If we now consider the ORD and CD spectra of phosvitin at pH 6.5, it appears that Ca^{2+} and Mg^{2+} affect the optical properties of the protein (Figure 4A,B). Although Mg^{2+} has a similar effect as previously observed for Na^{+} (Grizzuti and Perlmann, 1970), $[m']_{205}$ becomes less levorotatory when 17 or 117 Ca^{2+} is bound by phosvitin. Similarly, the fine structure of the dichroic bands in the wavelength range of 210–250 nm is affected in an adverse manner by Ca^{2+}. Thus these cations exert different effects on the spatial structure of the side chains of the phosvitin molecule.

In conclusion we should like to state that, although phosvitin in the pH range of 6.5–9.0 has an "unordered" conformation, ORD and CD measurements reveal that the nature of the counterions is of importance in controlling the polypeptide backbone conformation.

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Molecular Properties of the Cholinergic Receptor Purified from *Electrophorus electricus*†

Greg Biesecker

ABSTRACT: The cholinergic receptor protein from *Electrophorus electricus* was identified by the specific binding of a radioactively labeled snake neurotoxin, cobrotoxin, from *Naja naja atra*, solubilized from membrane fragments of the eel electric organ with detergent, and was purified on DEAE-cellulose and by affinity chromatography. The purified protein contained $2.2 \pm 0.3 \times 10^5$ g of protein/mol of toxin binding sites, gave a single band on an isoelectric focusing gel, and contained a single major polypeptide with a mol wt of 44,000. Results of cross-linking the tritiated toxin–receptor complex indicate that the mol wt 44,000 polypeptide is part of a mol wt 260,000 receptor molecule which binds the toxin.

According to the present theory of chemical transmission at cholinergic synapses (Eccles, 1964; Katz, 1969) depolarization of the postsynaptic membrane is initiated by the interaction of acetylcholine with a specific membrane component, the cholinergic receptor (AChR).[1] Numerous small molecules which either block or mimic the action of acetylcholine are thought to compete for a binding site on the receptor. In recent years small polypeptide neurotoxins which at very low concentrations irreversibly block synaptic transmission (Lee *et al.*, 1967; Lester, 1970) have been isolated from many snake venoms. Since their action is inhibited by cholinergic ligands, the snake neurotoxins are assumed to bind specifically to the receptor.

Because of its physiological importance, there have been many attempts to isolate and purify AChR (review articles: Rang, 1971; O'Brien *et al.*, 1972). Although the most direct method to identify the receptor is by its interaction with acetylcholine (Eldefrawi *et al.*, 1972), it is difficult to separate specific binding to the receptor from binding to acetylcholinesterase (whose catalytic sites must be blocked) and choline acetyltransferase and from nonspecific binding to other proteins. The much more specific binding of snake neurotoxins has proven a more reliable and easier means of identifying the cholinergic receptor. In addition to their irreversible actions *in vivo*, the snake neurotoxins specifically block the binding of acetylcholine (Eldefrawi *et al.*, 1972) and decamethonium (Changeux *et al.*, 1970) to the solubilized receptor and are in turn inhibited by several other cholinergic ligands (Meunier *et al.*, 1972).

Physical properties of crude preparations of detergent solubilized receptor labeled with radioactive neurotoxins have been investigated (Miledi *et al.*, 1971; Raftery *et al.*, 1971; Meunier *et al.*, 1971), and partial purification of the receptor has been achieved using isoelectric focusing (Eldefrawi and Eldefrawi, 1972) and several different affinity columns (Olsen *et al.*, 1972; Karlsson *et al.*, 1972; Schmidt and Raftery, 1973).

† From the Department of Chemistry, Yale University, New Haven, Connecticut 06520. *Received April 9, 1973.* This work was supported by U. S. Public Health Service Grant No. GM-04483 to J. H. Wang and U. S. Public Health Service Grant No. GM-12006-09 to F. M. Richards. This work forms part of the dissertation to be submitted by Greg Biesecker to Yale University in partial fulfillment of the requirements for the doctoral degree. The author has been supported as a doctoral candidate on U. S. Public Health Service Training Grant GM-711.

[1] Abbreviation used is: AChR, acetylcholine receptor.

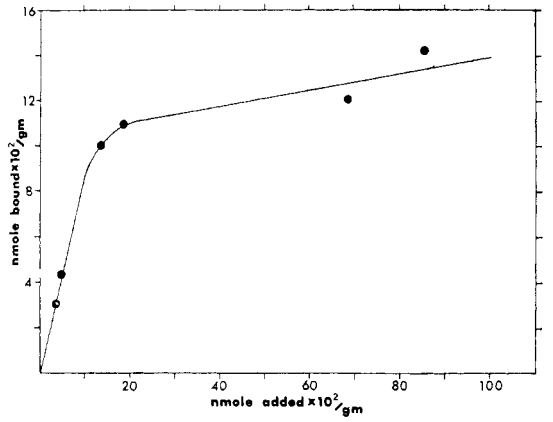


FIGURE 1: Binding of ^{14}C-labeled cobrotoxin to membrane fragments of electric organ. The receptor concentration was calculated per gram of organ.

