Isolation of Cholinergic Receptor Protein(s) from *Torpedo nobiliana* by Affinity Chromatography†

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ABSTRACT: The acetylcholine receptor (AChR) was purified 60-fold from the electroplax membranes of *Torpedo nobiliana* by affinity chromatography utilizing the principal neurotoxin of *Naja naja siamensis* as ligand. Displacement of the AChR from the Sepharose-bound toxin–AChR complex was far more difficult than when the complex was free in solution, suggesting that covalent linkage of the toxin to the Sepharose significantly changes the ligand–protein interaction. The purified AChR binds one molecule of neurotoxin per *ca.* 80,000 daltons of protein and shows bands corresponding to molecular weights 43,500, 38,500, 35,500, and 33,500 on sodium dodecyl sulfate polyacrylamide gels. Purification of the AChR using two other neurotoxins as ligands (from *Naja naja atra* or *Bungarus multicinctus*) yielded an AChR preparation with properties identical with those of the AChR isolated with *N. n. siamensis*. Since the three neurotoxins differ in amino acid content, molecular size, and affinity for AChR, it is likely that a unique population of protein molecules is selectively isolated from the tissue extracts, suggesting receptor multiplicity.

The selective isolation and purification of biologically important macromolecules by "affinity chromatography" exploit the unique biological property of these proteins to bind ligands specifically and reversibly (Cuatrecasas *et al.*, 1968). Elapid snake venoms contain polypeptide neurotoxins which possess the extraordinary properties of binding with very high affinity and with nearly total specificity to the nicotinic acetylcholine receptor (AChR)[1] found in excitable membranes at neuromuscular junctions (Boquet *et al.*, 1966; Lee and Chang, 1966). Hence these neurotoxins appear to be ideal ligands for the isolation and purification of the AChR *via* affinity chromatography. However, since the interaction between the toxin and AChR was generally regarded as irreversible (Miledi *et al.*, 1971; Meunier *et al.*, 1972), other ligands, notably quaternary nitrogen functions, were used in initial attempts to purify the AChR protein. The use of these selective adsorbants achieved extensive purification of the AChR protein from *Electrophorus electricus* (Olsen *et al.*, 1972), *Narcine entemedor* (Schmidt and Raftery, 1972), and *Torpedo californica* (Schmidt and Raftery, 1973). In each case, these purified preparations were inhomogeneous upon gel electrophoresis which suggested that the preparations were either contaminated with inert proteins or that more than one AChR was present in the tissue.

The recent observation that mice given sublethal doses of neurotoxins ($0.8–0.9 \times LD_{100}$) displayed severe dyspnea and yet recovered within a few hours (Karlsson *et al.*, 1971) was interpreted as being inconsistent with an irreversible interaction between the toxin and AChR. Consequently, an affinity column, using as ligand the principal neurotoxin of *Naja naja siamensis*, was employed to isolate the AChR (Karlsson *et al.*, 1972) from extracts of *Torpedo marmorata*. The free AChR was eluted from the column with carbamylcholine and two protein fractions, each of which retained high binding capacity for curare and the neurotoxin, were obtained. As was evident from the affinity chromatography pattern, these fractions were inhomogeneous and no additional purification procedures were reported.

We now report the preparation and use of three highly selective adsorbents to isolate and purify the AChR from *Torpedo nobiliana*. The adsorbents contained as ligands the neurotoxins from *Naja naja atra, Naja naja siamensis,* or *Bungarus multicinctus*.

We describe the gel electrophoretic characterization of the highly purified protein obtained using each of the three adsorbents and we discuss the significance of the observed identical heterogeneous patterns.

Materials and Methods

Sodium [^{3}H]borohydride was purchased from New England Nuclear, Boston, Mass. Lyophilized venoms of *Naja naja atra* (Formosan cobra), *Naja naja siamensis* (Thailand cobra), and *Bungarus multicinctus* (Formosan krait) were supplied by the Ross Allen Reptile Institute, Silver Springs, Fla. Cyanogen bromide activated Sepharose 4B, SP-Sephadex, DEAE-Sephadex, and Sephadex G-100 were purchased from Pharmacia, Uppsala, Sweden. *Torpedo nobiliana* was obtained from the Marine Biological Laboratory, Woods Hole, Mass.

