Acetylcholine Receptor

I. IDENTIFICATION AND BIOCHEMICAL CHARACTERISTICS OF A CHOLINERGIC RECEPTOR OF GUINEA PIG CEREBRAL CORTEX *

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H. BRUCE BOSMANN‡

From the Department of Pharmacology and Toxicology, School of Medicine and Dentistry, University of Rochester, Rochester, New York 14642

SUMMARY

Purified α-bungarotoxin was prepared from the venom of Bungarus multicinctus by carboxymethyl Sephadex C-50 chromatography and Sephadex G-25 chromatography. The material was found to be a pure polypeptide of molecular weight 8000. The purified α-bungarotoxin was acetylated with [3H]acetic anhydride, and the [3H]acetilated, purified α-bungarotoxin was called [3H]acetyl-α-bungarotoxin and was utilized to characterize and isolate an acetylcholine receptor from guinea pig cerebral cortex. With an isolated rat phrenic nerve diaphragm preparation, 1 × 10^-5 g per ml of [3H]acetyl-α-bungarotoxin caused a complete block of the indirectly stimulated rat hemidiaphragm in vitro, and this action was quantitatively and qualitatively similar to that of the unacetylated α-bungarotoxin. The [3H]acetyl-α-bungarotoxin had an isoelectric point of pH 9.3. [3H]Acetyl-α-bungarotoxin prepared in this manner had a specific activity of 6.36 Ci per mmole. Binding of [3H]acetyl-α-bungarotoxin was measured in a 0.1% Triton X-100, 0.1 M Tris (pH 7.6) homogenate of guinea pig cerebral cortex with an ultrafiltration cell apparatus. Binding of [3H]acetyl-α-bungarotoxin to the homogenate was saturable, showed optimum binding at pH 7.1 to 7.7, and was not inhibited by high concentrations (10^-2 M) of deoxyribonuclease, ribonuclease, lipase, phospholipase C, and neuraminidase were without effect. A receptor-[3H]acetyl-α-bungarotoxin complex was isolated and purified by centrifugation and Sephadex G-100, G-150, and G-200 column chromatography. The purified [3H]acetyl-α-bungarotoxin-receptor complex was homogeneous by polyacrylamide gel electrophoresis, had an isoelectric point of pH 4.8, and has a molecular weight of 94,000. The purified [3H]acetyl-α-bungarotoxin-receptor complex had the properties of protein, indicating that the receptor is probably a protein. Fractionation of the cerebral cortex showed an enrichment of the receptor in the synaptosomal fraction. Subsynaptosomal fractionation showed the synaptosome plasma membrane to contain the highest concentration of receptors. The original cerebral cortex homogenate bound 87.5 ± 7 pmol of [3H]acetyl-α-bungarotoxin per mg of protein, which, assuming one molecule of [3H]acetyl-α-bungarotoxin per receptor molecule, means that a 3 g (wet weight) guinea pig cerebral cortex contains 52.5 nmoles of receptor or 140 µg of receptor or 420 pg of protein. The purified [3H]acetyl-α-bungarotoxin-receptor complex contained 10.75 pmol of [3H]acetyl-α-bungarotoxin per mg of protein or 86 ± 4 µg of [3H]acetyl-α-bungarotoxin per mg of protein.

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The cholinergic receptor has been studied indirectly and directly by a variety of methods in a widely varied number of species. Cholinergic ligand binding to various tissue fractions has been studied primarily by equilibrium dialysis techniques and subsequent purification of the acetylcholine receptor-cholinergic ligand complex. At various times the acetylcholine receptor has been identified as a protein (1, 2), an acid mucopolsaccharide (glycosaminoglycan) (3), nucleoprotein (4), phospholipoprotein (5), and a special protolipid protein fraction (6).

The acetylcholine receptor, its isolation, and its characteristics are central to pharmacology from both a conceptual and a biochemical standpoint (7). From a conceptual viewpoint, postulation of receptor molecules allows actions of various classes of drugs to be explained in terms of a single molecular mechanism,
with drugs being either agonists or antagonists of the receptor. From a biochemical point of view, isolation of the cholinergic receptor allows the nature of the macromolecule responsible for mediating nerve conduction to be elucidated.

Recently, reports (2, 8) have appeared utilizing α-bungarotoxin, a polypeptide from the venom of the elapid snake Bungarus multicinctus (9-11) as an irreversible binder of the cholinergic receptor. Use of the polypeptide α-bungarotoxin to determine the cholinergic receptor is advantageous firstly because it binds tightly to the cholinergic receptor and secondly because it cannot be hydrolyzed by the enzyme cholinesterase (EC 3.1.1.7) which is ubiquitous in the various tissues in which the receptor would be expected to be found. The present report gives the isolation and characterization of a cholinergic receptor from guinea pig cerebral cortex utilizing tritiated α-bungarotoxin to identify the receptor. The cholinergic receptor of guinea pig cerebral cortex has properties unlike those of cholinergic receptors described in other tissues (12).

Materials and Methods

Materials—Lyophilized venom of B. multicinctus was purchased from the Miami Serpentarium, Miami, Florida. [3H]-Acetic anhydride (specific activity 400 Ci per mole) was purchased from New England Nuclear. Protease (EC 3.4.4.4.), trypsin (EC 3.4.21.18), lipase (hog pancreas) (EC 3.1.1.5), phospholipase C (EC 3.1.4.3), DNase (EC 3.1.4.5), and RNase (EC 3.1.4.5.16) were purchased from Worthington Biochemicals. All other biochemicals were purchased from Sigma Chemical Company. Drugs (crystalline) utilized for competition studies were the kind gift of Dr. Brian A. Hemsworth of this department.

Isolation of α-Bungarotoxin from Venom of B. multicinctus—The dry venom (812 mg) was dissolved in 20 ml of 0.005 M ammonium acetate buffer, pH 5.0, and placed on a column (1.8 x 80 cm) at 4°C of CM-Sephadex C-50 equilibrated with 0.005 M ammonium acetate buffer. A gradient was established by adding 0.9 M ammonium acetate buffer, pH 7.0, into a mixing flask containing 450 ml of 0.005 M ammonium acetate buffer, pH 5.0. The flow rate was 12 ml per hour. Samples of 4.5 ml were collected. After the fractionation pattern was determined, tubes 60 to 80 (90 ml) were lyophilized to 15 ml and applied to an exactly identical column and the procedure was repeated. Fractions 23 to 26 of this fractionation were placed on an exactly identical column and the procedure was repeated. Fractions 21 to 25 of this fractionation were considered to be the purified, tritiated acetyl-α-bungarotoxin and were lyophilized to 10 ml. This material, which is [3H]acetylated purified α-bungarotoxin, is referred to herein as [3H]acetyl-α-bungarotoxin. Utilizing a molecular weight of this material of 8000 (see below), 1 pmole of [3H]acetyl-α-bungarotoxin = 1.4 x 10^6 dpm. All measurements of radioactivity were made in Bray's solution (13) by adding 5% (v/v) of aqueous material to Bray's solution and counting in a Nuclear-Chicago liquid scintillation counter. Corrections were made for quenching, and disintegrations per min were converted to picomoles of [3H]acetyl-α-bungarotoxin.

Isoelectric Focusing—Isoelectric focusing was carried out utilizing the LKB 110-ml electrophoresis apparatus. Amphoteries were purchased from LKB. Runs were performed without added sample to determine amphotelyte contribution to the optical density at 580 nm; this contribution was subtracted from data presented herein. Runs were carried out for 24 or 48 hours; in typical runs starting parameters would be 300 volts, 4.5 ma or 335 volts, 8 ma and finishing parameters would be 370 volts, 1.3 ma or 365 volts, 1.2 ma. All runs were performed at 2°C. Runs containing radioactively labeled material were also fractionated and fractions were counted in Bray's solution (13).