This paper describes a method for the purification of the cholinergic receptor from *Electrophorus electricus* by affinity chromatography, using as an assay the binding of cobrotoxin from *Naja naja atra* (Yang, 1965). The purified receptor gives a different pattern of polypeptides on sodium dodecyl sulfate gel electrophoresis than that seen by Schmidt and Raftery (1973) for receptor purified from *Torpedo*, with a single major polypeptide at mol wt 44,000, intermediate to the value of 40,000 reported by Reiter *et al.* (1972) from affinity labeling data, and the value of 55,000 reported for a toxin–receptor complex in sodium dodecyl sulfate (Meunier *et al.*, 1972). An attempt was also made to determine the molecular weight of the receptor in detergent solution by cross-linking the toxin–receptor complex.

Materials and Methods

Cobrotoxin from *Naja naja atra* was purified from the crude venom (Sigma Chemical Co.) according to Yang (1964). The purified toxin moved as a single peptide of mol wt <10,000 on sodium dodecyl sulfate electrophoresis. Its LD_{50} was approximating 1–2 μg per mouse, measured by intraperitoneal injection. The molecular weight was taken as 7000 (Yang *et al.*, 1970) and the concentration was calculated using a molar extinction coefficient of 9.22×10^3 at 280 nm (Yang, 1965). A Cary 11 or Cary 15 was used for absorbance measurements.

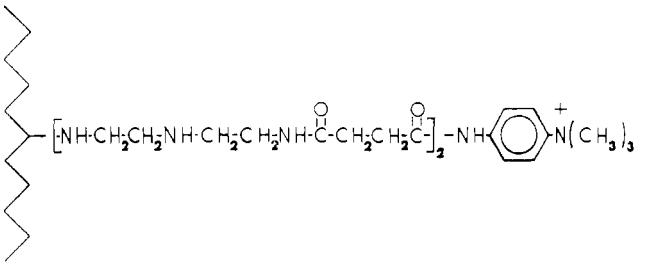
The toxin was labeled by partial methylation of the primary amino groups according to the procedure of Rice and Means (1971), using either [^{14}C]formaldehyde (New England Nuclear, 10 mCi/mmol) or NaB^3H_4 (Schwarz-Mann, 16,500 mCi/mmol). The labeled toxin was then desalted on a G-25 Sephadex column (Pharmacia, Sweden) and repurified on CM-cellulose (Whatman CM-52). [^{14}C]Cobrotoxin at 6 mCi/mmol and [^{3}H]cobrotoxin at 1.5 Ci/mmol, containing an average of ~0.5 methyl group per toxin, were obtained. Radioactivity was monitored using a Packard TriCarb Model 3002. The labeled protein retained full toxicity. A biologically inactive cobrotoxin derivative was made by oxidation with *N*-bromosuccinimide according to the method of Tu *et al.* (1971).

Electric eels (*Electrophorus electricus*), obtained from Paramount Aquarium, Ardsley, N. Y., were used a a source of synaptic material. The eels were cut into 10-cm sections, and the anterior portion of the main organ was removed, yielding 400–600 g wet weight of organ from a 3-ft eel. The organ was washed and stored in 0.1 M NaCl–0.01 M Tris (pH 8.0), 2–4°, prior to use, or washed in distilled water and frozen at −16°. The frozen material could be stored for several months with little loss of AChR.

Acetylcholinesterase (EC 3.1.1.7) was assayed using a Radiometer automatic titrator and buret. The assay solution of 4 mM acetylcholine chloride–0.1 M NaCl–40 mM $MgCl_2$, 22°, was maintained at pH 7.4 with 0.01 M NaOH. A specific activity of 660 mmol of acetylcholine hydrolyzed/mg of acetylcholinesterase per hr was used in the calculations (Kremzner and Wilson, 1964).

Protein was determined according to Lowry *et al.* (1951), using bovine serum albumin (Sigma Chemical Co.) as a standard.

Affinity Column. Trimethyl(*p*-aminophenyl)ammonium chloride hydrochloride was synthesized by the method of Traylor and Singer (1967), and coupled to Sepharose 6-B (Pharmacia) according to the method of Berman and Young (1971). The column was washed with 2 M guanidine·HCl for reuse.



Binding to Tissue. Portions of the electric organ were cut into ~0.5-cm pieces and homogenized in 3 vol of 0.1 M NaCl–0.01 M Tris (pH 8.0), 2–4°, with a Waring Blendor for 4 min at high speed. The homogenate was then spun at 12,000 rpm (23,500*g*) for 30 min in a Sorvall RC2-B centrifuge at 2–4°; the supernatant was discarded, and the precipitate drained. The precipitate contained 0.23 g of material/g of tissue.