Preparation of Electroplax and Excitable Membranes. Electric organs were excised from freshly killed ray and homogenized for 5 min in a Waring blender. The homogenate was lyophilized and stored at −70°. No loss of receptor activity was observed after prolonged storage of material processed in this manner.

Excitable membrane fractions were prepared in the following manner. Lyophilized electric organ tissue (250 mg) was suspended in 10 ml of modified Ringer's solution (Karlin, 1967), and homogenized in a Ten-Broeck hand homogenizer. The resulting suspension was centrifuged for 1 hr at 36,000*g* and the pellet was retained.

Extraction of Excitable Membranes. Receptor protein was extracted from excitable membranes by stirring with 1% Triton X-100 in Ringers solution (v/v) at room temperature for 1 hr,

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[1] Abbreviations used are: AChR, acetylcholine receptor; OV, ovalbumin; Ald, aldolase, Chym, chymotrypsinogen; RNase A, ribonuclease A.

followed by centrifugation for 1 hr at 36,000*g*. The resulting supernatant solution routinely contained 80% of the neurotoxin binding activity originally present in the excitable membranes.

Preparation of Neurotoxins. The principal neurotoxins of *B. multicinctus* (α-bungarotoxin) and *N. n. siamensis* were prepared by a modification of a chromatographic procedure reported by Brisbois *et al.* (1968). Lyophilized venom was dissolved in a minimal volume of 0.05 M sodium phosphate buffer (pH 6.0) and applied to a column of SP-Sephadex C-25 (1.5 × 40 cm) which had previously been equilibrated with the same buffer. Proteins were eluted from the column by a linear salt gradient (0.05 M sodium phosphate buffer (pH 6.0) to 1 M sodium chloride in 0.05 M sodium phosphate buffer (pH 6.0), total volume 1 l.). Examination of the fractions for toxicity in the rat phrenic nerve diaphragm preparation identified a single neurotoxic peak eluting from the column between 0.3 and 0.4 M salt. The fractions of the neurotoxic peak were combined, diluted to twice their original volumes with 0.05 M sodium phosphate buffer (pH 6.0), and applied to an SP-Sephadex C-25 column (1.5 × 40 cm) which had been previously equilibrated with 0.2 M sodium chloride in 0.05 M sodium phosphate (pH 6.0); a linear gradient formed from 0.2 to 0.5 M sodium chloride in 0.05 M sodium phosphate buffer (pH 6.0) (total volume 500 ml) displaced the pure neurotoxins from the resin. Cobratoxin was isolated from the venom of *N. n. atra* according to Yang (1964). Each neurotoxin was shown to be homogeneous by disc gel electrophoresis (Reisfeld *et al.*, 1962).

[^{3}H]Cobratoxin. Tritium was incorporated into cobratoxin by reductive formylation (Rice and Means, 1971) utilyzing sodium [^{3}H]borohydride as the reducing agent. After the reaction was complete, the pH was adjusted to 5 with 0.1 N HCl and the reaction mixture was dialyzed against distilled water in a Bio-Rad minibeaker. The resulting solution was directly applied to a column of SP-Sephadex (1.5 × 40 cm, equilibrated with 0.2 M sodium chloride in 0.05 M sodium phosphate buffer, pH 6.0) and the protein was eluted with a gradient of 0.2–1 M sodium chloride in 0.05 M sodium phosphate buffer (pH 6.0) (total volume 500 ml). The major radioactive peak possessed the same chromatographic properties as native cobratoxin. Disc gel electrophoresis (Reisfeld *et al.*, 1962) of the radioactive toxin displayed a single band which ran concurrently with native toxin. The gel was sectioned, dissolved in 30% H_2O_2, and counted in 10 ml of a scintillation fluid composed of one-third Triton X-100 and two-thirds Econofluor (New England Nuclear). All radioactivity corresponded in position to the visible protein band. Acid hydrolysis of the [^{3}H]cobratoxin followed by characterization of all radioactive species (Kakimoto and Akazawa, 1970; Tyihak and Vagujfalvi, 1970) demonstrated that only lysine residues had been modified. Greater than 98% of the radioactivity was present as dimethyllysine. Precipitation (see Assay Procedures) of the [^{3}H]toxin–receptor complex in the presence of excess solubilized receptor revealed that 70% of the toxin molecules retained AChR-binding ability. The neurotoxic properties of the [^{3}H]cobratoxin as judged by time to complete blockade in the rat phrenic nerve diaphragm preparation (Bilbring, 1946) were reduced to 75% of those of the native toxin. The specific radioactivity was 1.92 Ci/mmol which indicates an average of 1.5 tritiums per molecule (25% of the value expected for complete alteration of all lysines present).

