25 mM Tris (pH 8.0), and 50 mM NaCl. After centrifugation at 15,000 $\times$ *g* for 30 min, most of the deoxycholate was precipitated from this first supernatant fraction by making the solution 70–100 mM in $MgCl_2$ and centrifuging again at 15,000 $\times$ *g* for 30 min. Removal of the deoxycholate was necessary to prevent artifactual binding of decamethonium in the equilibrium dialysis assay. The second supernatant fraction contained 5–10 mg of protein per milliliter, high levels of acetylcholinesterase (specific activity, up to 7 moles of acetylthiocholine hydrolyzed per gram of protein per hour), and appreciable amounts of cholinergic receptor protein.

Binding of decamethonium in the presence and absence of 10 μM gallamine was stable for days at 0° in extracts dialyzed against 0.1 M $MgCl_2$, 0.1 M NaCl, and 0.01 M Tris, pH 8.0. No significant inactivation was observed in the course of decamethonium binding experiments carried out for 4–12 hr in the presence of Ringer's solution, pH 8.0, at 4°.

After concentration in the Diaflo system, the viscous extract contained 15–30 mg of protein per milliliter. Centrifugation at 65,000 $\times$ *g* (0.5 hr at 25,000 rpm in the SW 65K Spinco rotor) sedimented up to 50% of the total protein, including approximately the same fraction of acetylcholinesterase and decamethonium- and gallamine-binding material. This nonspecific aggregation was considerably reduced when the extract was centrifuged in the presence of 1% deoxycholate. The medium in which the receptor protein proved to be the most soluble was 1 M Tris (pH 8), 1 M NaCl, and 2% deoxycholate. In this buffer there was no loss of material binding both decamethonium and gallamine from the supernatant fraction after centrifugation for 1 hr at 100,000 $\times$ *g*. As will be reported in a subsequent paper, the solubilized receptor protein migrates on polyacrylamide gel electrophoresis, has a sedimentation velocity of 9.5 S during sucrose density gradient centrifugation, and can be purified by gel filtration.

TABLE 1

Binding of ^{14}C-decamethonium by a deoxycholate extract

The extract contained 16.6 mg of protein per milliliter. The specific activity of acetylcholinesterase was 7.05 moles of acetylthiocholine hydrolyzed per hour per gram of protein. If the specific activity of the pure protein is 750 times this value (20), 0.94% of the total protein in the extract was acetylcholinesterase.

	Radioactivity	Total decamethonium	Decamethonium bound
	cpm/100 μl	*μM*	*nmoles/g protein*
Equilibration buffer	4852	0.56	
Extract	8296	0.955	
Bound decamethonium	3444	0.395	23.9

Antagonism between decamethonium and various cholinergic agonists, cholinergic antagonists, and snake venom toxins. Binding of ^{14}C-decamethonium to concentrated deoxycholate extracts was conducted in Ringer's solution, pH 8.0. Table 1 shows that under these conditions a significant amount of radioactivity was retained inside the bag. The number of counts retained varied linearly with the concentration of extract, and retention was completely reversed when the dialysis bag was re-equilibrated against a large volume of decamethonium-free Ringer's solution.

In order to characterize the site(s), we studied quantitatively the influence on decamethonium binding of various characteristic effectors of the electroplax membrane *in vivo*. Figures 2–6 show the effects of three typical cholinergic antagonists (*d*-tubocurarine, gallamine, and hexamethonium) and two typical agonists (carbamylcholine and phenyltrimethylammonium). In these experiments the concentration of free decamethonium was kept constant at 0.56 μM and the concentration of nonradioactive cholinergic effector was varied. Bound decamethonium was displaced by each of these compounds, but the *total quantity* displaced by saturating levels of these cholinergic agents differed strikingly between compounds. Three, carbamylcholine, phenyltrimethylammonium, and hexamethonium, displaced all the decamethonium bound to the extract, which confirms the reversibility of decamethonium binding. Two of them, however, which are the most characteristic antagonists *in vivo*—*d*-tubocurarine and gallamine—displaced decamethonium only partially. The fraction displaced with a given preparation was the same at high levels of *d*-tubocurarine and gallamine, but varied between preparations,

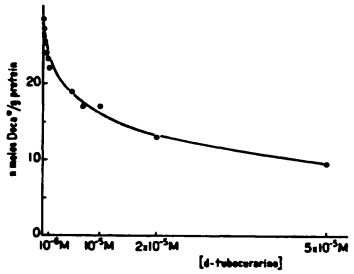

FIG. 2. *Antagonism by d-tubocurarine to binding of ^{14}C-decamethonium (Deca*)*

The conditions are described under MATERIALS AND METHODS. The concentration of free ^{14}C-decamethonium was 0.56 μM. The concentrations of *d*-tubocurarine given on the abscissa correspond to the total concentration of *d*-tubocurarine *added* to the solution at the beginning of the experiment, not exactly to the concentration of free *d*-tubocurarine.

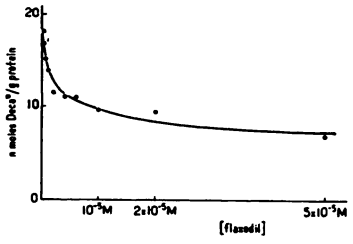

FIG. 3. *Antagonism by gallamine to binding of ^{14}C-decamethonium (Deca*)*

Conditions were the same as for Fig. 2, except that the extract was prepared from the organ of a different eel on a different day.