Polyacrylamide Gel Electrophoresis—Polyacrylamide gel electrophoresis was carried out by the mini-protein method of Laemmi et al. (14) as described previously (15), omitting the dialysis step to prevent loss of polypeptides. Suitable standards were always run simultaneously. Fifty μg (as protein) of each fraction were extracted at pH 7.1 in 0.1% sodium dodecyl sulfate and 0.1% 2-mercaptoethanol for 3 hours at 37°C. The samples were subjected to electrophoresis at 8 volts per cm for 2 hours in 125-mm 5% polyacrylamide gels which were 0.1 M phosphate and 0.1% sodium dodecyl sulfate. The electrophoresis buffer was 0.1 M phosphate buffer, pH 7.1, which is 1% sodium dodecyl sulfate. All gels were run toward the anode. Following electrophoresis the gels were stained for either protein or glycoprotein. For protein, the gels were fixed in 20% sulfosalicylic acid for 16 hours, stained with 0.2% Coomassie blue for 3 hours, and de-stained with several washes of 7% acetic acid. For glycoprotein, the gels were stained with a modified periodic acid Schiff technique exactly as described by Zacharias et al. (16). Gels containing radioactively labeled material were fractionated with a Savant (Savant Instruments Inc., Hicksville, N.Y.) autogel divider and fractions were counted in Bray's solution (13).

Binding Measurements—Binding measurements were made by a variety of methods including equilibrium dialysis, gel filtration, ultrafiltration, and sucrose gradient analysis. The method reported herein, which proved most reproducible and efficient was that utilizing the ultrafiltration cell method of Paulus (17). The method involved filtering of the solution of guinea pig cortex homogenate and [3H]acetyl-α-bungarotoxin through a Diaflo membrane (Amicon Corporation, Cambridge, Massachusetts) in a specially constructed ultrafiltration cell (Metaloglass Corporation, Boston, Massachusetts). The membrane on which the receptor-[3H]acetyl-α-bungarotoxin complex is deposited was then counted in Bray's solution (13) in the liquid scintillation spectrometer. The method is now described in detail. One guinea pig (300 g) was starved for 16 hours with water ad libitum and then killed by decapitation. The cerebral cortex was removed and homogenized immediately at 0°C in 5 volumes of 0.1% Triton X-100, 0.1 M Tris, pH 7.6; yield was about 600 mg (as
protein) per brain. A protein analysis (see below) was then performed on the sample, and the concentration was adjusted with 0.1% Triton X-100, 0.1 m Tris, pH 7.6, so that the 200 μl in the ultrafiltration cell would contain the desired amount of cortex protein (usually 200 μg). [H]Acetyl-α-bungarotoxin, usually 10 μl, 175 pmole (675 nM final concentration) was added and any competitive drugs, etc., were also added in a test tube. This solution (200 μl always) was then pipetted into the eight channels of the upper block of the ultrafiltration cell, the sample ports were sealed with screw plugs, and 40 p.s.i. of N₂ was applied to force the mixture through 7-mm UM-10 Diaflo membrane discs (also supplied from Metalloglass Corporation, Boston, Massachusetts). Filtration was continued until complete in all channels (usually 2 hours) and while the apparatus was still under pressure the channels were washed as described by Paulus (17). The filter discs were removed and placed in Bray's solution (13) for counting. For each run duplicate samples without protein were run to correct for a small amount of solution (3 μl) which is retained by the Diaflo membrane and for the nonspecific binding of the [H]acetyl-α-bungarotoxin (less than 0.2% added cpm) to the Diaflo membranes. All data reported herein have been corrected for this nonspecific activity.

Isolation of Receptor [H]Acetyl-α-bungarotoxin Complex—All procedures were carried out at 4°C. For these experiments, three cerebral cortices were extracted exactly as given above in 0.1% Triton X-100, 0.1 m Tris, pH 7.6, and 1 ml, 17.5 nmole, of [H]acetyl-α-bungarotoxin was added with stirring at 4°C. The material after 4 hours was centrifuged at 20,000 × g for 1 hour. The supernatant fluid (10 ml) was placed on a Sephadex G-100 column (2.5 × 90 cm), equilibrated, and eluted with 0.1% Triton X-100, 0.1 m Tris, pH 7.6. A flow rate of 80 ml per hour was maintained, and samples of 5 ml were collected. Radioactivity was determined in Bray’s solution, and optical density at 280 nm was determined against a 0.1% Triton X-100 blank. Fractions 15 to 23 of this fractionation were dialyzed, lyophilized to 10 ml, and placed on a Sephadex G-150 column prepared and eluted exactly as above. A flow rate of 00 ml per hour was maintained and samples of 8 ml were collected. Fractions 14 to 21 of this fractionation were dialyzed, lyophilized, and placed on a Sephadex G-200 column prepared and eluted exactly as above. A flow rate of 30 ml per hour was maintained and fractions of 9 ml were collected. Fractions 29 to 33 were considered to be the purified receptor-[H]acetyl-α-bungarotoxin complex. In each instance fractions were chosen to maximize quantity of material as well as specific activity.

Synaptosoma and Subsynaptosomal Fraction Isolation—Synaptosomes (nerve ending particles, Fraction P₂) and subsynaptosomal fractions were isolated exactly by the methods of Whitaker et al. (18-21) as described (22) at 0-4°C from guinea pig cerebral cortex (brain stem transected between superior and inferior colliculi). Male guinea pigs, 300 g, fasted for 16 hours before death were the source of cerebral cortex. Twelve guinea pigs were used for each experiment, yielding a wet weight of cortex of approximately 30 g. The procedures used in these experiments are outlined in schematic form in Fig. 1. In each instance of fractionation, if the fraction was present as a pellet it was suspended in 0.1% Triton X-100, 0.1 m Tris buffer, pH 7.6. If the fraction was in sucrose it was diluted 1:3 with 0.1 m Tris buffer, pH 7.6, and centrifuged out of solution at 40,000 × g for 10 min before resuspension in 0.1% Triton X-100, 0.1 m Tris buffer, pH 7.6.

Phosphorus—Phosphorus was determined by the method of Chen, Toribara, and Warner (23).

Protein—Protein in the various samples was determined by the method of Lowry et al. (24). Samples were prepared by precipitation with 1% phosphotungstic acid in 0.5 N HCl, washing three times with 5% trichloroacetic acid and washing once with diethyl ether-ethanol (1:2, v/v). The resulting residue was dissolved in 1 ml of 0.2 N NaOH and analyzed. Crystalline bovine serum albumin was used as standard.

Monoamine Oxidase (EC 1.4.5.2)—Monoamine oxidase activity was measured with benzylamine as a substrate by the method of Tabor, Tabor, and Rosenthal (25).

Acid Phosphatase (EC 3.1.3.2)—Acid phosphatase activity at pH 4.3 was measured with p-nitrophenyl phosphate as substrate by the method of De Duve (26).

Esterase (EC 3.1.1.1)—This enzyme was determined with p-nitrophenyl acetate as substrate by the method of Bier (27).

5’-Nucleotidase (EC 3.1.3.5)—The 5’-nucleotidase activity was assayed with 5’-AMP as substrate by the method of Bosmann and Pike (28).

Succinic Dehydrogenase (EC 1.3.99.1)—This enzyme was determined by the method previously described (29).
Uridine Diphosphatase (EC 3.6.1.6)—The uridine diphosphatase activity was determined by the method of Bosmann (30).