Preparation of Selective Adsorbents. Neurotoxins were coupled to cyanogen bromide activated Sepharose 4B essentially according to Axen *et al.* (1967). Neurotoxin (3–4 mg) was added per g of dry gel (or 1 mg/ml of swollen gel vol) and the coupling reaction was allowed to proceed at room temperature for 1 hr in 0.1 M sodium borate buffer (pH 8.5). The resin suspension was made 1 M in ethanolamine and the reaction was continued for an additional 1 hr. The coupling reaction was determined to be 92% complete and approximately 10% of the coupled toxin residues retained the ability to bind soluble AChR protein.

Assay for Toxin–AChR Complex. The extent of [^{3}H]toxin–AChR complex formation was determined by a modification of the Franklin and Potter (1972) method. Solubilized AChR preparation was added to a solution containing 0.025 M potassium phosphate buffer (pH 6.7), 0.1 M sodium chloride, 0.25% Triton X-100, 1.2 mg of bovine serum albumin, and 0.3 mg of γ-globulin (final volume was 1 ml). Binding was initiated by the addition of 2.1 μg of [^{3}H]cobratoxin (1.3×10^6 dpm) to give a final toxin concentration of 3×10^{-7} M. After the reaction mixture has been allowed to stand at room temperature for 1 hr, 4.0 ml of 37% saturated ammonium sulfate solution in 0.05 M potassium phosphate (pH 6.5) was added, mixed well, and the resulting precipitate was immediately collected on Millipore filters. The precipitate was (1) washed with 10 ml of 30% saturated ammonium sulfate, (2) transferred (together with the filter) to a plastic liquid scintillation counting vial, and (3) digested for 1 hr at 50° with 1 ml of Protosol (New England Nuclear). The resulting solution was decolorized by the addition of 0.1 ml of 30% hydrogen peroxide followed by incubation for 1 hr at 50°. The solutions were neutralized with 0.05 ml of glacial acetic acid and 15 ml of scintillation fluid (previously described) was added to each and the samples were counted.

Affinity Chromatography. Affinity columns (0.5 × 4.5 cm) were prepared in glass wool stoppered Pasteur pipets. To ensure maximal adsorption of the AChR, solubilized AChR preparations were cycled through the column twice. The column was then washed with the following series of eluents: 3.0 ml of modified Ringers, 3.0 ml of 1.0 M sodium chloride in 0.05 M potassium phosphate buffer (pH 7.0), and 3.0 ml of 0.05 M potassium phosphate buffer (pH 7.0). Each wash solution contained 1% Triton X-100.

The affinity column was connected in series to a Pasteur pipet column (0.5 × 4.5 cm) of DEAE-Sephadex C-25. A solution of 0.1 M carbamylcholine chloride in 0.01 M potassium phosphate buffer (pH 7.0) and 1% Triton X-100 was then cycled from a reservoir, through the columns and returned to the reservoir by means of a Technicon pump (flow rate, 0.25 ml/min). During elution the temperature was maintained at 4°. The DEAE-Sephadex column was removed from the system afer specified time periods (1, 2, or 3 days) and was washed with 5.0 ml of 0.01 M potassium phosphate buffer (pH 7.0)–0.1% Triton X-100 to completely remove the cycling eluent. The purified AChR was displaced from the DEAE-Sephadex with 0.5 M sodium chloride in 0.01 M potassium phosphate buffer (pH 7.0)–0.1% Triton X-100. Fractions of 1.0 ml were collected; 70–80% of the recovered AChR was obtained in the first milliliter of eluent.