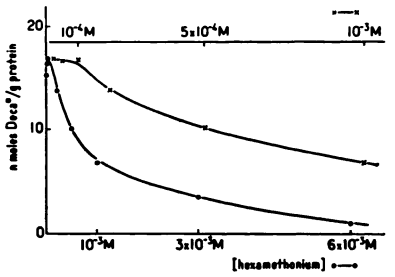

FIG. 4. *Antagonism by hexamethonium to binding of ^{14}C-decamethonium (Deca*)*

Conditions were the same as for Fig. 3.

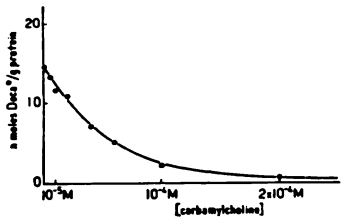

FIG. 5. *Antagonism by carbamylcholine to binding of ^{14}C-decamethonium (Deca*)*

Conditions were the same as for Fig. 3, except that the concentration of free ^{14}C-decamethonium was 0.86 μM.

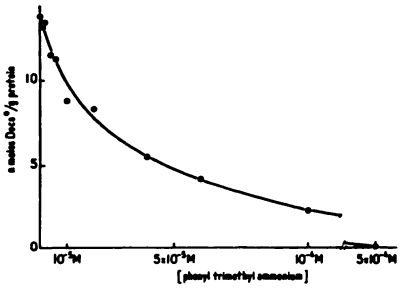

FIG. 6. *Antagonism of phenyltrimethylammonium to binding of ^{14}C-decamethonium (Deca*)*

Conditions were the same as for Fig. 3.

from 30% (below 30% the preparation was discarded) to 70%.

We then tested the effect of two toxins, purified from snake venom, known to be irreversible blocking agents of the response of various muscle preparations to cholinergic agonists (21–24). The first was α-bungarotoxin, a polypeptide of 8000 mol wt. Changeux, Kasai, and Lee (25) have shown that α-bungarotoxin acts *in vivo*, on the electroplax, or *in vitro*, on excitable microsacs, as an irreversible blocking agent, and *partially* displaces the binding of decamethonium to the extract. Figures 7 and 8 show that the α-toxin of *Naja nigricollis* (12, 26), purified by Boquet and associates (13), plays the same role. The α-toxin was an irreversible antagonist of the isolated electroplax and blocked irreversibly, but partially, the binding of radioactive decamethonium to the deoxycholate extract. Interestingly, the same displacement was obtained with the toxin from *N. nigricollis* and from *B. multicinctus*.

It has never been possible to show any effect of the two toxins on the catalytic site of acetylcholinesterase, neither on the binding of the substrates or inhibitors, nor on the catalytic activity of the enzyme (21, 25); thus these α-toxins constitute reagents highly specific for the cholinergic receptor site (21, 25).

As shown in Fig. 8, an excess of α-toxin from *N. nigricollis* displaced decamethonium bound to our extract almost to the same extent as an excess of *d*-tubocurarine or gallamine. No further displacement was observed in the presence of both α-toxin and gallamine. Although they are structurally unrelated, *d*-tubocurarine, gallamine, and the toxins had the same action *in vivo* and *in vitro*. The toxins, however, acted irreversibly. We therefore tentatively considered as bound to the physiological receptor sites of acetylcholine the molecules of decamethonium which were displaced in the presence of 0.56 μM free decamethonium by 10 μM gallamine, or 10 μM *d*-tubocurarine, or after 30 min of exposure of the extract to 20 μg/ml of snake toxin.

In contrast, the displacement of bound decamethonium by two typical agonists, carbamylcholine and phenyltrimethylammonium, and by an antagonist, hexamethonium, was complete. Interestingly, these three compounds are all potent inhibitors of the catalytic site of acetylcholinesterase under the same conditions as used in the binding experiments. Thus the molecules of bound decamethonium which were *not* displaced by *d*-tubocurarine, gallamine, or the α-toxins but were displaced by carbamylcholine, phenyltrimethylammonium, or hexamethonium were indeed bound to catalytic centers of acetylcholinesterase present in our extracts.

Strong support for this interpretation came from selective heat denaturation (Fig. 9). A deoxycholate extract was heated at

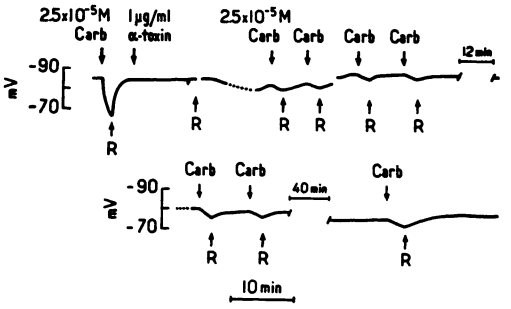

FIG. 7. *Irreversible effect of α-toxin from N. nigricollis on response (R) of the isolated electroplax to a cholinergic agonist, carbamylcholine (Carb)*

Electrical potential was measured on the isolated electroplax as described by Higman, Podleski, and Bartels (5).

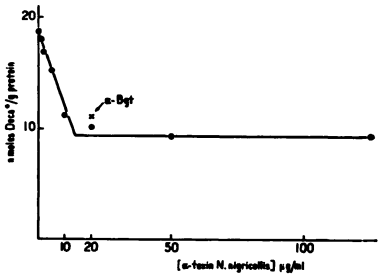

FIG. 8. *Irreversible antagonism by α-toxin from N. nigricollis to binding of ^{14}C-decamethonium (Deca*) to a deoxycholate extract*

The aqueous solution of toxin was added to the extract, and the mixture was gently agitated at room temperature for 1 hr. The concentration of protein in the extract was 20.9 mg/ml. α-Bgt, α-bungarotoxin.