Acetylcholinesterase (EC 3.1.1.7) Cholinesterase activity was estimated by the method of Hemsworth (31) by a null point titration method using the Radiometer (Copenhagen) pH-stat unit (burette units ABU12; titration assembly TTA31; titrator TTT11; pH meter PHM28; and recorder SBR2) with a 5-ml water-jacketed reaction chamber for automatic titration of 0.01 N NaOH from the 0.25-ml burette. The temperature was maintained throughout at 37°C by a thermostated circulating water bath (Heto T6TB water bath).

pH Measurements—pH measurements were performed with a Beckman research model pH meter. Corrections for solution temperature were made.

Rat Phrenic Nerve-Diaphragm Preparation—In order to determine activity of the various preparations at the neuromuscular junction, isolated phrenic nerve-diaphragm preparations of the rat (32) were utilized. Both hemidiaphragms were mounted in McEwen’s (33) solution at 37°C by the method of Hemsworth and Foddes (34). The muscles were excited by supramaximal rectangular pulses of 0.1 msec duration applied to the phrenic nerve. Nerve stimulation was carried out at one stimulation per sec. The contractions were recorded by means of a Grass force displacement transducer on a Beckman dynograph.

Reversibility of Binding—Reversibility of binding was measured in the following manner. Samples containing 200 μg (as protein) of the 0.1% Triton X-100, 0.1 M Tris, pH 7.6, extract of guinea pig cerebral cortex, and 175 pmole of [3H]acetylα-bungarotoxin were prepared and ultrafiltered as described above. After the ultrafiltration the material on the membrane was subjected to dialysis against 0.1% Triton X-100, 0.1 M Tris, pH 7.6, at 4°C for varying periods. After adjustment of the volume by lyophilization the amount of bound [3H]acetylα-bungarotoxin was then determined on the material in the dialysis sac, as described above. Also at 90 hours dialysis the material in the dialysis sac was exposed to a further 175 pmole of [3H]acetyl-α-bungarotoxin determined. Similarly the receptor-[3H]acetylα-bungarotoxin complex (1 μg in 200 μl) was extracted with 200 μl of HCCl₃-CH₃OH (2:1, v/v) and the lipid solvent removed. The 200-μl aqueous layer remaining was then ultrafiltered with the ultrafiltration cell to determine the amount of bound [3H]acetylα-bungarotoxin.

Treatment of Purified Receptor-[3H]Acetylα-bungarotoxin Complex with Various Detergents and Solvents—The isolated receptor-[3H]acetylα-bungarotoxin complex (1 μg as protein) was treated with a variety of solvents and detergents in an attempt to dissociate the receptor-[3H]acetylα-bungarotoxin complex. The receptor-[3H]acetylα-bungarotoxin was placed in the appropriate concentration of salt or detergent for 14 hours at 25°C, and the resulting solution was ultrafiltered in the ultrafiltration cell as described above to determine the amount of bound [3H]acetylα-bungarotoxin.

**RESULTS**

Purification of α-Bungarotoxin from *B. multicinctus* Venom—The lyophilized venom of *B. multicinctus* was subjected to gel chromatography on CM-Sephadex C-50. The data in Fig. 2 indicate that elution of the venom with increasing concentrations of ammonium acetate with a pH gradient resulted in nine distinct bands of material, labeled with Roman numerals in Fig. 2. Fraction II, which contained the polypeptide of interest, α-bungarotoxin (9-11), was eluted in tubes 60 to 80 of this fractionation. Fraction II was selected because it was most active and present in fairly large quantity. Pooling of these fractions and rerunning of the CM-Sephadex C 50 column gave the data presented in Fig. 3. It is evident from the data in Fig. 3 that the Fraction II, α-bungarotoxin, is a homogeneous fraction on CM-Sephadex C-50. This material (Fractions 63 to 75) was then applied to a column of Sephadex G-25, as shown in Fig. 4. The polypeptide eluted in the void volume of the Sephadex G-25 and in one clean peak, as shown in Fig. 4. This material, purified α-bungarotoxin, was utilized in these studies.

[3H]Acetylation of Purified α-Bungarotoxin to Yield [3H]Acetylα-bungarotoxin—The purified α-bungarotoxin was reacted with [3H]acetic anhydride as described under “Materials and Methods.” The purification system utilized to remove unreacted [3H]acetic anhydride was repeated Sephadex G-25 chromatography, and the results are given in Fig. 5. Successive passages through Sephadex G-25 caused the [3H]acetylated polypeptide [3H]acetylα-bungarotoxin to be freed from unreacted [3H]acetic anhydride. The material in Panel D of Fig. 5 was considered to be [3H]acetylα-bungarotoxin. The material was [3H]acetylated α-bungarotoxin and is referred to as [3H]acetylα-bungarotoxin. It had an activity of 1.4 × 10⁶ dpm per pmole of [3H]acetylα-bungarotoxin (assuming a molecular weight of 8000, see below).

Isoelectric Focusing—Isoelectric focusing of the venom of *B. multicinctus* yielded nine distinct bands with absorbance at 280
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Fig. 2. Gel chromatography of the venom of Bungarus multicinctus on CM-Sephadex C-50. Venom, 812 mg, was dissolved in 20 ml of 0.005 M ammonium acetate buffer, pH 5.0, placed on a column of CM-Sephadex C-50, and eluted with a pH, ammonium acetate gradient, as described under "Materials and Methods." The flow rate was 12 ml per hour, and samples of 4.5 ml were collected.

nm. These nine bands are not unlike the nine fractions found with fractionation on CM-Sephadex C-50 (Fig. 2). The nine bands had the following isoelectric points: 2.1, 3.0, 3.7, 4.2, 4.5, 5.3, 5.8, 6.3, 8.0, and 9.3, as shown in the bottom panel of Fig. 6. The middle panel of Fig. 6 indicates that the α-bungarotoxin had an isoelectric point of 9.3, and thus it is the venom constituent with the highest isoelectric point. The top panel indicates that the [3H]acetyl-α-bungarotoxin had an isoelectric point of 9.3. Thus acetylation did not alter the isoelectric point of the α-bungarotoxin. Furthermore, the top panel indicates that all the [3H]radioactivity was associated with the [3H]acetyl-α-bungarotoxin; there was no free [3H]acetic anhydride present in the preparation. That the [3H]acetyl-α-bungarotoxin has an isoelectric point at pH 9.3 means that at neutral pH (see below and Fig. 16) the molecule will carry a positive charge. This may be one basis for the binding of the α-bungarotoxin to the acetylcholine receptor since many cholinergic ligands (including acetylcholine) contain quaternary nitrogens which carry a positive charge throughout a wide pH range.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis—The photograph of the sodium dodecyl sulfate polyacrylamide gel electrophoresis pattern of the B. multicinctus venom is given in Fig. 7A. The gel contains four distinct bands and one very broad dark band, probably containing the five other proteins found on CM-Sephadex C-50 chromatography and electrofocusing. The gel stains heavily for protein with Coomassie brilliant blue but did not stain for glycoprotein with the periodic acid-Schiff technique. Gel B in Fig. 7 is of Fraction II of the CM-Sephadex C-50 chromatography. It contains one distinct band and one very broad dark band. Gel C in Fig. 7 is of the [3H]acetyl α-bungarotoxin. It contains one distinct band staining with Coomassie brilliant blue.

The data presented in Fig. 8 indicate that upon fractionation of a gel similar to that of Fig. 7C with a Savant autogel divider, all of the radioactivity is associated with the [3H]acetyl-α-bungarotoxin protein band.