Protein was determined by amino acid analysis on a Beckman Model 121 amino acid analyzer according to the procedure of Shih and Hash (1971) and by the method of Lowry *et al.* (1951). Acetylcholinesterase activity was determined by measuring the rate of acetylthiocholine hydrolysis as described by Ellman *et al.* (1961).

Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate gel electrophoresis was performed according to the procedure of Weber and Osborn (1969). Molecular weights were established by coelectrophoresis with standards. Densitometric

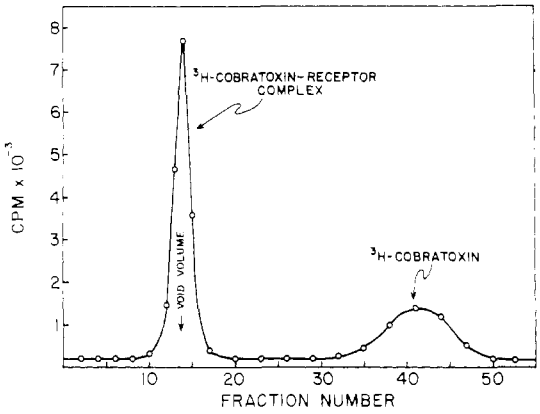

FIGURE 1: Chromatography of solubilized membrane protein on Sephadex G-100. A preparation of crude *Torpedo* electroplax membranes was maximally labeled with [H³]cobratoxin, then washed extensively by centrifugation to remove free toxin. The membranes were extracted with Triton X-100 as described in Methods and the solubilized protein obtained after centrifugation was submitted to gel chromatography. Fractions of 5.0 ml were collected and radioactivity was determined.

traces were obtained using a Gilford spectrophotometer with a Model 2410 linear transport attachment.

Results

Formation of [³H]Cobratoxin-AChR Complex. The elution profile obtained by chromatography of solubilized [³H]cobratoxin-AChR complex (see legend, Figure 1) on Sephadex G-100 is shown in Figure 1. A clean separation of [³H]cobratoxin-AChR complex from [³H]cobratoxin is achieved by this procedure. An identical pattern is obtained if the [³H]cobratoxin is added to the extract after the excitable membranes have been solubilized. As indicated in Figure 2, formation of the [³H]cobratoxin-AChR complex is linear with respect to added solubilized membrane protein over the concentration range examined. The extent of complex formation is greatly decreased when either *d*-tubocurare, carbachol, or the neurotoxin of *N. n. siamensis* are included in the assay mixture. As

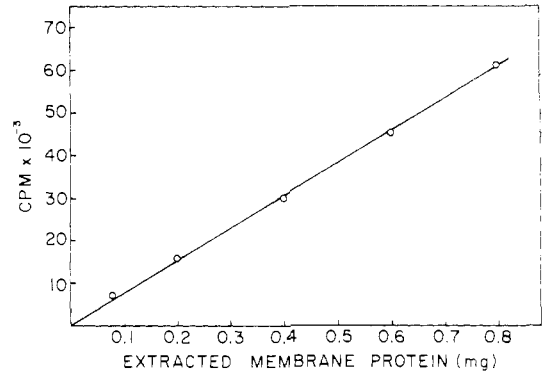

FIGURE 2: Formation of [³H]toxin-AChR complex. Increasing amounts of extracted membrane protein were added to 3.0×10^{-7} M [³H]cobratoxin and assayed for complex formation as described in Methods.

seen in Table I, saturated concentrations of *d*-tubocurare (10^{-2} M) only reduce complex formation by 62% whereas high levels of carbachol and *N. n. siamensis* neurotoxin essentially abolish [³H]cobratoxin-AChR complex formation. Of the latter two ligands, the neurotoxin is by far the more potent, completely inhibiting complex formation when added at a concentration 20 times that of the [³H]cobratoxin. High salt and lysosyme, a protein of low molecular weight and high p*I*, have no measurable effect on complex formation.