48° for various times and binding of decamethonium was measured in the presence and absence of 10 μM gallamine. The activity of acetylcholinesterase on acetylthiocholine was followed in parallel. Inactivation of the enzyme and loss of the sites which bind decamethonium in the presence of gallamine followed the same time course. This result supports the assumption that in the presence of 10 μM gallamine decamethonium binds almost exclusively to the catalytic site of acetylcholinesterase. On the other hand, the rate of inactivation of the sites from which decamethonium is displaced by gallamine was much slower than that of the catalytic site of the enzyme. After heating for 10 min at 48° the extract still contained about 50% of the initial amount of decamethonium- and gallamine-binding sites but only 5% of the acetylcholinesterase active sites. This gives additional support to our tentative assumption that two distinct classes of decamethonium-binding sites are present. Furthermore, the observation that all the bound decamethonium can be assigned to either one or the other of these two classes of sites suggests that only these sites were detected.

Interpretation of the binding data was facilitated by quantitative measurement of the dissociation constants of the various cholinergic agents tested. The binding of decamethonium as a function of increasing concentration of the agonist was followed in the presence and absence of α-toxin. The binding curve obtained in the presence of 20 μg/ml of α-bungarotoxin approximates a hyperbola, and half-saturation occurs around 1 μM. This is very close to the K_i of decamethonium for the active site of acetylcholinesterase measured under the same experimental conditions with acetylthiocholine as the substrate (Table 2). From the differences between the results obtained in the absence of toxin and in its presence, one can draw a curve for the binding of decamethonium to sites which are blocked by the toxin (Fig. 10). This curve approximates a hyperbola, and half-saturation occurs with this preparation at 0.8 μM free decamethonium, which is in the range expected for the dissociation constant of decamethonium bound to the cholinergic receptor site (Table 2). The "intrinsic" dissociation constants of decamethonium for the two classes of binding sites are thus in complete agreement with the K_i of the ligand for the catalytic site of acetylcholinesterase and with its "apparent" affinity for the excitable membrane.

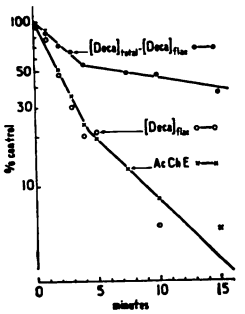

FIG. 9. *Effect of heat treatment on binding of ^{14}C-decamethonium to a deoxycholate extract*

Extract (0.45 ml) containing 20 mg of protein per milliliter was heated at 48° for the indicated times and then immediately cooled to 0°. On each sample were measured the activity of acetylcholinesterase (AcChE) with acetylthiocholine as the substrate and the capacity to bind ^{14}C-decamethonium in the presence ($[Deca]_{flux}$) and absence ($[Deca]_{total}$) of 10 μM gallamine. The specific activity of acetylcholinesterase in the extract was 9 moles of acetylthiocholine hydrolyzed per hour per gram of protein.

From the curves of antagonism represented in Figs. 2–6 we attempted to determine the "intrinsic" dissociation constants of the various cholinergic agonists and antagonists tested for the two classes of sites present in our extracts. Experiments were carried out under conditions (see MATERIALS AND METHODS) in which at least 70% of the bound decamethonium was displaced by an excess of either snake venom toxin, i.e., in which decamethonium was bound largely, but not exclusively, to the cholinergic receptor sites. Since an appreciable number of acetylcholinesterase catalytic sites were still present in our extracts, the values computed from these curves are not precisely the "intrinsic" dissociation constants of the cholinergic effectors for the sites blocked by toxins but can be regarded only as first approximations of these dissociation constants. Dissociation constants were estimated by plotting the reciprocal of the

TABLE 2

Comparison of "intrinsic" and "apparent" dissociation constants of cholinergic agonists and antagonists
Acetylcholinesterase activity was measured in Ringer's solution.

Compound	"Apparent" dissociation constants			"Intrinsic" dissociation constants		
	Isolated electroplax (membrane potential) (22°)[a]	Excitable microsacs ($^{22}Na^+$ efflux)[b]		Deoxycholate extract (^{14}C-decamethonium binding)	Acetylthiocholine hydrolysis (competitive inhibition)	
		22°	4°	(4°)	4°	22°
	μM	μM		μM	μM	
Agonists						
Decamethonium	1.2	1.2	1.2	0.8	2.7	2.3
Carbamylcholine	30	40	100	18.3	42	17
Phenyltrimethylammonium	13	20	40	13	73	57
Antagonists						
d-Tubocurarine	0.16	0.15	0.15	2.6	61	50
Gallamine	0.3	0.3	0.3	2.2	140	130
Hexamethonium	30	62		352	420	400

[a] From refs. 5, 17, 19, and 27.
[b] From ref. 28.