The exponential relationship between molecular weight and migration of proteins in sodium dodecyl sulfate polyacrylamide gel electrophoresis (35, 36) provides a simple and accurate means of estimating molecular weights of unknown proteins. The basis of this separation according to molecular weight is still unknown but may be due to conformational changes of the proteins and masking of the protein's original charge by the sodium dodecyl sulfate, leaving only size or molecular weight as a parameter that is acted upon by the exponential sieving effect of the polyacrylamide gel. This principle was applied in the present system and utilizing known molecular weight standards run simultaneously, the curve in Fig. 9 was prepared. It is apparent that from these data the [3H]acetyl-α-bungarotoxin has an approximate molecular weight of 8000. This value is in agreement with that found for α-bungarotoxin by Changeux, Kasai, and Lee (8).

Activity of [3H]Acetyl-α-bungarotoxin at Neuromuscular Junc-
Fig. 3. Gel chromatography of α-bungarotoxin on CM-Sephadex C-50. Fraction II from the gel chromatography shown in Fig. 2 (tubes 60 to 80) was lyophilized to 5 ml and applied to an identical column. Elution was accomplished as described under "Materials and Methods" and the legend to Fig. 2. The flow rate was 12 ml per hour and samples of 4.5 ml were collected.

Fig. 4. Gel chromatography of α-bungarotoxin on Sephadex G-25. Fractions 63 to 75 of the CM-Sephadex C-50 chromatography (Fig. 3) were pooled, lyophilized to 15 ml, placed on a Sephadex G-25 column (2.5 × 90 cm), equilibrated, and eluted with glass-distilled water. Fractions of 5 ml were collected with a flow rate of 60 ml per hour.
Reversibility of Binding of [3H]Acetyl-α-bungarotoxin to 0.1% Triton X-100, 0.1 M Tris, pH 7.6, Extracts of Guinea Pig Cerebral Cortex—The data in Fig. 13 demonstrate that with long periods of dialysis it was possible to dissociate the receptor and the [3H]acetyl-α-bungarotoxin. At 90 hours dialysis 5.0 pmoles of [3H]acetyl-α-bungarotoxin were bound compared to 17.5 pmoles bound at 0 hours; that this decrease was not due to proteolytic or other degradation of the binding macromolecule is demonstrated by the ability of this 90-hour dialyzed material to bind its full complement of [3H]acetyl-α-bungarotoxin (Add in Fig. 13). This reversibility is at first appearance in disagreement with the report of irreversible binding by Chang and Lee (9). However, three points may explain these reports: (a) irreversibility was demonstrated (9) with a neuromuscular junction tissue preparation which adds a diffusion constant to the dissociation constant; (b) the large number of acetyl groups of the [3H]acetyl-α-bungarotoxin may render it a less effective binder; or (c) to be totally irreversibly bound a covalent bond must be formed, and it is conceivable that in tissue studies such bonding might occur enzymatically, which would not occur in the nonionic detergent extract studied herein. In any instance the data in Fig. 13 demonstrate that the [3H]acetyl-α-bungarotoxin binding to the 0.1% Triton X-100, 0.1 M Tris, pH 7.6, extract was reversible.

Binding Specificity—The specificity of binding of the guinea pig cerebral cortex, 0.1% Triton X-100, 0.1 M Tris, pH 7.6, homogenates was determined by comparing the inhibition of receptor-[3H] acetyl-α-bungarotoxin formation in the presence of...
FIG. 7. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of various fractions of Bungarus multicinctus venom. Polyacrylamide gel electrophoresis was carried out as described under "Materials and Methods." All gels were stained with Coomassie brilliant blue. There was no staining with the periodic acid-Schiff technique (see "Materials and Methods"). The gels were 5% polyacrylamide; 0.1% sodium dodecyl sulfate, pH 7.1, in samples, gels, and buffers. Samples were loaded on the top of the gel and migrated toward the bottom, which is the anode (+).

A, B. multicinctus venom. B, Fraction II of the CM-Sephadex C-50 column (Figs. 2 and 3). C, [3H]acetyl-α-bungarotoxin (Fractions 21 to 25, Panel D, Fig. 6).

FIG. 8. Polyacrylamide gel electrophoresis of [3H]acetyl-α-bungarotoxin. Electrophoresis was performed as described under “Materials and Methods” and the legend to Fig. 7. The top portion shows a schematic representation of a gel stained with Coomasie brilliant blue of [3H]acetyl-α-bungarotoxin. The bottom portion indicates the radioactivity present as determined by gel slicing with a Savant autogel divider.

cholinergic and noncholinergic ligands (Table II). The data indicate that d-tubocurarine was an effective inhibitor of the formation of the receptor-[3H]acetyl-α-bungarotoxin complex and can be considered to compete for the binding site(s) (see below). Even at 1 x 10^{-4} M, d-tubocurarine caused a 52% inhibition of [3H]acetyl-α-bungarotoxin binding; at 10^{-4} M a 99.8% inhibition of [3H]acetyl-α-bungarotoxin binding occurred (Table II).

FIG. 9. Electrophoretic migration as a function of molecular weight. Gel electrophoresis with authentic standards was performed as described under “Materials and Methods.” a, insulin, approximate molecular weight 6,000; b, [3H]acetyl-α-bungarotoxin, approximate molecular weight 8,000 (see Fig. 8); c, hemoglobin monomer, approximate molecular weight 16,000; d, hemoglobin dimer, approximate molecular weight 32,000; e, hemoglobin trimer, approximate molecular weight 48,000; f, hemoglobin tetramer, approximate molecular weight 64,000; and g, purified receptor-[3H]acetyl-α-bungarotoxin complex, approximate molecular weight 94,000 (see Fig. 20).

FIG. 10. Maximal twitches of isolated rat hemidiaphragm elicited indirectly by stimulation of the phrenic nerve at frequencies of 1 per 5 sec. At the arrow 10^{-4} g of [3H]acetyl-α-bungarotoxin per ml was added to the bath. Similar patterns were obtained with α-bungarotoxin (see Fig. 6). Gallamine, which has a similar electropharmacologic activity as d-tubocurarine also caused a dramatic inhibition of [3H]acetyl-α-bungarotoxin binding. Decamethonium and carbachol, two other drugs thought to be antagonists of acetylcholine binding to the cholinergic receptor also drastically inhibited [3H]acetyl-α-bungarotoxin binding. Acetylcholine itself also competed with the [3H]acetyl-α-bungarotoxin for binding sites; however, removal of the acetyl group to give choline reduced the competition greatly; at 10^{-4} M choline little competit-
Fig. 11. Relationship between protein concentration of 0.1% Triton X-100, 0.1 M Tris, pH 7.6, extract of guinea pig cerebral cortex and the amount of [3H]acetyl-α-bungarotoxin bound. Each value is the mean ± 1 S.D. from more than seven duplicate estimations on separate guinea pig cerebral cortices. The 0.2 ml of 0.1% Triton X-100, 0.1 M Tris, pH 7.6, contained in every instance 175 pmoles of [3H]acetyl-α-bungarotoxin. Experiments were performed as described under “Materials and Methods.”

Table I

Binding of [3H]acetyl-α-bungarotoxin to proteins and tissue homogenates

In all instances the reaction mixture contained 200 μg as protein of the designated homogenate or protein, 175 pmoles of [3H]acetyl-α-bungarotoxin, and 0.1% Triton X-100, 0.1 M Tris, pH 7.6. Experiments were performed as described under “Materials and Methods.” Means ± 1 S.D.

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<tr>
<th>Sample</th>
<th>pmoles of [3H]acetyl-α-bungarotoxin bound</th>
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<tbody>
<tr>
<td>Guinea pig cerebral cortex</td>
<td>17.8 ± 0.4</td>
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<tr>
<td>Guinea pig liver</td>
<td>0.2 ± 0.01</td>
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<tr>
<td>Guinea pig intestine</td>
<td>0.1 ± 0.01</td>
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<tr>
<td>Bovine serum albumin</td>
<td>0</td>
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<td>Fatnin</td>
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</table>

Fig. 12. Amount of [3H]acetyl-α-bungarotoxin bound in the presence of excess 0.1% Triton X-100, 0.1 M Tris, pH 7.6, extract of guinea pig cerebral cortex. The 0.2 ml of 0.1% Triton X-100, 0.1 M Tris, pH 7.6, contained in every instance 10 pmoles of [3H]acetyl-α-bungarotoxin. Experiments were performed as described under “Materials and Methods.”