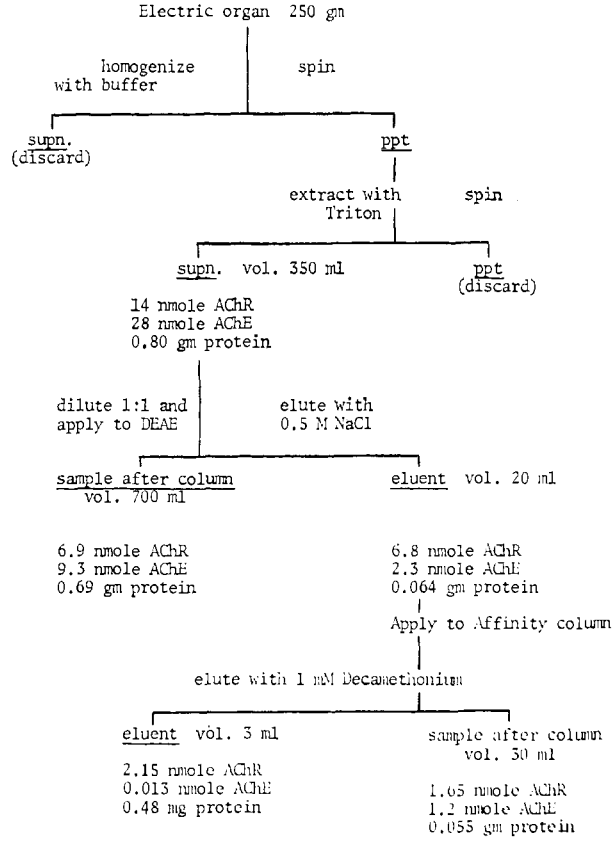
Samples of the tissue fragments were measured into 10-ml centrifuge tubes and suspended in 0.1 M NaCl–0.01 M Tris (pH 8.0), 2–4°, and aliquots of labeled toxin were added. The suspension was incubated overnight (20 hr) at 2–4° with periods of occasional stirring and then spun at 15,000 rpm (27,000*g*) for 15 min. The precipitate was resuspended in cold buffer and incubated for 1 hr and then spun as before. The precipitate was dissolved by heating in 2 ml of Protosol (New England Nuclear) for 2 hr at 60°, and then counted in 15 ml of Bray's solution (Bray, 1960) after incubation for 2 days at 2–4°, to eliminate chemiluminescence (Figure 1).

Binding to Solubilized Material. The homogenized tissue was suspended in 1.5 vol of 1.5% Triton X-100 (Packard)–0.1 M NaCl–0.01 M Tris–10% glycerol (pH 8.0), 2–4°, with a Teflon and glass hand homogenizer (Thomas) and stirred for 3 hr. The suspension was then spun at 100,000*g* for 1 hr in a Beckman Model L2-65B ultracentrifuge, the supernatant was decanted, and the precipitate discarded.

Labeled toxin was added to a sample of the supernatant, incubated for 15 min, and then applied to a Sephadex G-50 column equilibrated with 0.5% Tween 20–0.1 M NaCl–0.01 M Tris (pH 7.8), 24°. Fractions were collected and counted in Bray's solution (Figure 2). There are two separate peaks, the AChR–cobrotoxin complex emerging in the void column, and the free toxin at the back of the column. The G-50 column was used to assay for AChR during the purification.

Purification of AChR (Chart I). Portions of the electric organ were homogenized, spun, and extracted with 1.5 vol

CHART I



of detergent solution as before. The extract was spun at 23,500g in a Sorvall centrifuge for 2 hr. The supernatant contained 5.4×10^7 g of protein per mol of toxin binding sites. The sample was filtered through a 0.45-μ Millipore filter and then brought to pH 7.5 with 0.1 N HCl and applied to a DEAE-cellulose (Whatman) column equilibrated with 1.5% Triton–0.1 M NaCl–0.01 M Tris–10% glycerol (pH 7.5) (vol 7–10 ml; flow rate, 20 ml/hr), or first diluted 1:1 with solvent and then applied to the column. All operations were carried out at 2–4°.

Approximately 50% of the AChR bound when the undiluted solution was applied and up to 75–80% when first diluted. The remainder of the AChR cound be recovered by reapplication to a fresh or regenerated DEAE column.

The column was washed with 30–50 ml of 5% Triton–0.1 M NaCl–10 mM Tris–10% glycerol (pH 7.5) and then eluted with 0.5 M NaCl–5% Triton–0.01 M Tris–10% glycerol (pH 7.5). About 20 ml of the void peak was collected and dialyzed against 5% Triton–0.1 M NaCl–0.01 M Tris–40 mM $MgCl_2$–10% glycerol (pH 7.5). The AChR was recovered in almost quantitative yield, purified six- to tenfold.