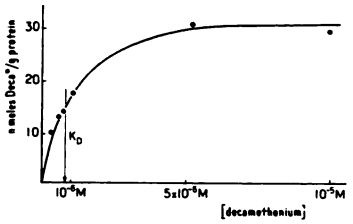

FIG. 10. *Specific binding of ^{14}C-decamethonium (Deca*) to cholinergic receptor sites present in a deoxycholate extract*

The values plotted on the ordinate correspond to the *differences* between data collected in the absence of any antagonist and after 60 min of exposure of a deoxycholate extract to an excess of α-bungarotoxin. The concentration of protein was 21 mg/ml. Above 0.5 μM decamethonium the concentration of ^{14}C-decamethonium was kept constant and the solution was supplemented with nonradioactive decamethonium.

amount of decamethonium bound as a function of increasing concentration of effector. The tangent of the curve at its origin was drawn, and on this line the concentration of effector (I_{50}) giving 50% displacement of decamethonium was measured. The dissociation constant K was then calculated by the relation

$$K = I_{50} \times \frac{K_{deca}}{K_{deca} + \text{free decamethonium}}$$

with K_{deca} = 0.8 μM. This constant thus concerns the part of the curve which is located near its origin, i.e., the sites presenting the highest affinity for the effectors considered. As expected, with *d*-tubocurarine or gallamine, compounds which displace decamethonium only partially, a similar value was obtained when the residual amount of decamethonium bound at saturating levels of antagonist was subtracted from all points on the curve.

In Table 2, we have compared these "intrinsic" dissociation constants with the "apparent" dissociation constants of the same compounds measured either on the isolated electroplax by following steady-state membrane potentials (5, 19) or on excitable microsacs by following $^{22}Na^+$ efflux (28). Kinetic data are also presented for acetylcholinesterase using acetylthiocholine in Ringer's solution at 4° and 22°.

The "intrinsic" affinities of the three agonists tested were in agreement with their

"apparent" affinities, although a systematic deviation was noticeable. The "apparent" affinities were smaller in each case than the "intrinsic" values. The "intrinsic" affinities of these agonists for the catalytic site of acetylcholinesterase were similar in magnitude to the "apparent" affinities of the same compounds for the excitable membrane. In other words, with these agonists, the decamethonium-binding sites of the cholinergic receptor cannot be distinguished easily from those of the catalytic unit of acetylcholinesterase. Indeed, we have mentioned that these three agonists completely displace decamethonium bound to *both* the cholinergic receptor site and the catalytic site of the enzyme.

This is also the case for one antagonist, hexamethonium, but the situation is different with *d*-tubocurarine and gallamine. With these two antagonists, the "intrinsic" affinities measured by equilibrium dialysis were 23 and 63 times higher, respectively, than those for the catalytic site of acetylcholinesterase, and antagonism was incomplete. For *d*-tubocurarine and gallamine a plateau was reached at concentrations less than 10^{-5} M; at this concentration minimal binding is to be expected at the catalytic center of acetylcholinesterase. These high "intrinsic" affinities of *d*-tubocurarine and gallamine were still less than their "apparent" affinities estimated from the membrane response. Nevertheless, the common finding that a class of decamethonium-binding sites possesses a high affinity for *d*-tubocurarine and gallamine and is irreversibly blocked by the snake toxins seems sufficient to identify them as the cholinergic receptor sites.

From these data the *numbers* of cholinergic receptor sites present in our extracts can readily be calculated. The values obtained varied noticeably from one extract to another. The total number of decamethonium-binding sites blocked by the α-toxin in the preparation used in the experiment represented in Fig. 8 was 24.7 nmoles/g of protein, but this value was rather high, and in some experiments extracts contained 10 times fewer sites per gram of protein. As shown in Table 3, the number of receptor sites can be estimated by the binding of either radioactive decamethonium or the toxin, assuming the toxin to be an exclusive and irreversible reagent for the receptor (25). Interestingly, these two numbers are similar, although in both cases the number of toxin molecules was found to be larger than the number of decamethonium-binding sites blocked by the toxin.

TABLE 3

Number of toxin- and decamethonium-binding sites and catalytic sites of acetylcholinesterase in deoxycholate extracts

The data for α-bungarotoxin are those of Changeux *et al.*, (25); the data for *Naja* toxin are those of Fig. 8. Numbers of catalytic sites were estimated from the value of the specific activity of acetylcholinesterase in the extract, assuming that 750 moles of acetylthiocholine are hydrolyzed by 1 g of pure enzyme per hour (20). The molecular weight of the enzyme was assumed to be 260,000, with four catalytic sites per molecule. In the experiment with α-bungarotoxin the extract contained 18 mg of protein per milliliter and the specific activity of the enzyme was 5.85 moles/hr/g of protein. In the experiment with the α-toxin the concentration of protein in the extract was 20.9 mg/ml and the specific activity of the enzyme was 5.25 moles/hr/g of protein.

	α-Bungarotoxin	α-Toxin of *N. nigricollis*
	nmoles/g protein	
No. of toxin molecules for complete blockade	152	110
No. of ^{14}C-decamethonium-binding sites blocked by toxin	40	24.7
No. of acetylcholinesterase molecules	30	27
No. of acetylcholinesterase catalytic sites	120	108

The number of catalytic sites of acetylcholinesterase present in our preparation can be computed from the specific activity of the pure protein [assuming a molecular weight of 260,000 and a specific activity of 750 moles of acetylcholine per gram of protein per hour (20), and four catalytic sites per molecule of mol wt 260,000 (29)]. Table 3 shows that the number of catalytic sites is close to the number of toxin molecules required to block decamethonium binding at the cholinergic receptor site. In addition, the

table shows that the number of acetylcholinesterase catalytic centers is similar to but somewhat greater than the number of cholinergic receptor sites.