Reversal of [³H]Cobratoxin-AChR Complex. The ability of various neuroactive agents to reverse the [³H]cobratoxin-AChR complex is summarized in Table II. Carbachol, decamethonium, and *d*-tubocurare very efficiently displace the toxin from the complex. Since the extent of complex reversal is not linear with respect to the concentration of displacing agent, no direct comparisons can be made concerning the relative efficiencies of these three ligands to displace cobratoxin from the complex. The neurotoxins of *N. n. siamensis* and *B. multi-*

TABLE I: Inhibition of AChR-[³H]Toxin Complex Formation by Various Ligands.[a]

Ligand	Concn (M)	Inhibn (%)
	1×10^{-4}	25
Carbachol	0.1	78
	0.5	97
d-Tubocurare	1×10^{-3}	49
	1×10^{-2}	62
N. n. Siamensis neurotoxin	1×10^{-8}	49
	5×10^{-7}	83
	1×10^{-5}	96
Lysosyme	1×10^{-4}	0
NaCl	0.5	0

[a] Ligands were added to the standard assay mixture to give the ligand concentrations listed and the reaction was incubated for 1 hr at room temperature as described in Methods. [³H]Cobratoxin was added (5×10^{-7} M) and the reaction was incubated for an additional hour. The final volume was 2.0 ml. The per cent inhibition was determined by the decrease in precipitable counts compared to the assay without added ligand.

TABLE II: Reversal of AChR-[³H]Toxin Complex by Various Ligands.[a]

Ligand	Concn (M)	Reversal (%)
Carbachol	0.01	25
	0.1	45
	0.5	73
Decamethonium	0.05	43
	0.1	48
d-Tubocurare	0.005	41
	0.05	61
N. n. Siamensis neurotoxin	5×10^{-6}	23
	5×10^{-5}	56
α-Bungarotoxin	5×10^{-6}	22
	5×10^{-5}	64
Lysosyme	1×10^{-4}	0
NaCl	0.5	0

[a] [³H]Toxin-AChR complex was prepared by incubating the standard assay mixture for 1 hr as described in Methods. Ligands were added to the reaction mixture to give the ligand concentrations listed and the reaction was incubated at room temperature for an additional hour. The final volume was 2.0 ml. The per cent reversal was determined by the decrease in precipitable counts compared to the assay without added ligand. [³H]Cobratoxin was present at 5×10^{-7} M.

TABLE III: Results of Continuous Elution of Sepharose-Bound Toxin–AChR Complex with 0.1 M Carbachol at 4°.[a]

Time Period (hr)	Recov (% of Bound AChR)	Equiv Wt (daltons)/ Molecule of Toxin Bound
0–24	40.1	82,000
24–48	12.2	76,000
48–72	4.1	

[a] A solubilized preparation of excitable membrane protein was subjected to affinity chromatography as described in Methods. DEAE-Sephadex columns were removed after 24 hr and replaced with fresh columns. The isolated AChR was eluted from the DEAE-Sephadex (see Methods), and the per cent recovery and equivalent weight of the purified AChR were determined. Cycling solution was 0.1 M carbachol–0.01 M potassium phosphate–1% Triton X-100 (pH 7.0).

cinctus effectively reversed the complex when added at a concentration 100 times that of the [^{3}H]cobratoxin. High salt and lysosyme (at concentrations 200 times that of the cobratoxin) have no measurable effect on complex reversal.

When the complex was partially reversed with carbachol and the assay mixture was applied directly to a column of DEAE-Sephadex C-25, both the undissociated complex and the free AChR were retained by the resin. When the free AChR was eluted from the column with high salt and assayed for [^{3}H]cobratoxin–AChR complex formation (see Assay Procedures), it was verified that no AChR activity was lost during complex formation and reversal.