Fig. 13 (left). Reversibility of binding of [3H]acetyl-α-bungarotoxin to 0.1% Triton X-100, 0.1 M Tris, pH 7.6, extract of guinea pig cerebral cortex. Samples were assayed as described under “Materials and Methods” and in each instance the 0.2 ml of 0.1% Triton X-100, 0.1 M Tris, pH 7.6, extract contained 200 μg (as protein) of guinea pig cerebral cortex and 175 pmoles of [3H]acetyl-α-bungarotoxin. After ultrafiltration the material on the filter was subjected to dialysis against 0.1% Triton X-100, 0.1 M Tris, pH 7.6, for the times indicated. At each time period, the amount of bound [3H]acetyl-α-bungarotoxin was determined on 206 μl adjusted by lyophilization. The (+) denoted by Add indicates a sample dialyzed for 90 hours to which 175 pmoles of [3H]acetyl-α-bungarotoxin were readded. It is obvious that the macromolecule retained its ability to bind [3H]acetyl-α-bungarotoxin after 90 hours of dialysis.

Fig. 14 (right). Binding of [3H]acetyl-α-bungarotoxin to 0.1% Triton X-100, 0.1 M Tris, pH 7.6, extracts of guinea pig cerebral cortex at various concentrations of [3H]acetyl-α-bungarotoxin. Each value is the mean of seven or more duplicate estimations on separate guinea pig cerebral cortices. The 0.2 ml of 0.1% Triton X-100, 0.1 M Tris, pH 7.6, extract contained in every instance 200 μg (as protein) of guinea pig cerebral cortex. Experiments were performed as described under “Materials and Methods.” Boil refers to extracts boiled for 10 min before addition of [3H]acetyl-α-bungarotoxin; –protein refers to assays performed minus the cerebral cortex protein.
TABLE II

<table>
<thead>
<tr>
<th>System</th>
<th>Percentage of control</th>
<th>System</th>
<th>Percentage of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (8.75 x 10^-6 M [H]acetyl-α-bungarotoxin)</td>
<td>100 ± 4⁵</td>
<td>+ 10^-6 M Gallamine triethiodide</td>
<td>67 ± 4</td>
</tr>
<tr>
<td>+ 10^-6 M d-Tubocurarine</td>
<td>48 ± 3</td>
<td>+ 10^-6 M Gallamine triethiodide</td>
<td>37 ± 2</td>
</tr>
<tr>
<td>+ 10^-5 M d-Tubocurarine</td>
<td>28 ± 2</td>
<td>+ 10^-5 M Gallamine triethiodide</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>+ 10^-4 M d-Tubocurarine</td>
<td>0.4 ± 0.02</td>
<td>+ 10^-4 M Dexamethonium bromide</td>
<td>58 ± 4</td>
</tr>
<tr>
<td>+ 10^-4 M Acetylcholine chloride</td>
<td>70 ± 4</td>
<td>+ 10^-4 M Dexamethonium bromide</td>
<td>48 ± 4</td>
</tr>
<tr>
<td>+ 10^-3 M Acetylcholine chloride</td>
<td>37 ± 3</td>
<td>+ 10^-4 M Dexamethonium bromide</td>
<td>1 ± 0.08</td>
</tr>
<tr>
<td>+ 10^-3 M Acetylcholine chloride</td>
<td>21 ± 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ 10^-4 M Choline chloride</td>
<td>100 ± 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ 10^-5 M Choline chloride</td>
<td>88 ± 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ 10^-4 M Choline chloride</td>
<td>76 ± 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ 10^-4 M Carbachol chloride</td>
<td>62 ± 2</td>
<td>+ 10^-4 M Propanpheline bromide</td>
<td>100 ± 2</td>
</tr>
<tr>
<td>+ 10^-3 M Carbachol chloride</td>
<td>36 ± 4</td>
<td>+ 10^-4 M Propanpheline bromide</td>
<td>90 ± 3</td>
</tr>
<tr>
<td>+ 10^-4 M Carbachol chloride</td>
<td>11 ± 1</td>
<td>+ 10^-4 M Propanpheline bromide</td>
<td>71 ± 4</td>
</tr>
<tr>
<td>+ 10^-4 M Atropine sulfate</td>
<td>100 ± 4</td>
<td>+ 10^-4 M Serotonin</td>
<td>102 ± 1</td>
</tr>
<tr>
<td>+ 10^-4 M Atropine sulfate</td>
<td>82 ± 3</td>
<td>+ 10^-4 M Serotonin</td>
<td>101 ± 3</td>
</tr>
<tr>
<td>+ 10^-4 M Atropine sulfate</td>
<td>64 ± 5</td>
<td>+ 10^-4 M Serotonin</td>
<td>116 ± 5</td>
</tr>
</tbody>
</table>

⁵ The control reaction mixture contained in 200 μl, 175 pmoles of [H]acetyl-α-bungarotoxin and 200 μg (as protein) of guinea pig cerebral cortex extract. The mixture was 0.1% Triton X-100 and 0.1 M Tris, pH 7.6. The control activity was 17.5 ± 1.6 pmoles bound. Experiments were performed as described under "Materials and Methods."

at 10^-4 M serotonin slight enhancement of binding of [H]acetyl-α-bungarotoxin occurred. The data of Table II indicate that the binding of [H]acetyl-α-bungarotoxin is indeed quite specific and that the receptor being studied in this system is more nicotinic than muscarinic because of the high inhibition with d-tubocurarine and like compounds and low inhibition by atropine and like compounds. The fact that atropine did not inhibit the binding more substantially is surprising.

Binding of [H]Acetyl-α-bungarotoxin in Presence of Physostigmine—Physostigmine is known to bind acetylcholinesterase catalytic and allosteric sites rather tightly so that inhibition of [H]acetyl-α-bungarotoxin binding in the presence of physostigmine might indicate binding to acetylcholinesterase as opposed to a distinct receptor. That this was not the case is shown by the data in Table III. Only at 10^-3 M physostigmine did any inhibition of binding of [H]acetyl-α-bungarotoxin occur; at lower concentrations the binding was not affected.

Saturation of Binding of [H]Acetyl-α-bungarotoxin to Cerebral Cortex Homogenate—The saturation of the binding of [H]acetyl-α-bungarotoxin to the 0.1% Triton X-100, 0.1 M Tris, pH 7.6, homogenates of guinea pig cerebral cortex are given in Fig. 14. Saturation occurred above about 150 pmoles of [H]acetyl-α-bungarotoxin; at this level about 17 pmoles of [H]acetyl-α-bungarotoxin were bound to the 200 μg of protein in the extract. Boiling of the extract diminished binding of the [H]acetyl-α-bungarotoxin, and this binding by the boiled extracts did not illustrate saturation (Fig. 14). These data may mean that the binding of the [H]acetyl-α-bungarotoxin to the binding macromolecule may be more dependent on the amino acid sequence (or other primary structure) or charge on the macromolecule as opposed to tertiary structure. Assays without extract protein showed slight, linear, nonspecific binding of the [H]acetyl-α-bungarotoxin to the Diablo filters. Repploting of the data of Fig. 14 according to the method of Scatchard (37), as shown in Fig. 15, indicates that two binding sites exist with dissociation constants of 6.01 x 10^-9 M and 2.41 x 10^-7 M. Calculation of the binding site concentrations gives for the high affinity site 60 picomoles per mg of protein or 12 nmoles per g of cerebral cortex wet weight and for the low affinity site 130 pmoles per mg of protein or 26 nmoles per g of cerebral cortex wet weight.