Chemical nature of the molecule binding decamethonium, gallamine, and α-toxins. The capacity to bind decamethonium at the level of the sites blocked by gallamine and the α-toxins was preserved after concentration of the extract on a Diaflo membrane (XM 50). The cholinergic receptor sites are thus carried by a macromolecule of molecular weight larger than 50,000 daltons. As shown in Table 4, this structure is thermosensitive and acid-labile and is no longer active upon digestion with Pronase; it is thus a protein or, at least, contains a protein moiety.

Separation of polypeptide chains carrying cholinergic receptor site and catalytic site of acetylcholinesterase. The α-toxin from *N. nigricollis* was coupled to Sepharose granules following the technique described by Axen, Porath, and Ernback (14). Table 5 shows the results of an experiment in which the cholinergic receptor protein was adsorbed to the Sepharose α-toxin. A concentrated deoxycholate extract was mixed with an equal volume of settled Sepharose α-toxin and incubated at room temperature for 30 min; then the mixture was centrifuged at low speed and the supernatant fluid was assayed for acetylcholinesterase activity and for binding of ^{14}C-decamethonium in the presence and absence of 10^{-5} M gallamine.

TABLE 4

Some properties of the cholinergic receptor macromolecule

Samples of an extract containing 18 mg of protein per milliliter were subjected to the treatment indicated and then dialyzed at pH 8.0 in the presence of 0.56 μM ^{14}C-decamethonium or 0.56 μM ^{14}C-decamethonium and 10 μM gallamine.

Treatment	Decamethonium bound			Protein
	0.56 μM free decamethonium	0.56 μM free decamethonium + 10 μM gallamine	Antagonism	
	nmoles/ml		%	*mg/ml*
Controls	352	180	48	18
2 min, 100°	18	—[a]		
5 min, pH 4.75	17	—[a]		
Pronase, 8 mg/ml, 3 hr	61	—[a]		7

[a] Negligible.

TABLE 5

Differential adsorption of cholinergic receptor protein to α-toxin from N. nigricollis coupled to Sepharose 4B

One milliliter of decanted Sepharose toxin or Sepharose alone was suspended in 1.0 ml of extract containing 20–25 mg of protein per milliliter in 30 mM sodium citrate (pH 8.0) and 1% deoxycholate. The suspension was slowly agitated for 30 min at 20°, then cooled to 0°, and centrifuged at 2000 rpm in a Sorvall SS 34 rotor at 4°. Acetylcholinesterase and ^{14}C-decamethonium were assayed in the supernatant fraction.

Supernatant	Acetylcholinesterase	^{14}C-Decamethonium displaced by 10 μM gallamine	Protein
	moles/hr/g protein	*nmoles/g protein*	*mg/ml*
Sepharose 4B + 1% deoxycholate	9.1	3.0 ± 0.4	15.1
Sepharose 4B α-toxin + 1% deoxycholate	8.0	0.3 ± 0.3	14.2

After exposure to the Sepharose toxin the extract lost 75–100% of its capacity to bind decamethonium at the level of the cholinergic receptor site, less than 12% of its acetylcholinesterase activity, and about 6% of its total protein content. Thus selective adsorption of the cholinergic receptor protein on Sepharose toxin granules physically separates it, *in vitro*, from acetylcholinesterase (7). This result is in agreement with the observations that 1) decamethonium bound to a preparation of acetylcholinesterase which contained the various isozymes A, C, and D of Massoulié and Riéger (30), was not significantly displaced by 10^{-5}M gallamine; 2) acetylcholinesterase (Sigma) was not adsorbed to the Sepharose toxin (Table 5).

The cholinergic receptor site and the catalytic site of acetylcholinesterase are thus distinct protein units. This finding (7) has been confirmed by Miledi *et al.* (8) with *Torpedo*.

DISCUSSION

The experiments presented above illustrate the method that we have followed to

obtain (a) a binding test characteristic of the physiological receptor site of acetylcholine and (b) a procedure for extraction of the macromolecule(s) which carries this site.

A method of rapid equilibrium dialysis was selected to measure the reversible binding of cholinergic agents. Decamethonium, a cholinergic agonist, was used as the radioactive ligand, since it presents a high "apparent" affinity *in vivo* for the excitable membrane of the eel electroplax and it is not hydrolyzed by acetylcholinesterase. At the ionic strength of physiological Ringer's solution, little, if any, nonspecific binding of decamethonium occurs. Extraction of membrane fragments with deoxycholate yields a soluble crude preparation of proteins which contains two major classes of specific decamethonium-binding sites. The first constitutes the catalytic site of the enzyme acetylcholinesterase, a protein present in large amounts in our extracts. Decamethonium bound to this site is displaced by carbamylcholine and various competitive inhibitors of the enzyme. It is not displaced by *d*-tubocurarine or gallamine, two antagonists *in vivo*, except at concentrations above 10^{-5} M). This would also be predicted from the K_i estimated by kinetic experiments. In addition, *d*-tubocurarine and gallamine antagonize the binding of decamethonium to another class of sites and at concentrations which are compatible with the "apparent" affinity of these compounds, measured *in vivo* with the isolated electroplax, or *in vitro* with excitable microsacs. Binding of decamethonium to this last class of site is also blocked, selectively and irreversibly, by two polypeptidic toxins purified from snake venoms: α-bungarotoxin (21) from *B. multicinctus* and the α-toxin from *N. nigricollis*. These toxins are considered the most selective reagents for the cholinergic receptor site. The parallelism between the effect of these toxins and that of typical cholinergic antagonists, such as *d*-tubocurarine and gallamine, strongly supports the conclusion that the decamethonium-binding site on which they act is the cholinergic receptor site. We thus have an assay *in vitro* for the specific binding of a cholinergic agonist to the physiological receptor of acetylcholine.