Affinity Chromatography. Although several neuroactive agents efficiently displace the toxin from the toxin–AChR complex when the complex is free in solution, once the toxin is covalently bound to the Sepharose matrix, reversal conditions change immensely. Dissociation studies were carried out using suspensions of toxin-bound Sepharose under incubation conditions of time, temperature, toxin concentrations, etc., similar to those employed for the dissociation studies of the free complex. Carbachol (0.5 M), decamethonium (0.3 M), *d*-tubocurare (0.02 M), formic acid (0.5 M) and sodium chloride (0.15 M), guanidine (6 M), mercaptoethanol (0.05 M), and dithioerythritol (0.05 M) were totally ineffective when employed to displace AChR from the adsorbent bound complex. Because of the inefficient AChR displacement in these batchwise experiments, a cycling system of continuous elution was designed (see Experimental Section) to increase AChR yield. Carbachol (0.1 M) was selected as eluent since higher carbachol concentration precluded retention of the AChR protein on the DEAE-Sephadex column.

Under the conditions employed, one cycle of the solubilized AChR preparation through the affinity column was sufficient to remove 90% of the available AChR activity. Further cycling of the same solubilized preparation never yielded greater than 96% adsorption even though the capacity of the column had not been exceeded. As noted earlier, approximately 10% of the immobilized cobratoxin retained the ability to bind AChR.

Recovery of free AChR from the DEAE-Sephadex column after continuous elution of the affinity column with 0.1 M carbachol is shown in Table III. Approximately one-half of the bound AChR material is displaced by the carbachol, the greatest portion being eluted within the first 24 hr. Recoveries of as high as 55% of bound AChR have been observed for a 24-hr elution.

Attempts to further purify the receptor protein by elution of the DEAE-Sephadex column with a stepwise gradient from 0.15 to 0.5 M sodium chloride (0.05 M steps) gave only a single protein peak, eluting between 0.2 and 0.3 M salt.

Although different adsorbent preparations containing the same or different neurotoxins (cobratoxin, α-bungarotoxin, and *N. n. siamensis* neurotoxin) were essentially identical in capcity and percentage of active AChR-binding toxin molecules, some variation was observed in the efficiency with which AChR was eluted from batch to batch. Adsorbents which contained cobratoxin or *N. n. siamensis* neurotoxin yielded essentially the same percentage recovery; however binding of the AChR to the α-bungarotoxin–Sepharose adsorbent was significantly more difficult to reverse. (Recovery of active AChR from these columns was approximately one-half that observed with the other two neurotoxins.)

The typical two-step purification procedure is described in Table IV. The isolated AChR protein has been purified 60-fold and has an equivalent weight of approximately 80,000 daltons per molecule of toxin bound. The acetylcholine esterase activity of this purified material is extremely low, representing about 0.005% by weight of the total recovered protein. High levels of acetylcholine esterase activity are present in the crude extract of the excitable membranes but all the esterase activity applied to the affinity column is recovered in the nonadsorbed fractions.

If AChR protein is adsorbed to an affinity column and the adsorbent is eluted with several column volumes of 6 M guanidine, the dialysed guanidine eluent contains protein but very little AChR activity. If such a guanidine-treated affinity column was inserted into the continuous elution system and eluted for 24 hr with 0.1 M carbamylcholine, no protein or toxin binding material was collected by the DEAE-Sephadex column. Apparently the guanidine pretreatment eluted the AChR from the affinity column but irreversibly denatured it in the process.

Gel Electrophoresis. The electrophoretic pattern of purified AChR protein isolated from an adsorbent containing as ligand *N. n. siamensis* neurotoxin is shown in Figure 3. In the presence of sodium dodecyl sulfate, at least five polypeptide species are present, one band with an apparent molecular weight of 43,500, a grouping of three bands at molecular weights of 38,500, 35,500, and 33,500, and a major band observed at a position on the gel which indicate molecular weights greater

TABLE IV: Purification of Acetylcholine Receptor.

Fraction	Protein (mg)	Act. (pmol of Toxin Bound)	Sp Act. (pmol/mg)	Purifcn -fold	Recov (%)
Membrane suspension	35.2[a]	6.87	0.195		
Triton X-100 extract	17.6[a]	5.48	0.311	1.6	79.8
Purified receptor	0.242[b]	2.96	12.2	62	43.1

[a] By Lowry. [b] By amino acid analysis.