The AChR sample from the DEAE column was then applied to the affinity column equilibrated with 5% Triton–0.1 M NaCl–0.04 M $MgCl_2$–10% glycerol–0.01 M Tris (pH 7.5) (volume, 1 ml; 1.15 cm × 1 cm; flow rate, 2–3 ml/hr). The column was then washed with 10–20 ml of solvent. The column was eluted with the same solvent as above but containing 1 mM decamethonium bromide (flow rate, 1 ml/hr) and the void peak containing the AChR was collected (volume, 2–3 ml). The purified AChR contained $2.2 \pm 0.3 \times 10^5$ g of protein/mol of cobrotoxin binding sites, for an overall purification of 256-fold. The AChR was concentrated by adsorption onto a 1-ml column of DEAE-cellulose, followed by elution

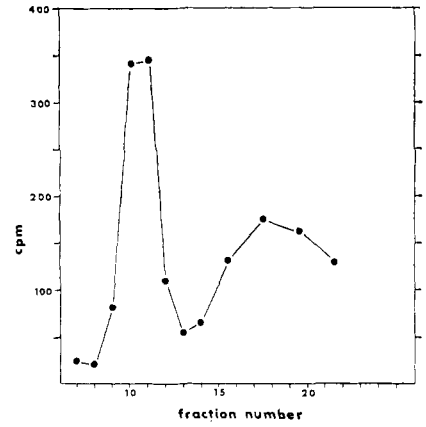


FIGURE 2: Sephadex G-50 column assay for solution binding of ^{14}C-labeled cobrotoxin. To a 5-ml aliquot of the Triton extract was added 50 μl of ^{14}C-labeled-cobrotoxin (0.1 mg/ml) and the sample was applied to a G-50 column (2 cm × 22.5 cm). Fractions (3 ml) were collected and 1 ml was counted in 10 ml of Bray's solution. The toxin–receptor complex emerged in the void volume, the free toxin at the back.

with 0.5 M NaCl–1.5% Triton–10 mM Tris (pH 7.5) and dialysis.

Sodium dodecyl sulfate gel electrophoresis was run according to Weber and Osborn (1969). The protein was denatured by addition to 100 μl of protein of a 50-μl aliquot of freshly made 30% sodium dodecyl sulfate–11% β-mercaptoethanol, followed by incubation at 40° for 18 hr. The samples were run on 10% acrylamide–0.18% bisacrylamide gels. The gels were soaked in 5% Cl_3CCOOH for 20 min and then stained with Coomassie Brilliant Blue. Cytochrome *c*, chymotrypsinogen, ovalbumin, and bovine serum albumin were used for molecular weight standards.

Isoelectric focusing was run similar to the procedure of Wrigley (1971). A pH gradient from 2 to 10 was set up in 3.75% acrylamide–0.375% bisacrylamide gels containing 2% carrier ampholytes (LKB, Sweden) and 2% Triton X-100. The upper reservoir buffer contained 0.4% ethylenediamine and 2% Triton; the lower buffer contained 0.2% H_2SO_4 and 2% Triton. The sample containing sucrose was applied to the top of the gel; then a solution of 2% ampholytes and 2% Triton X-100 was layered on top of the sample. The gels were run at room temperature.

Receptor–Toxin Cross-Linking. (A) The receptor–toxin complex was cross-linked with either glutaraldehyde or *p*-difluorodinitrobenzene. To 50 μl of 0.1 mg/ml of receptor in 0.5% Brij-35–0.1 M NaCl–50 mM sodium phosphate (pH 7.7), 23°, was added 20 μl of tritiated toxin solution to give a slight molar excess of toxin. The sample was incubated for 2 hr and then cross-linking reagent was added. The reaction mixture was then incubated overnight. The next day 10 μl of ethanolamine at 60 mg/ml, pH 8.0, was added to quench any remaining reagent. The samples were then denatured and run on sodium dodecyl sulfate gel electrophoresis as previously described. Bovine serum albumin at 5 mg/ml was cross-linked with 1% glutaraldehyde to use in addition to the other standards. The gels were fractionated on a Gilson Aliquogel Fractionator and then soaked in ~0.5 ml of a 0.1-mg/ml Pronase solution overnight and counted in 10 ml of Bray's solution.

(B) Tritiated cobrotoxin (100 μl) at 0.12 mg/ml was mixed with 100 μl of 0.5 M Na_2CO_3 (pH 9.1), 2–4°. Two microliters of 10% glutaraldehyde was then added, and the sample was allowed to react for 2 min at 2–4°. The reacted toxin, 4 μl,

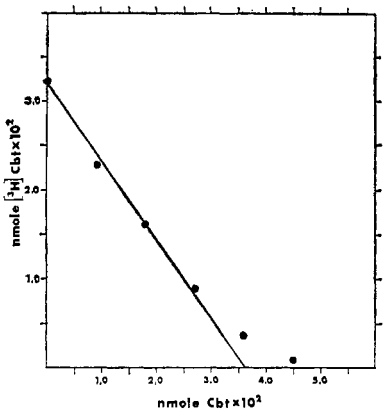


FIGURE 3: Blockage of tritiated cobrotoxin binding by native toxin. To a 0.4-ml sample of assay solvent containing 10^{-7} M receptor was added cobrotoxin, and the sample was incubated for 30 min. Excess tritiated cobrotoxin was then added and the sample was quickly applied to a Sephadex column (0.9 cm × 9 cm; flow rate, 40 ml/hr). Fractions (0.5 ml) were collected and counted in 4 ml of Bray's solution. The tritiated toxin bound was calculated from the void peak.

was added to 30 μl of 0.23 mg/ml of receptor in 0.3% Brij-35–0.3% Triton–0.1 M NaCl–40 mM sodium phosphate–7% glycerol (pH 7.7), at 2–3°. The reaction was quenched after 30 min by the addition of 3 μl of ethanolamine solution. After standing overnight the sample was denatured and run on sodium dodecyl sulfate electrophoresis as before. The gel fractions were counted in 20 ml of Bray's solution.