It is worthwhile to emphasize that cholinergic antagonists such as *d*-tubocurarine and gallamine displace *in vitro* the binding of a cholinergic agonist, decamethonium. In other words, the classical antagonism between a depolarizing and a blocking agent, observed with the excitable membrane, can be repeated with the soluble receptor macromolecule present in our extracts. In addition, the "intrinsic" affinities measured with our binding assay are similar to the "apparent" affinities recorded *in vivo*. However, a slight but consistent discrepancy exists between the two sets of results. The "intrinsic" affinities of the agonists were systematically higher than their "apparent" affinities, and the reverse was true for the antagonists. The significance of this difference is not yet clear, since in these experiments the free concentration of antagonist added was never measured directly. Assuming that this result is correct, an explanation for the apparent discrepancy can be proposed in the framework of the general theory of the allosteric transition of the cholinergic receptor (1). The isolated receptor macromolecule could be thought of as stabilized, *in solution*, in a state or conformation which would preferentially bind the agonist, i.e., in a state close to that adopted by the receptor when the membrane is depolarized by acetylcholine.

According to the same theory, the displacement of decamethonium by the cholinergic antagonists observed with our soluble extracts would be mediated by an allosteric transition of the receptor macromolecule. This type of displacement should be contrasted with the displacement of decamethonium by cholinergic agonists, which may be understood strictly on the basis of steric hindrance. The fact that the snake venom toxins block the binding of both agonists and antagonists and that *d*-tubocurarine protects against toxin binding has suggested to us that all these compounds may bind to overlapping sites on the receptor macromolecule (25).

The macromolecule which carries the cholinergic receptor site is thermolabile and digested by proteolytic enzymes. It is, as previously predicted by Nachmansohn (3), a protein, or, at least, possesses a protein moiety. In addition, the receptor protein is adsorbed selectively to snake venom α-toxin covalently bound to Sepharose, and can thus be readily separated from acetylcholinester-

ase. The catalytic site of acetylcholinesterase and the cholinergic receptor site thus are located on different polypeptide chains. Nothing more is known about the topographical relationships of these two protein units once integrated within the excitable membrane. Our extraction procedure leads to the dissociation of these elementary units, but alternative methods might preserve their association (30).

Several years ago, one of us showed that at low ionic strength acetylcholinesterase strongly binds a number of cholinergic agents, and analysis of kinetic data suggested that regulatory sites for cholinergic agents were present on the enzyme surface (18). This point was recently confirmed by Kitz and his associates (31). It is now clear that these effects disappear at the ionic strength at which we assay the cholinergic receptor sites and thus seem unrelated to a hypothetical function of acetylcholinesterase as the cholinergic receptor protein. However, the recent report that this enzyme is found in more than one form having strikingly different sedimentation velocities (30) indicates that acetylcholinesterase is not a single molecular species. Again, hypothetical association of some catalytic units of acetylcholinesterase with the receptor protein cannot be completely ruled out. In this respect Kato *et al.* (32) have found that a partially purified preparation of squid acetylcholinesterase contains an atropine-binding site distinct from the catalytic site.

Finally, our findings may be compared with published results on the much-debated subject of the cholinergic receptor. Early claims by Chagas *et al.* (33) and Ehrenpreis (34) were subsequently withdrawn (35, 36). The problem of isolation of the specific cholinergic receptor was restated by Beychok (37), who emphasized that two major criteria had to be fullfilled by the fraction suspected of containing the cholinergic receptor protein: (a) homogeneity of the binding sites and (b) agreement of the thermodynamic binding constant of the cholinergic effector measured by equilibrium dialysis with the "apparent" binding constant estimated *in vivo* from dose-response curves.

The work of O'Brien and associates (38–40) has been carried out largely with insoluble membrane fragments from the electric tissue of *Torpedo*. These authors claimed "partial solubilization" of the binding protein, but the "solubilized" activity did not migrate on electrophoresis. Unfortunately, almost nothing is known of the pharmacological properties of *Torpedo* electroplax *in vivo*. Assuming that the electroplax of this elasmobranch has properties similar to the subsynaptic membrane of the neuromuscular junction from higher vertebrates, and in particular that muscarone is a specific ligand of the cholinergic receptor site, it becomes plausible that binding occurs at least partly at the level of the cholinergic receptor sites present in membrane fragments. In their last paper, these authors presented a few results obtained with a particulate fraction of *E. electricus*, but distinction between binding at the cholinergic receptor site and at the catalytic site of acetylcholinesterase was not explicitly discussed, particularly with decamethonium as a ligand. The very recent studies of Miledi, Molinoff, and Potter (8) have been carried out, as well, with *Torpedo* tissue.

Nevertheless, there exists general agreement between the results obtained with *Torpedo* and *Electrophorus* on the following points. (a) Decamethonium alone is a relatively nonspecific reagent. (b) Snake venom α-toxins are highly specific reagents for the cholinergic receptor site (8, 21, 25). (c) The receptor contains a protein moiety, as judged by its degradability by proteinases. (d) Various detergents can solubilize receptor activity. (e) Acetylcholinesterase and receptor activities are clearly separable.