Binding of [H]Acetyl-α-bungarotoxin to Enzyme-treated Guinea Pig Cerebral Cortex Homogenate—The data in Table IV indicate
Fig. 15. Scatchard plot of the data in Fig. 14. The data give a dissociation constant $K_d$ of $6.61 \times 10^{-7}$ M and a dissociation constant $K_2$ of $2.41 \times 10^{-7}$ M.

**Table IV**

**Binding of $[^{3}H]$acetyl-$\alpha$-bungarotoxin to enzyme-treated guinea pig cerebral cortex extracts**

Each 200 µg of 0.1% Triton X-100, 0.1 M Tris, pH 7.6 extract of guinea pig cerebral cortex was incubated with 50 µg of the indicated enzyme at 37° for 1 hour. Binding of $[^{3}H]$acetyl-$\alpha$-bungarotoxin was then determined as described under “Materials and Methods.” Means ± S.D.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Picosoles of $[^{3}H]$acetyl-$\alpha$-bungarotoxin bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pronase</td>
<td>3.1 ± 0.3</td>
</tr>
<tr>
<td>Trypsin</td>
<td>2.7 ± 0.4</td>
</tr>
<tr>
<td>Trypsin then soybean trypsin inhibitor</td>
<td>2.6 ± 0.3</td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>17.9 ± 0.6</td>
</tr>
<tr>
<td>Lipase</td>
<td>18.2 ± 0.4</td>
</tr>
<tr>
<td>Phospholipase C</td>
<td>17.7 ± 0.3</td>
</tr>
<tr>
<td>DNase</td>
<td>18.3 ± 0.1</td>
</tr>
<tr>
<td>RNase</td>
<td>17.9 ± 0.5</td>
</tr>
<tr>
<td>No addition (control)</td>
<td>18.0 ± 0.2</td>
</tr>
</tbody>
</table>

that only the proteolytic enzymes were able to diminish the binding capacity of the guinea pig cerebral cortex homogenates; neuraminidase, lipase, phospholipase C, DNase, and RNase were ineffective. The data with the proteases suffer, obviously, since the action of the protease could be directed against the $[^{3}H]$acetyl-$\alpha$-bungarotoxin instead of the receptor. To partially alleviate this problem the homogenate was incubated with trypsin for 1 hour at 37° and then a 10 fold excess of soybean trypsin inhibitor was added to inhibit the trypsin activity; as shown in Table IV the binding of $[^{3}H]$acetyl-$\alpha$-bungarotoxin was still greatly decreased after this treatment. In any event, phospholipase C and lipase caused no decrease in binding, indicating that lipid may not be essential for binding; DNase and RNase caused no decrease in binding, indicating that nucleic acid may not be essential for binding, and neuraminidase caused no decrease in binding, indicating that sialic acid may not be essential for binding.

**Binding of $[^{3}H]$Acetyl-$\alpha$-bungarotoxin to 0.1% Triton X-100, 0.1 M Tris, pH 7.6 Homogenate of Guinea Pig Cerebral Cortex as Function of pH**—The binding of $[^{3}H]$acetyl-$\alpha$-bungarotoxin had an optimum from pH 7.1 to pH 7.7, as shown in Fig. 16. Below pH 6.2 or above pH 8.6 less than 50% of the optimal binding occurred.

**Purification of $[^{3}H]$Acetyl-$\alpha$-bungarotoxin-Receptor Complex**—The 0.1% Triton X-100, 0.1 M Tris, pH 7.6 homogenate was reacted with $[^{3}H]$acetyl-$\alpha$-bungarotoxin, centrifuged, and purified by successive gel filtrations as described under “Materials and Methods.” The Sephadex G-100 gel chromatography elu-
Gel chromatography of receptor-[αH]acetyl-α-bungarotoxin complex on Sephadex G-150. The material from the Sephadex G-100 fractionation (Fig. 17) was fractionated on Sephadex G-150. Details of the fractionation are described under "Materials and Methods." The heavy line indicates the fractions placed onto Sephadex G-200 (Fig. 19).

The material from the Sephadex G-100 fractionation (Fig. 17) was fractionated on Sephadex G-150. Details of the fractionation are described under "Materials and Methods." The heavy line indicates the fractions placed onto Sephadex G-200 (Fig. 19).

The data in Table V indicate that while in the 0.1% Triton X-100, 0.1 M Tris, pH 7.6 extract of guinea pig cerebral cortex the picomoles bound per mg of protein was 87.5, the purified receptor-[αH]acetyl-α-bungarotoxin complex from the Sephadex G-200 column had 10.75 nmoles of [αH]acetyl-α-bungarotoxin bound per mg of protein. It should be noted that this material is purified but may not be absolutely pure.

Polyacrylamide Gel Electrophoresis of Purified Receptor-[αH]Acetyl-α-bungarotoxin Complex—The data in Fig. 20 indicate that the purified receptor-[αH]acetyl-α-bungarotoxin complex migrated as one protein band stainable with Coomassie brilliant blue on polyacrylamide gel electrophoresis. All of the radioactivity was associated with this band. Referring to the molecular weight versus migration graph (Fig. 9) it is evident that the purified receptor-[αH]acetyl-α-bungarotoxin complex had an approximate molecular weight of 94,000. Subtracting the molecular weight of [αH]acetyl-α-bungarotoxin of 8000 and assuming one [αH]acetyl-α-bungarotoxin molecule per complex, one is left with a receptor molecular weight of 86,000 (if one assumes both binding sites are on the same molecule (see Fig. 15) one is left with a molecular weight of 78,000; see "Discussion").

Isoelectric Focusing of Purified Receptor-[αH]Acetyl-α-bungarotoxin Complex—The data in Fig. 21 indicate essentially one protein peak for the purified [αH]acetyl-α-bungarotoxin-receptor complex; all of the radioactivity was associated with this peak. The data in Fig. 22 demonstrate one coincident protein and radioactivity peak for the purified [αH]acetyl-α-bungarotoxin-receptor complex utilizing a much narrower ampholyte pH range. The purified receptor-[αH]acetyl-α-bungarotoxin-receptor complex utilizing a much narrower ampholyte pH range. The purified receptor-[αH]acetyl-α-bungarotoxin-receptor complex had an isolectric point of pH 4.8. This complex isolectric point is in contrast to the [αH]acetyl-α-bungarotoxin isolectric point of 9.3. Assuming the major contribution to the complex isolectric point is the receptor isolectric point, it is obvious that the receptor must have a very low isolectric point below pH 4.8. Thus at neutral pH the receptor would carry a net negative charge while the [αH]acetyl-α-bungarotoxin has a net positive charge. This may be a partial basis for the interaction.

Enzyme Activity and Phosphorus Content of Purified Receptor-[αH]Acetyl-α-bungarotoxin Complex—The data in Table VI indicate that the purified receptor-[αH]acetyl-α-bungarotoxin complex was free of any measurable acetylcholinesterase or monoamine oxidase activity or any other enzyme activity which might be suspected to be present in the cerebral cortex. Also the purified receptor-[αH]acetyl-α-bungarotoxin complex contained no measurable phosphorus which might be thought to be present if the receptor were a phospholipoprotein.