Results

The cholinergic receptor concentration in a preparation of electroplax membrane fragments was calculated from the saturatable portion of the cobrotoxin binding curve (Figure 1) to be 102 ± 10 pmol/g of electric organ. The receptor could be completely solubilized (91 ± 5 pmol/g) with Triton X-100, using a Sephadex G-50 column to assay for toxin binding in solution (Figure 2). The amount of receptor obtained from the electric organ in ten preparations from six eels ranged from 54 to 109 pmol/g of organ, with an average of 78 pmol; preparations from different eels had a much greater variation in the receptor concentration than samples from any single eel. Samples of frozen organ, which were used within 2 months, showed no loss of activity compared to fresh, unfrozen organ.

Gel filtration was the most accurate and reliable method to measure toxin binding; assays were reproducible within 2–3%. As shown in Figure 3, binding of the labeled toxin was quantitatively blocked by native cobrotoxin. *N*-Bromo-

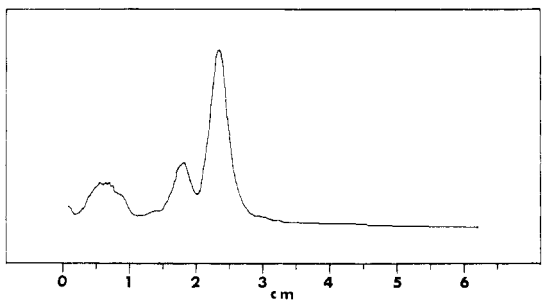


FIGURE 4: Sodium dodecyl sulfate gel electrophoresis of the receptor. A 50-μl sample containing 20 μg of protein was applied to the gel. The marker dye front was 6.5 cm from the top of the gel, which is to the left of the diagram.

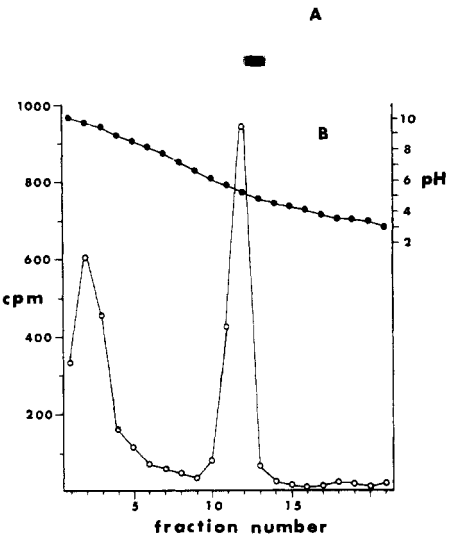


FIGURE 5: Isoelectric focusing of receptor and toxin–receptor complex. (A) A 200-μl sample of the AChR in 2% Triton, stained after soaking in 5% trichloroacetic acid with Coomassie Brilliant Blue. The protein band at pH 4.7 was cut from a parallel unstained gel and the receptor recovered (in 50% yield) by soaking in pH 7 buffer. (B) A 100-μl sample of tritiated toxin–receptor complex in 2% Triton was applied to the gel. The gel was cut into 0.5-cm slices, heated to 60° in 1 ml of 5% (w/w) H_2O–Protosol for 2 hr, and then counted. Binding of the toxin shifted the p*I* of the receptor 0.5 pH unit to pH 5.2. The pH gradient was determined by fractionating an equivalent gel.

succinimide-oxidized toxin did not block binding of the labeled toxin, demonstrating the correlation between binding and biological activity, since biological activity is also abolished when the tryptophan residue of the toxin is modified (Tu *et al.*, 1971; Chang and Hayashi, 1969).

The amount of toxin bound for a given sample of receptor was constant for increasing ionic strength but was increased five- to tenfold in low salt (0.02 M phosphate), presumably due to increased nonspecific electrostatic interaction, since the toxin was also strongly bound to sodium cholate micelles in low salt. In this work the receptor was identified and considered active by its binding of labeled cobrotoxin under near physiological ionic strengths and pH.

The receptor is stable for many weeks under the proper conditions (0.1–0.2 M ionic strength, pH 6–9, 2–4°, 1.5% Triton, and 10% glycerol), retaining 50% activity even after 6 months. However, under other conditions it was easily inactivated. Both the receptor and toxin–receptor complex were rapidly and irreversibly inactivated in 1 M guanidine·HCl and pH below 4.7. The receptor was also less stable in salt above 0.5 M and in 5% Triton; a 60% loss of activity resulted after 0.5 hr in 1 M NaCl and after 4 weeks in 5% Triton. With the addition of glycerol to a concentration of 10%, the receptor was completely stable for several days, sufficient time for elution from the DEAE-cellulose column and dialysis.

The purification procedure and yields for one sample are outlined in Chart I. The receptor was purified sixfold on DEAE-cellulose, with recovery of 90–95% of the activity adsorbed on the column. A further 42-fold purification was obtained using the affinity column, with a 40–50% yield of activity, to give an overall purification of 256-fold. Although ideally use of the affinity column alone should completely purify the receptor, higher overall yields and purity resulted if it was partially purified initially on DEAE-cellulose.