The cholinergic proteolipid receptor of De Robertis and associates (41–43) has a high affinity for various cholinergic agonists and antagonists. However, under the conditions described by those authors the binding is irreversible: moreover, the binding experiments were carried out in the presence of organic solvents, and the dissociation constants measured by them can hardly be compared with the apparent dissociation constants obtained *in vivo* in the presence of Ringer's physiological solution. In addition, although their material appears to have been chromat-

ographically homogeneous, several classes of binding sites for cholinergic ligands were present.

In our case the criterion of homogeneity is not fulfilled, but we have clearly distinguished specific from nonspecific binding. In addition, the "intrinsic" affinities of the various effectors tested agree generally with their "apparent" affinities *in vivo*.

Finally, comparison of the number of receptor sites reported by the various authors might be an excellent indication of the specificity of binding. In our soluble preparation we have found 10–100 nmoles of decamethonium-binding sites per gram of protein. The number of toxin molecules required for complete blockade of decamethonium receptor sites falls in the same range but is always higher. Either the toxin is not an exclusive reagent for the cholinergic receptor site or several toxin molecules are required for complete blockade of a decamethonium receptor site. It is interesting to recall that decamethonium is a bisquaternary ligand and consequently might bind to two acetylcholine receptor sites.

The number of catalytic sites of acetylcholinesterase, estimated on the basis of the presently available data on the structure of the enzyme, is (25) similar to the number of receptor sites estimated with the snake venom toxins. Interestingly, the numbers of muscarone-binding sites and acetylcholinesterase catalytic sites found by O'Brien are very similar to ours and to those of Miledi. The density of cholinergic receptor sites per gram of tissue protein thus seems to be of the same order of magnitude in *Torpedo* and *Electrophorus* electric tissue. The values obtained by Karlin *et al.* (44), using an affinity-labeling reagent, are lower. However, they studied the whole electroplax, and our experiments were performed with a preparation enriched in receptor protein.

The results of Waser (45) are in agreement with those mentioned above. He has esti-

TABLE 6

Comparison of numbers of cholinergic receptor sites found by various authors with different preparations derived from electric tissue

Authors	Preparation	Ligand	Technique	No. of receptor sites
				nmoles/g protein
O'Brien *et al.* (39)	Membrane fragments from *T. marmorata*	^{3}H-Muscarone	Equilibrium dialysis	109
Karlin *et al.* (44)	Single electroplax from *E. electricus*	^{3}H-4-Maleimidobenzyltrimethylammonium	Affinity labeling after reduction	0.3–0.9
De Robertis *et al.* (42)	Proteolipid from *E. electricus*	^{14}C-Acetylcholine	Column fractionation	25,000–250,000
De Robertis and Fiszer de Plazas (43)	Purified membrane fragments from *E. electricus*	^{14}C-Hexamethonium; dimethyl-(+)-^{14}C-tubocurarine	Centrifugation	15,000 2,600
Changeux *et al.* (6, 25)	Preparation of soluble receptor protein from *E. electricus*	^{14}C-Decamethonium; displacement by α-bungarotoxin	Equilibrium dialysis	40 152
Kasai and Changeux (28)	Purified excitable membrane fragments from *E. electricus*	^{14}C-Decamethonium; displacement by α-bungarotoxin	Ultracentrifugation	59 108
Miledi *et al.* (8)	Membrane fragments from *T. marmorata*	^{131}I-α-Bungarotoxin	Ultracentrifugation	197

mated that mouse diaphragm contains 5–10 × 10^6 receptor complexes in the postsynaptic membrane of one end plate. The number of synapses per electroplax of *E. electricus* is 50,000, according to Nachmansohn (3). Assuming a 1:1 correspondence between an electroplax synapse and a diaphragm end plate, there would be 2.5–5 × 10^{11} receptors per electroplax according to Waser, which is close to the 1.6–4.5 × 10^{11} receptors per electroplax reported by Karlin *et al.* (44).

The numbers reported by De Robertis and co-workers are strikingly different. However, the receptor proteolipid represents a very small proportion of the total protein content of the electroplax, and this might explain the high specific activities reported (see Table 6). Analysis of the data published by La Torre, Lunt, and De Robertis (41) shows that the number of proteolipid molecules per gram of fresh tissue is of the order of 6.8 × 10^{14} in *Torpedo* and about half this value in *Electrophorus*. The former number appears to be in agreement with those proposed by Changeux *et al.* (25) and Miledi *et al.* (8) for α-bungarotoxin with *Electrophorus* (2.4 × 10^{14} sites/g of fresh tissue) and *Torpedo* (6.6 × 10^{14} sites/g of fresh tissue), respectively.

It is plausible, but not certain, that in all the cases cited above the authors followed binding at the level of the physiological cholinergic receptor.