Treatment of Purified Receptor-[αH]Acetyl-α-bungarotoxin Complex with Various Agents—Treatment of the receptor-[αH]acetyl-α-bungarotoxin complex with detergents only caused dissociation of the complex at 10% (Table VII) and even at this extremely high concentration only less than 30% dissociation occurred. On the other hand, 8 M urea was an effective agent causing dissociation of receptor and [αH]acetyl-α-bungarotoxin. These data again demonstrate that the binding was reversible and not irreversible as might be the case if a covalent bond was formed.
Fig. 20. Polyacrylamide gel electrophoresis of the purified receptor-[\(^{3}H\)]acetyl-\(\alpha\)-bungarotoxin complex. The top portion of the graph represents the Coomassie brilliant blue staining of the gel. The dotted lines represent where [\(^{3}H\)]acetyl-\(\alpha\)-bungarotoxin alone would run in the gel (see Fig. 8); there was no staining of the gel in this area. The only staining of the gel occurred in Fractions 14, 15, and 16. The bottom portion of the graph represents the counts per min found after fractionation of the gel with the Savant autogel divider. Details of polyacrylamide gel electrophoresis are described under "Materials and Methods."

Fig. 21. Isoelectric focusing of the purified receptor-[\(^{3}H\)]acetyl-\(\alpha\)-bungarotoxin complex. Isoelectric focusing utilizing ampholytes of pH 3 to 10 was carried out as described under "Materials and Methods."

Fig. 22. Isoelectric focusing of the purified receptor-[\(^{3}H\)]acetyl-\(\alpha\)-bungarotoxin complex. Isoelectric focusing utilizing ampholytes of pH 3 to 5 was carried out as described under "Materials and Methods."
TABLE VI
Enzyme activity and phosphorus content of purified receptor-[\(^{3}\!\!H\)acetyl-\(\alpha\)-bungarotoxin complex

Data for the enzymes are micromoles per hour per mg of protein. Purified receptor-[\(^{3}\!\!H\)acetyl-\(\alpha\)-bungarotoxin as protein (10 \(\mu\)g) was utilized in each assay. Experiments were performed as described under “Materials and Methods.” 0 indicates no detectable activity.

<table>
<thead>
<tr>
<th>Enzyme activity</th>
<th>Original homogenate</th>
<th>Purified receptor-[(^{3}!!H)acetyl-(\alpha)-bungarotoxin]</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-Nucleotidase</td>
<td>0.4</td>
<td>0</td>
</tr>
<tr>
<td>Acetylcholinesterase</td>
<td>3416</td>
<td>0</td>
</tr>
<tr>
<td>Monoamine oxidase</td>
<td>48</td>
<td>0</td>
</tr>
<tr>
<td>Succinic dehydrogenase</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>Uridine diphosphatase</td>
<td>0.9</td>
<td>0</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>3.9</td>
<td>0</td>
</tr>
<tr>
<td>Esterase</td>
<td>2.9</td>
<td>0</td>
</tr>
<tr>
<td>Phosphorus ((\mu)g/mg protein)</td>
<td>1.7</td>
<td>0</td>
</tr>
</tbody>
</table>

TABLE VII
Treatment of purified receptor-[\(^{3}\!\!H\)acetyl-\(\alpha\)-bungarotoxin complex with various agents

Experiments were performed with the purified receptor-[\(^{3}\!\!H\)acetyl-\(\alpha\)-bungarotoxin complex (see Fig. 19) as described under “Materials and Methods.” Means \(\pm\) 1 S.D. One \(\mu\)g of receptor-[\(^{3}\!\!H\)acetyl-\(\alpha\)-bungarotoxin complex (as protein) was utilized in these experiments.

<table>
<thead>
<tr>
<th>Agent</th>
<th>Picomoles of [(^{3}!!H)acetyl-(\alpha)-bungarotoxin bound per (\mu)g of protein]</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>11.0 (\pm) 0.9</td>
</tr>
<tr>
<td>0.1% Triton X-100</td>
<td>11.1 (\pm) 1.0</td>
</tr>
<tr>
<td>1% Triton X-100</td>
<td>11.1 (\pm) 0.7</td>
</tr>
<tr>
<td>10% Triton X-100</td>
<td>7.3 (\pm) 0.6</td>
</tr>
<tr>
<td>0.1% Sodium dodecyl sulfate</td>
<td>11.2 (\pm) 0.7</td>
</tr>
<tr>
<td>1% Sodium dodecyl sulfate</td>
<td>10.8 (\pm) 0.8</td>
</tr>
<tr>
<td>10% Sodium dodecyl sulfate</td>
<td>8.9 (\pm) 0.6</td>
</tr>
<tr>
<td>1 M Urea</td>
<td>9.7 (\pm) 0.6</td>
</tr>
<tr>
<td>8 M Urea</td>
<td>1.7 (\pm) 0.2</td>
</tr>
</tbody>
</table>

TABLE VIII
Treatment of purified receptor-[\(^{3}\!\!H\)acetyl-\(\alpha\)-bungarotoxin complex with various enzymes and lipid solvents

Experiments were performed with the purified receptor-[\(^{3}\!\!H\)acetyl-\(\alpha\)-bungarotoxin complex (see Fig. 19) as described under “Materials and Methods.” Means \(\pm\) 1 S.D. The amount of receptor-[\(^{3}\!\!H\)acetyl-\(\alpha\)-bungarotoxin in each experiment was 1 \(\mu\)g (as protein).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Picomoles of [(^{3}!!H)acetyl-(\alpha)-bungarotoxin bound per (\mu)g of protein]</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>11.0 (\pm) 0.9</td>
</tr>
<tr>
<td>Phospholipase C</td>
<td>10.8 (\pm) 1.1</td>
</tr>
<tr>
<td>Lipase</td>
<td>11.2 (\pm) 0.7</td>
</tr>
<tr>
<td>DNase</td>
<td>10.9 (\pm) 0.8</td>
</tr>
<tr>
<td>RNase</td>
<td>11.0 (\pm) 0.7</td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>10.7 (\pm) 0.8</td>
</tr>
<tr>
<td>HCCl(_3)-CH(_2)OH (2:1, v/v)</td>
<td>10.2 (\pm) 0.0</td>
</tr>
</tbody>
</table>

Treatment of Purified Receptor-[\(^{3}\!\!H\)acetyl-\(\alpha\)-bungarotoxin] Complex with Enzymes and Lipid Solvents—The data in Table VIII demonstrate that phospholipase C, lipase, DNase, RNase, and neuraminidase were not effective in dissociating the receptor-[\(^{3}\!\!H\)acetyl-\(\alpha\)-bungarotoxin complex as might be the case if lipid, nucleic acid, or sialic acid were important in the binding of the [\(^{3}\!\!H\)acetyl-\(\alpha\)-bungarotoxin to the receptor. Furthermore, upon extraction of the complex with HCCl\(_3\)-CH\(_2\)OH the complex did not dissociate and continued to be associated with the aqueous layer of the extract.

Synaptosomal and Subsynaptosomal Localization of Binding of [\(^{3}\!\!H\)acetyl-\(\alpha\)-bungarotoxin]—The data of Fig. 23 demonstrate that 200 \(\mu\)g of Fraction P, the synaptosomal fraction, bound more [\(^{3}\!\!H\)acetyl-\(\alpha\)-bungarotoxin than the 0.1% Triton X-100, 0.1 M Tris homogenate of guinea pig cerebral cortex (i.e. 36 pmol of [\(^{3}\!\!H\)acetyl-\(\alpha\)-bungarotoxin versus 17.5 pmol of [\(^{3}\!\!H\)acetyl-\(\alpha\)-bungarotoxin). Thus the receptors were preferentially located in the synaptosomes compared to the whole cerebral cortex. When the synaptosomes were lysed and subsynaptosomal fractions prepared, binding was found throughout the gradient in each of the fractions. However, the highest amount of binding occurred in the synaptosome plasma membrane fraction (79 pmol per 200 \(\mu\)g of protein or 395 pmol per mg of protein; cf. Table V). Binding was also high in Fraction G, the synaptosome membrane fraction. Low binding and hence low concentration of receptor occurred in Fraction D, synaptic vesicles; E, myelin and microsomes; H, damaged synaptosomes; and I, intraneural mitochondria. Fraction O, the soluble frac-
tion, had the lowest binding of $[^{3}H]$acetyl-$\alpha$-bungarotoxin (Fig. 23).