The major difficulty encountered in purifying the receptor

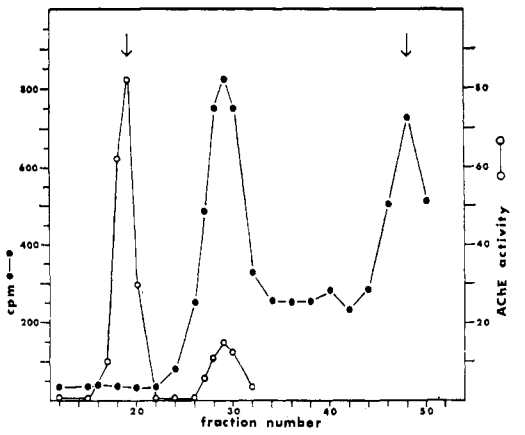


FIGURE 6: Gel filtration in Sepharose 6-B of the tritiated cobrotoxin–receptor complex. Tritiated cobrotoxin was added to a 1-ml sample of purified receptor and the solution applied to a Sepharose 6-B column (0.9 cm × 43 cm; flow rate, 4 ml/hr). The solvent was 2% Triton–0.15 M NaCl–0.01 M Tris (pH 7.2), 23°. Fractions (0.6 ml) were collected, assayed for acetylcholinesterase, and then counted in 4 ml of Bray's solution. Arrows mark the column void volume were most of the acetylcholinesterase emerges, and the back of the column where the free tritiated toxin appears.

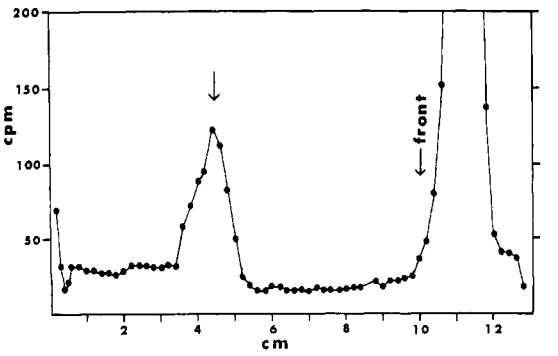


FIGURE 7: Sodium dodecyl sulfate gel electrophoresis of cross-linked toxin–receptor complex. The complex was treated with 1% glutaraldehyde and run on 3.3% × 0.12% acrylamide gels. The gels were cut into 1-mm fractions. The arrows mark the marker dye front and the tritiated toxin–receptor peak. Chymotrypsinogen, ovalbumin, and cross-linked bovine serum albumin were used as standards.

was its low stability. Purification procedures such as gradient elution of chromatography column, and isoelectric focusing resulted in a considerable (50% or greater) loss of activity. The best results were obtained using a small column, with a single step elution instead of a gradient.

The purified receptor contained only a small amount of cholinesterase activity. Calculated from the specific activity the molar ratio was 0.6% and assuming a mol wt of 260,000 (Lenzinger *et al.*, 1969), there was 0.7% by weight.

The receptor was purified 256-fold from the detergent extract of the tissue; the sample contained 2.2 ± 0.3 × 10^5 g of protein per toxin binding site. On sodium dodecyl sulfate gel electrophoresis (Figure 4) the major polypeptide has a mol wt of 44,000 ± 3000, similar in size to the receptor subunit labeled with Karlin's affinity reagent (Reiter *et al.*, 1972). There are minor bands at mol wt 50,000 and 95,000–110,000.

The purified receptor focused in a single protein band of p*I* = 4.7 (Figure 5); the toxin–receptor complex focused at p*I* = 5.2 (Raftery *et al.*, 1971).

Application of a sample of receptor–toxin complex to a Sepharose 6-B column (Figure 6) gave results consistent with previous reports (Raftery *et al.*, 1971; Meunier *et al.*, 1972). The complex moved with an apparent mol wt of 500,000, emerging behind the peak of aggregated acetylcholinesterase at the void volume. A smaller peak of acetylcholinesterase activity coincided with the receptor peak.

However, because of the presence of detergent the apparent molecular weight determined by gel filtration cannot be assumed to indicate the true size of the receptor. In order to obtain a more reliable determination of the receptor molecular weight, the toxin–receptor complex was treated with either glutaraldehyde or *p*-difluorodinitrobenzene, using a low protein concentration (0.1 mg/ml) to minimize intermolecular cross-linking. Multiples of bovine serum albumin up to tetramer were obtained by cross-linking and used for high molecular weight standards. The cross-linked complex gave a single peak at mol wt 260,000 ± 25,000 on sodium dodecyl sulfate gel electrophoresis (Figure 7). The size of the high molecular weight peak was a function of cross-linked concentration.