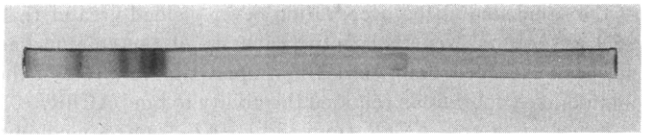

FIGURE 3: Gel electrophoresis of purified AChR. AChR was purified by continuous elution affinity chromatography (see Methods) utilizing the neurotoxin of *N. n. siamensis* as ligand. Approximately 20 μg of the purified protein were electrophoresed as described in Methods. The gel was developed from left to right.

than 70,000. This latter may represent multimers of the smaller species. Identical gel patterns were observed for AChR protein isolated from adsorbents utilizing α-bungarotoxin and cobratoxin as the adsorbing ligands (see Figure 4). When the protein fraction which was eluted from the affinity column by 6 M guanidine was examined by gel electrophoresis, an electrophoretic pattern identical with Figure 3 was obtained.

Discussion

Affinity Chromatography. The investigation reported here offers a feasible approach for the rapid isolation of milligram quantities of AChR. Although displacement of the AChR from the Sepharose-bound toxin–AChR complex was not nearly as facile in our hands as previously reported by Karlsson *et al.* (1972), the continuous elution technique provides for ample recovery of bound AChR.

Successful application of affinity chromatography depends in large part on how closely experimental conditions permit the ligand–protein interaction to simulate the reactions observed when the components are free in solution. Examination of the amino acid sequences of the three neurotoxins employed as ligands shows that each had a lysine residue which is in close proximity to one of the residues thought to play a central role in toxin–AChR interaction (Harrington and Brady, unpublished data). Hence, covalent linkage to the Sepharose resin through the ϵ-amino group of this lysine would probably preclude formation of the toxin–AChR complex. Chang *et al.* (1971) reported that this same lysine is the most reactive lysine in the cobratoxin molecule; predominant coupling of the toxin to the resin through this residue would explain the observation that only 10% of the toxin molecules retain the ability to bind to AChR after they are coupled to the Sepharose. It is also apparent that covalent linkage of the toxin–AChR complex to the Sepharose significantly changes the ligand–protein interactions since displacement of the AChR is more readily accomplished when the complex is free in solution. No efforts were made to vary the solid carrier, to introduce a "bridge" between the toxin and resin or to determine which residue in the toxin molecule was best suited for linkage to the resin in order to produce more facile displacement of AChR. Variation of these functions and consideration of any stereochemical alteration of the ligand resulting from its covalent linkage to the water-insoluble carrier may better simulate those conditions which permit complex reversal in free solution.

Receptor Purification. As indicated in Figure 3, isolation of the AChR by affinity chromatography as described herein does not give rise to a unique polypeptide species. The three neurotoxins which are employed as ligands for the chromatography vary in amino acid content, molecular size, and affinity for the AChR. It is striking that the protein species isolated by means of each of these immobilized toxins (see Figure 4) exhibit identical pattern upon sodium dodecyl sulfate gel electrophoresis strongly arguing that a unique population of protein molecules is being selectively isolated. The high selectivity of the method is demonstrated by the essentially complete exclusion of acetylcholineesterase from the adsorbent and by the observation that apparently only AChR protein binds to the adsorbent since elution with 6 M guanidine yields a protein fraction with an electrophoretic pattern identical with that obtained with 0.1 M carbachol, a cholinergic ligand.

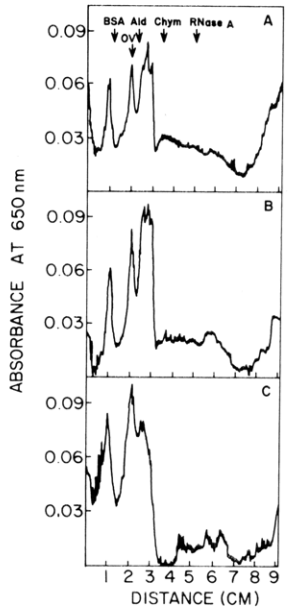

FIGURE 4: Gel electrophoresis of purified AChR. AChR was purified by continuous elution affinity chromatography (see Methods) utilizing as ligands the neurotoxin of *N. n. siamensis* (A), cobratoxin (B), and α-bungarotoxin (C). Approximately 20 μg of the purified AChR were electrophoresed and scanned as noted in Methods.