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REFERENCES

1. J.-P. Changeux, T. R. Podleski and J. C. Meunier, *J. Gen. Physiol.* **54**, 225S (1969).
2. B. Belleau, V. Di Tullio and Y. H. Tsai, *Mol. Pharmacol.* **6**, 41 (1970).
3. D. Nachmansohn, "Chemical and Molecular Basis of Nerve Activity." Academic Press, New York, 1959.
4. E. Schoffeniels and D. Nachmansohn, *Biochim. Biophys. Acta* **26**, 1 (1957).
5. H. Higman, T. R. Podleski and E. Bartels, *Biochim. Biophys. Acta* **75**, 187 (1963).
6. J.-P. Changeux, M. Kasai, M. Huchet and J. C. Meunier, *C. R. Hebd. Seances Acad. Sci. Paris* **270**, 2864 (1970).
7. J. C. Meunier, M. Huchet, P. Boquet and J.-P. Changeux, *C. R. Hebd. Seances Acad. Sci. Paris* **272**, 117 (1971).
8. R. Miledi, P. Molinoff and L. Potter, *Nature* **229**, 554 (1971).
9. W. Gilbert and B. Müller-Hill, *Proc. Nat. Acad. Sci. U. S. A.* **56**, 1891 (1966).
10. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
11. C. Y. Lee, C. C. Chang, T. H. Chiu, P. J. S. Chiu, T. C. Tseng and S. Y. Lee, *Naunyn-Schmiedebergs Arch. Exp. Pathol. Pharmakol.* **259**, 360 (1968).
12. P. Boquet, Y. Izard, M. Jouanet and J. Meaume, *C. R. Hebd. Seances Acad. Sci. Paris* **262**, 1134 (1966).
13. E. Karlsson, D. Eaker and J. Porath, *Biochim. Biophys. Acta* **127**, 505 (1966).
14. R. Axen, J. Porath and S. Ernback, *Nature* **214**, 1302 (1967).
15. J.-P. Changeux, T. R. Podleski and L. Wofsy, *Proc. Nat. Acad. Sci. U. S. A.* **58**, 2063 (1967).
16. J.-P. Changeux, W. Leuzinger and M. Huchet, *FEBS Lett.* **2**, 77 (1968).
17. H. G. Mautner, E. Bartels and G. D. Webb, *Biochem. Pharmacol.* **15**, 187 (1966).
18. J.-P. Changeux, *Mol. Pharmacol.* **2**, 369 (1966).
19. J.-P. Changeux and T. R. Podleski, *Proc. Nat. Acad. Sci. U. S. A.* **59**, 944 (1968).
20. W. Leuzinger and A. L. Baker, *Proc. Nat. Acad. Sci. U. S. A.* **57**, 446 (1967).
21. C. Y. Lee and C. C. Chang, *Mem. Inst. Butantan Sao Paulo* **33**, 555 (1966).
22. P. Boquet, Y. Izard and A. M. Ronsseray, *C. R. Hebd. Seances Acad. Sci. Paris* **271**, 1456 (1970).
23. F. Tazieff-Depierre and J. Pierre, *C. R. Hebd. Seances Acad. Sci. Paris* **263**, 1785 (1966).
24. H. Lester, *Nature* **227**, 727 (1970).
25. J.-P. Changeux, M. Kasai and C. Y. Lee, *Proc. Nat. Acad. Sci. U. S. A.* **67**, 1241 (1970).
26. P. Boquet, Y. Izard, M. Jouannet and J. Meaume, *in* "Animal Toxins," p. 293. Ruffel, F. E. and Saunders, P. R. eds. Pergamon Press, New York, 1967.
27. A. Karlin and M. Winnik, *Proc. Nat. Acad. Sci. U. S. A.* **60**, 668 (1968).
28. M. Kasai and J.-P. Changeux, *C. R. Hebd. Seances Acad. Sci. Paris* **270**, 1400 (1970).
29. L. Kremzner and I. B. Wilson, *Biochemistry* **3**, 1902 (1964).

30. J. Massoulié and F. Riéger, *Eur. J. Biochem.* **11**, 441 (1969).
31. R. J. Kitz, L. M. Braswell and S. Ginsburg, *Mol. Pharmacol.* **6**, 41 (1970).
32. G. Kato, J. Yung and M. Ihnat, *Mol. Pharmacol.* **6**, 588 (1970).
33. C. Chagas, E. Penna-Franca, K. Nishie and E. J. Garcia, *Arch. Biochem. Biophys.* **75**, 251 (1958).
34. S. Ehrenpreis, *Biochim. Biophys. Acta* **44**, 561 (1960).
35. C. Chagas, *Ann. N. Y. Acad. Sci.* **81**, 345 (1959).
36. S. Ehrenpreis, *Nature* **201**, 887 (1964).
37. S. Beychok, *Biochem. Pharmacol.* **14**, 1249 (1965).
38. R. D. O'Brien and L. P. Gilmour, *Proc. Nat. Acad. Sci. U. S. A.* **63**, 496 (1969).
39. R. D. O'Brien, L. P. Gilmour and M. E. Eldefrawi, *Proc. Nat. Acad. Sci. U. S. A.* **65**, 438 (1970).
40. M. E. Eldefrawi, A. T. Eldefrawi and R. D. O'Brien, *Mol. Pharmacol.* **7**, 104 (1970).
41. J. L. La Torre, G. S. Lunt and E. De Robertis, *Proc. Nat. Acad. Sci. U. S. A.* **65**, 716 (1970).
42. E. De Robertis, G. Lunt and J. La Torre, *Mol. Pharmacol.* **7**, 97 (1970).
43. E. De Robertis and S. Fiszer de Plazas, *Biochim. Biophys. Acta* **219**, 388 (1970).
44. A. Karlin, J. Prives, W. Deal and M. Winnik, *Ciba Found. Symp. Molecular Properties of Drug Receptors*, 247 (1970).
45. P. G. Waser, *Ciba Found. Symp. Molecular Properties of Drug Receptors*, 59 (1970).