When the toxin alone was first treated with glutaraldehyde for 2 min, then complexed to the receptor to increase toxin–receptor cross-linking relative to intrareceptor cross-linking, an additional peak containing almost 20% of the cross-linked toxin was found at mol wt 48,000 (Figure 8). The toxin alone when treated with glutaraldehyde under the same conditions gave no high molecular weight multiples. The toxin is presumably cross-linked to the mol wt 44,000 polypeptide seen

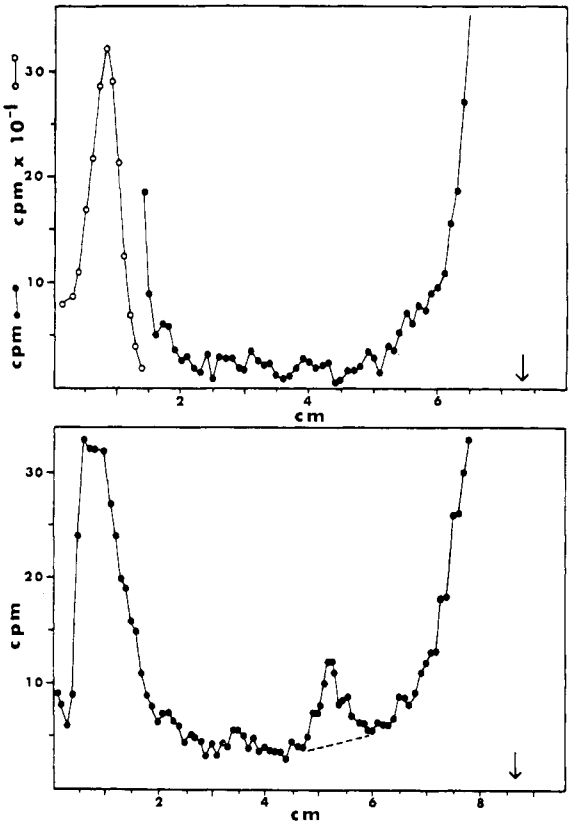


FIGURE 8: Sodium dodecyl sulfate gel electrophoresis of cross-linked toxin–receptor complex. (Top) The complex was allowed to react with 0.1% glutaraldehyde and run on a 5% × 0.17% acrylamide gel. The gel was cut into 0.5-mm fractions. (Bottom) The sample obtained when cobrotoxin was allowed to react with glutaraldehyde prior to forming a complex with receptor (Materials and Methods) was run on a 5% × 0.17% acrylamide gel. The gel was cut into 0.5-mm fractions. The arrows mark the positions of the marker dye front.

directly on the sodium dodecyl sulfate gel, since under the stringent denaturing conditions used for the cross-linking experiments no toxin–sodium dodecyl sulfate complex was ever seen. The results indicated, although do not prove, that the mol wt 44,000 polypeptide is contained in a higher molecular weight aggregate which binds the toxin.

Discussion

The cholinergic receptor was identified by the specific binding of labeled cobrotoxin, and the identification confirmed by competition experiments with native and oxidized toxin.

It must be stressed that toxin binding assays are not necessarily significant when performed in other than physiological salt and pH conditions. In low salt (*e.g.*, 0.02 M phosphate) the very basic toxin will bind to many anions, such as sodium cholate micelles, losing all specificity. The large increase in the amount of toxin bound by membrane fragments of eel electric organ and detergent extract of the organ if assayed in low salt must result from this type of nonspecific interaction and lead to erroneously high values for the receptor concentration. The toxin binding was found to be constant in salt concentrations of 0.1 M and above all assays were performed in this range.

Affinity chromatography is especially useful for purification of membrane protein because it requires only an accessible ligand binding site, and because salt concentrations and pH can be maintained at suitable levels. Receptor binding to the affinity column involved strong ligand–protein interaction, since the receptor remained on the column up to 0.35–0.4 in ionic strength, but was eluted in one column volume with 1 mM decamethonium.

The purified protein contained $2.2 \pm 0.3 \times 10^5$ g of protein per toxin binding site, compared with 5.0×10^5 per binding site reported by Olsen *et al.* (1972) for receptor for *Electrophorus electricus* purified using a different affinity column. Schmidt and Raftery (1973) have reported obtaining a receptor preparation from *Torpedo californica* which contained 1.6×10^5 g of protein per toxin binding site, although the sample gave multiple polypeptide bands in sodium dodecyl sulfate electrophoresis. One of the major polypeptides in their preparation with a mol wt of 42,000 may correspond to the major polypeptide at mol wt 44,000 reported here. There were no other similarities between the two gel patterns, which may reflect a difference in the receptor molecule isolated from the two different sources. From densitometry of the sodium dodecyl sulfate gel the mol wt 44,000 species makes up greater than 70% of the total protein; however, quantitative estimates of purity based on the gel may be unreliable since the protein does not stain well. An equivalent amount of soluble protein (*e.g.*, bovine serum albumin) gives 2–5 times greater staining density, and the amounts of protein in the different polypeptides may not be directly proportional to the amounts of dye bound.

The results presented in this paper for sodium dodecyl sulfate electrophoresis and toxin–receptor cross-linking, along with the results of affinity labeling (Reiter *et al.*, 1972) and electrophoresis of the toxin–receptor complex under nondenaturing conditions (Meunier *et al.*, 1972), strongly suggest that the receptor contains a peptide of mol wt 40,000–45,000, although the toxin binding species in solution and in the membrane is indicated to be much larger, and thus a complex of polypeptides.