**Discussion**

The results describe the isolation and characterization of a mammalian cerebral cortex acetylcholine receptor. The receptor as described herein seems to be protein in nature although the relatively crude methods utilized cannot rule out the possibility of the receptor being a complex protein, i.e. a glycoprotein or lipoprotein. The results, furthermore, clearly demonstrate that the receptor is membrane associated (i.e. synaptosomal plasma membrane) and in vivo the receptor might be present as a membrane lipoprotein complex. De Robertis, Lunt, and La Torre (6), utilizing extraction with chloroform-methanol, have reported a special proteolipid protein fraction in electroplax of *Electrophorus electricus* (for work of De Robertis and coworkers on other tissue see review (38)) which is thought to be an acetylcholine receptor. In this light, it is of interest that in the present study extraction with chloroform-methanol yielded the receptor-$[^{3}H]$acetyl-$\alpha$-bungarotoxin in the aqueous and not the lipid fraction. Eldefrawi, Eldefrawi, and O'Brien (5) have reported that in *Torpedo marmorata*, acetylcholine receptors were phospholipoproteins, while in *Musca domestica* L. brains the receptors were proteins. Changuey et al. (9) recently reported that a receptor which binds $\alpha$-bungarotoxin in *E. electricus* is a protein while Millesi, Molinoff, and Potter (2) have stated that a single membrane protein of *T. marmorata* binds $\alpha$-bungarotoxin (although no data on chemical nature were given). The present results indicate that in mammalian brain the receptor is a protein. Moreover, the receptor described herein, because of its selective interaction with $\beta$-tubocurarine and gallamine, seems to be of a nicotinic nature. It should be noted that although the present data seem to fulfill criteria for an acetylcholine receptor, the term binding macromolecule might be more appropriate until such time as direct correlation between in vivo pharmacologic function and the in vitro data presented herein can be made.

The molecular weight of the present receptor is about 80,000 if one assumes 1 molecule of $[^{3}H]$acetyl-$\alpha$-bungarotoxin bound per molecule of receptor. The two binding sites found with the Scatchard plot (Fig. 15) could be one protein with two sites or two proteins each with a single site. The fact that 1 n mole of the purified $[^{3}H]$acetyl-$\alpha$-bungarotoxin-receptor complex contains 86 pg of $[^{3}H]$acetyl-$\alpha$-bungarotoxin indicates that 1 molecule of $[^{3}H]$acetyl-$\alpha$-bungarotoxin is probably bound to one receptor molecule. Finally, since the dissociation constants vary by a factor of 4 the equilibrium would be such that the majority of the stronger binding sites would be preferentially filled. Previous molecular weight estimates of acetylcholine receptors include 88,000 and 180,000 for the proteins of *T. marmorata* (2) and 40,000 for the special proteolipid protein fraction of *E. electricus* (6). The presently described receptor seems to be very similar to that of molecular weight 88,000 of *T. marmorata*.

Few estimates of the concentration of acetylcholine receptors in tissues have been published. The value of 0.1 n mole per g, wet weight, of the electric organ of *E. electricus* (39) and 18 nmoles per g, wet weight, of rat brain (40) have been roughly estimated. A value of 1 n mole per g of *Torpedo* electroplax has also been reported (2, 5, 41), as has 0.01 to 0.7 n mole per g for a particulate fraction of *E. electricus* (42), and 2 to 3 nmoles per g of head for housefly brain (43). A guinea pig cerebral cortex weighs 3 g, wet weight, of which 600 mg are Lowry et al. (24) protein. The cerebral cortex homogenate contains (Table V) 87.5 ± 7 nmoles per mg of protein. Thus one cerebral cortex contains 52.2 nmoles of receptor, or 17.5 nmoles per g, wet weight, of guinea pig cerebral cortex. This value is surprisingly close to that reported for rat brain. It is of interest that the isoelectric point of the receptor and $[^{3}H]$acetyl-$\alpha$-bungarotoxin are quite different and that at neutral pH the $[^{3}H]$acetyl-$\alpha$-bungarotoxin would have a net positive charge and the receptor a net negative charge. This is seen in the pH binding curve (Fig. 16); as the pH approaches 9.0 and thus the $[^{3}H]$acetyl-$\alpha$-bungarotoxin loses its positive charge, binding is greatly decreased.

The findings of the acetylcholine receptor in the synaptosome membrane fractions are of interest since it is at this site that the receptor may exert its physiological and pharmacological action. Whether the finding of binding at other subsynaptosomal sites is a consequence of fraction contamination or actually reflects the in vivo situation is not known.

It should be noted that the pH profile (Fig. 16) of $[^{3}H]$acetyl-$\alpha$-bungarotoxin binding differs strongly from that presented by O'Brien and Gilmour (40) for the binding of muscarine to a cholinergic receptor in *Torpedo* electroplax. The particular contrast is that at elevated pH of 8 to 10 O'Brien and Gilmour (40) had essentially maximal binding, while in the present study binding decreased above pH 8. The difference could be explained on the different isoelectric properties of the muscarine and the $[^{3}H]$acetyl-$\alpha$-bungarotoxin. Whether the finding of binding at other subsynaptosomal sites is a consequence of fraction contamination or actually reflects the in vivo situation is not known.

It should be noted that the pH profile (Fig. 16) of $[^{3}H]$acetyl-$\alpha$-bungarotoxin binding differs strongly from that presented by O'Brien and Gilmour (40) for the binding of muscarine to a cholinergic receptor in *Torpedo* electroplax. The particular contrast is that at elevated pH of 8 to 10 O'Brien and Gilmour (40) had essentially maximal binding, while in the present study binding decreased above pH 8. The difference could be explained on the different isoelectric properties of the muscarine and the $[^{3}H]$acetyl-$\alpha$-bungarotoxin. Whether the finding of binding at other subsynaptosomal sites is a consequence of fraction contamination or actually reflects the in vivo situation is not known.

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**References**

Additions and Corrections

Vol. 247 (1972) 130-145

In Bosmann, H. Bruce. Acetylcholine Receptor. I. Identification and Biochemical Characteristics of a Cholinergic Receptor of Guinea Pig Cerebral Cortex.

Page 130, Line 16 in the left-hand column, for 6.36 Ci per mmole read:

0.63 Ci per mmole

Page 131, left-hand column, third line in last paragraph, for 100 mCi (25.5 mg) read:

1 Ci (255 mg)

Page 131, right-hand column, Line 7, for 1.4 X 10^4 dpm read:

1.4 X 10^4 dpm

Page 132, right-hand column, Line 4, for 17.5 nmoles read:

175 nmoles

Page 133, right-hand column, 4th line from bottom, for 1.4 X 10^4 dpm per pmole read:

1.4 X 10^4 dpm per pmole

Page 139, Table II, add to footnote a:

Experiments with acetylcholine chloride were performed in the presence of 10^{-4} M eserine.

Page 141, legend to Fig. 17, Line 4, for 17.5 nmoles read:

175 nmoles

We suggest that subscribers photocopy these corrections and insert the photocopies at the appropriate places where the article to be corrected originally appeared. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.
Acetylcholine Receptor: I. IDENTIFICATION AND BIOCHEMICAL CHARACTERISTICS OF A CHOLINERGIC RECEPTOR OF GUINEA PIG CEREBRAL CORTEX
H. Bruce Bosmann


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