Contamination arising from the ion-exchange properties in the resin is extremely unlikely since material adsorbed to the column in this manner would be removed by the molar sodium chloride wash which precedes the continuous elution process. Although impurities resulting from hydrophobic interactions with the adsorbent cannot be completely ruled out, they are highly unlikely since their appearance would require the specific reversal of the hydrophobic interactions by 0.1 M carbachol, a cholinergic ligand. A more likely explanation for the presence of more than one molecular species in our preparation is receptor multiplicity. Plurality of binding sites has been reported for two species of *Torpedo* (Raftery *et al.*, 1972; Eldefrawi *et al.*, 1971) and hereterogeneity on sodium dodecyl sulfate gels has been previously observed for material purified by affinity chromatography employing covalently bound quaternary ammonium functions as ligands (Schmidt and Raftery, 1973). Purified α-bungarotoxin–AChR complex, obtained by using α-bungarotoxin to displace AChR from an affinity column containing cobratoxin as ligand, shows heterogeneity after sodium dodecyl sulfate electrophoresis (Raftery, 1973). As in the present study, the major species are in the 35,000–45,000 molecular weight range.

A third and as yet unconsidered explanation of the heterogeneity is the concept that the AChR protein is tightly associated in the membrane with specific non-toxin-binding proteins. Treatment with Triton X-100 might lead to cosolubilization such that the integrity of the protein–protein interactions is undisturbed. The associated proteins could be carried through the affinity chromatography purification and eluted from the DEAE-Sephadex column still attached to the AChR. Treatment for sodium dodecyl sulfate gel electrophoresis would then disrupt the association and upon electrophoresis this specific population of non-toxin-binding proteins would be revealed.

Finally, it is possible that the several species observed upon

sodium dodecyl sulfate gel electrophoresis are a result of proteolytic hydrolysis of the AChR protein while in the crude solubilized preparation or during the extended period of time required for the affinity chromatography. However, when the affinity chromatography was carried out at room temperature, the recovery of AChR was greater, but no change in the electrophoretic pattern of the isolated protein was observed.

An equivalent weight of 80,000 daltons/molecule of toxin bound is consistently obtained by this procedure and is lower than that observed by Karlsson (140,000 and 300,000) after affinity chromatography on insolubilized *N. n. siamensis* neurotoxin and by Raftery (160,000) after affinity chromatography on a quaternary ammonium function. It is identical with that suggested by Miledi and Potter for material purified by sucrose gradient centrifugation. Since the majority of isolated protein falls in the molecular weight range of 33,000–43,000 unless more than one toxin molecule binds to a single polypeptide, the lowest equivalent weight possible would probably also be in this range. Since we observe four species of approximately equal quantity in this range, an equivalent weight of 80,000 would appear to require that more than one species be capable of binding toxin, again arguing for receptor multiplicity. Attempts to answer the question of multiple toxin-binding protein by subjecting [^{3}H]cobratoxin–purified AChR complex to sodium dodecyl sulfate gel electrophoresis (with and without mercaptoethanol) invariably led to dissociation of the complex. The question of how many of the observed species are capable of binding neurotoxin therefore remains unanswered. Numerous disc gel electrophoresis systems were also investigated but none proved suitable for analysis of the purified AChR or the AChR–toxin complex. Although only one protein band was observed in the absence of sodium dodecyl sulfate, with all systems tried, it was noted that the protein did not sufficiently penetrate into the gel to allow for satisfactory separation of protein components; perhaps this was a result of the formation of large aggregates in Triton X-100. We observed a single peak of purified AChR in the molecular weight range greater than 200,000 when the material was applied to a calibrated Sephadex G-200 column. Experiments are now in progress to ascertain how many of the observed species are capable of binding [^{3}H]toxin.

The AChR appears to be a major constituent of the electroplax membrane protein. Based on an equivalent weight of 80,000 daltons, the 60-fold purification suggests that as much as 1.5% of membrane protein is AChR.

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