The apparent molecular weight of the receptor determined by gel filtration of 500,000–600,000 (Figure 6) may be a considerable overestimate due to detergent binding, which is known to occur with membrane proteins; for example, rhodopsin appears as a complex of mol wt 300,000 in digitonin solution, although the actual mol wt is 40,000 (Hubbard, 1954). In order to determine the actual molecular weight of the protein the tritiated toxin–receptor complex was cross-linked and then run on sodium dodecyl sulfate electrophoresis. Cross-linking the complex in effect converts the toxin to a covalent affinity label.

The sodium dodecyl sulfate gel pattern of the cross-linked toxin–receptor complex shows a single 260,000 mol wt peak, which is also much larger than any of the peptides seen directly on sodium dodecyl sulfate gel electrophoresis and must be a complex of smaller subunits.

To analyze the cross-linking results it must be kept in mind that the toxin–receptor complex is a molecular aggregate composed of at least two kinds of subunits. For such proteins the reactivity between one kind of subunit can be much different than the other. For example, the β oligomers of tryptophan synthetase $\alpha_2\beta_2$ complex are cross-linked with dimethyl suberimidate, but the α protomers are not (Davies and Stark, 1970). Even if the proteins had the same intrinsic reactivity, their widely different sizes would make their probability of reaction very different. The results of cross-linking the toxin–receptor indicate that the reaction rate for cross-linking the receptor subunits is much faster than for toxin–receptor coupling, so that under conditions where the toxin is linked to the receptor, the receptor is already extensively reacted, and all that is seen on the sodium dodecyl sulfate gel is the fully cross-linked complex.

A mol wt of 260,000 would mean that the receptor species seen on gel filtration, which was run under conditions similar to the cross-linking conditions, would be associated with approximately an equivalent amount of detergent.

Further work is needed to determine whether or not the receptor is a complex of a single type of polypeptide, and also to determine the number of toxin binding sites contained in the receptor species. Such answers are necessary to decide on criteria of purity and also fundamental to the elucidation of the receptor's function *in vivo*.

That the solubilized receptor forms a specific complex in solution is a strong indication that the same complex is present *in vivo*. The receptor is large enough to span the membrane and its solubility characteristics indicate that it is deeply imbedded in the bilayer and could possibly be exposed on both sides of the membrane. Whether or not the receptor functions physiologically as an actual ion pore will require further work using systems where its function can be measured. The fact that such a protein can be isolated and purified, with its properties confirmed by several researchers, is strong evidence for the ion permeability properties of the membrane residing in a protein constituent.

Acknowledgments

The author is indebted to Drs. F. M. Richards and J. H. Wang, in whose laboratories this work was performed, for their valuable advice and encouragement and especially thanks Dr. Richard Henderson for many fruitful discussions.

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Tryptophan Quantitation by Magnetic Circular Dichroism in Native and Modified Proteins†

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ABSTRACT: Magnetic circular dichroism (MCD) has been used to determine the tryptophan content of proteins. The intense positive band near 293 nm, uniquely characteristic of tryptophan, allows the direct and quantitative measurement of this amino acid with high sensitivity and accuracy under conditions which preserve the integrity of the native protein structure and function. Tryptophan determinations of 17 proteins have been made and the results compared with those obtained by other recognized procedures. Solvent perturbation and denaturation studies indicate that the environment of the indole chromophore minimally affects the intensity of the band but significantly shifts the wavelength maximum necessitating the identification of the exact wavelength of λ_{max} in the quantitation process. The effects of tryptophan side chain and indole ring substituents have also been examined. The MCD spectra of ring-substituted indoles vary widely and can be used to identify and quantitate tryptophan derivatives and to monitor the chemical modification of this residue in proteins. The reaction of tryptophan with *N*-bromosuccinimide and of tryptophan and the tryptophyl residues in lysozyme with *o*-nitrophenylsulfenyl chloride have been examined in this regard.

Much of the remarkable progress in protein chemistry over the past years has been due to the development of accurate and convenient methods for the qualitative and quantitative analysis of amino acids. Determination of tryptophan, however, has been a persistent problem. This is particularly unfortunate since this residue contributes significantly to the optical, stereooptical, and fluorescent properties of proteins. Tryptophanyl residues have been postulated to be involved in enzyme catalysis and substrate binding (Glickson *et al.*, 1971); Robbins and Holmes, 1972), and their spectral characteristics are considered to be important gauges of protein conformation (Donovan, 1969; Herskovits and Sorenson, 1968a,b; Wetlaufer, 1962; Kauzmann, 1959).

We have previously reported (Holmquist, 1971) on the

† From the Biophysics Research Laboratory, Department of Biological Chemistry, Harvard Medical School, and the Division of Medical Biology, Peter Bent Brigham Hospital, Boston, Massachusetts. *Received June 29, 1973.* This work was supported by Grants-in-Aid GM-15003 and GM-02123 from the National Institutes of Health, of the Department of Health, Education, and Welfare.