Proc. Nat. Acad. Sci. USA
Vol. 70, No. 12, Part I, pp. 3636–3640, December 1973

The Affinity-Labeling of Partially Purified Acetylcholine Receptor from Electric Tissue of *Electrophorus*

(affinity chromatography/4-(*N*-maleimido)-benzyltrimethylammonium/labeling kinetics/membrane proteins/subunit molecular weight)

ARTHUR KARLIN AND DAVID COWBURN*

Department of Neurology, College of Physicians and Surgeons, Columbia University, New York, N.Y. 10032

Communicated by Lyman C. Craig, August 10, 1973

ABSTRACT **The receptor for acetylcholine was partially purified by affinity chromatography of an extract in Triton X-100 of membrane fragments from electric tissue. The receptor was assayed, after its reduction with dithiothreitol, by reaction with the affinity-alkylating agent, [*methyl*-^{3}H]4-(*N*-maleimido)-benzyltrimethylammonium iodide. Alternative labeling procedures, one useful for routine assay of picomole quantities of receptor and the other for labeling larger quantities of receptor, are described. The purified receptor specifically incorporated about 3 nmol of label per mg of protein. This incorporation was blocked by pretreatment of the receptor with *Naja naja siamensis* neurotoxin. The rate of the affinity reaction was similar to that found with membrane fragments and with intact electroplax. Furthermore, as in intact electroplax, [^{3}H]4-(*N*-maleimido)-benzyltrimethylammonium iodide reacted 5000-fold faster with the reduced receptor than did [^{14}C]*N*-ethylmaleimide. When purified receptor was labeled with [^{3}H]4-(*N*-maleimido)-benzyltrimethylammonium iodide and subjected to electrophoresis on polyacrylamide gels in dodecyl sulfate and dithiothreitol, three major protein bands were observed. Only one of these, however, contained ^{3}H activity; its mobility indicated a molecular weight of 40,000.**

The receptor for acetylcholine in electric tissue of *Electrophorus electricus* has a readily reduced disulfide bond in the vicinity of the acetylcholine-binding site. After reduction, at least one of the resulting SH groups is affinity-alkylated by quaternary ammonium alkylating agents three to four orders of magnitude faster than by similar alkylating agents lacking a quaternary ammonium group (1, 2). One quaternary ammonium alkylating agent, tritiated 4-(*N*-maleimido)-benzyltrimethylammonium iodide [$C_4H_2O_2N \cdot C_6H_4 \cdot CH_2 \cdot N^+(CH_3)_3$ I^-; MBTA], has been used to label receptor in intact single cells (electroplax) (3, 4) and in subcellular membrane fragments (1, 2) from electric tissue, in which both specific and nonspecific labeling occur. Based on physiological evidence (5–7), specific labeling is taken as that which is dependent on prior reduction by dithiothreitol, is saturable, and is blocked by reversible cholinergic ligands, by snake neurotoxins, and by prior specific affinity-reoxidation of the receptor by dithiobischoline. The specifically labeled component in both cells and membrane fragments, behaves electrophoretically on Na dodecyl sulfate (SDS)–polyacrylamide gels like a polypeptide of molecular weight about 40,000 (4). This component was inferred to be a subunit of the receptor containing all or part of the acetylcholine-binding site.

Abbreviations: AChE, acetylcholinesterase; MBTA, 4-(*N*-maleimido)-benzyltrimethylammonium iodide; NEM, *N*-ethylmaleimide; SDS, sodium dodecyl sulfate.

* Present address: Rockefeller University, New York, N.Y. 10021.

Several groups, using the binding of snake neurotoxins (8) as an assay for the receptor, have solubilized and purified the toxin-binding component from electric tissue of *Electrophorus* (9–11) and of *Torpedo* (12–14). The reported activities are 2–10 nmol of toxin bound per mg of protein. The apparent molecular weights of the polypeptide components have been determined in some cases by SDS–polyacrylamide gel electrophoresis followed by staining. Two major components of molecular weights 54,000 and 42,000 of receptor purified from *Electrophorus* (11) and three major components of molecular weights 42,000, 35,000, and 26,000 from *Torpedo californica* (14) have been reported.

The affinities of neurotoxins and of cholinergic antagonists for the purified toxin-binding component are similar to those for membrane fragments and to those inferred from dose-response data (15). But agonists are reported to bind with markedly different affinities to the purified receptor (12, 15).

We have partially purified the receptor by affinity chromatography, assaying it by the affinity-alkylation reaction. We find that the rate of reaction of MBTA with the partially purified receptor is unchanged compared with intact cells and with membrane fragments and that although three major stained bands are found on SDS–acrylamide gel electrophoresis of labeled receptor, only one of these, of an apparent molecular weight of 40,000, is labeled. Affinity labeling combined with SDS–gel electrophoresis is uniquely suited to the determination of which of several subunits differing in molecular weights bear a reactive site. In addition, the rate of the affinity-reaction should be a sensitive indicator of the integrity of the affinity-labeled site.

MATERIALS AND METHODS

The principal neurotoxin of *Naja naja siamensis* venom (Miami Serpentarium) was purified (16). The affinity gel was prepared by reacting a homocysteinyl derivative of agarose (Affinose 401, Bio-Rad), reduced with 2 mM dithiothreitol in 50 mM Tris·HCl (pH 8), with the *p*-nitrophenyl ester of *p*-carboxytrimethylammonium iodide (17), 2 mM in 100 mM PO_4^{-3} (pH 7) for 30 min, to obtain 4% agarose–NH-$(CH_2)_3NH(CH_2)_3NHCOCH(NHCOCH_3)CH_2CH_2SCOC_6H_4$-$N^+(CH_3)_3$ at 1 μeq/g of packed gel. SH was assayed with dithiobis-(2-nitrobenzoate) (18) before and after acylation.

A membrane fraction of main organ electric tissue was prepared by a modification of a previous method (19). To 600 g of fresh tissue, 2400 ml of 0.89 M sucrose was added, and the mixture was homogenized in a 4-liter Waring commercial blendor on "low" for 10 sec. This treatment is sufficient to disperse the tissue, leaving large, multinucleated cell fragments with connected nerve nets. The homogenate

was centrifuged in a L2 65B centrifuge (Beckman) set at 19,000 rpm for 35 min (4°). A 19 rotor was used throughout. The pellets plus 1200 ml of 0.89 M sucrose were homogenized on "low" for 5 sec and centrifuged as before. The pellets plus 1200 ml of 0.3 M sucrose were homogenized on "high" for about 30 sec and checked by phase microscopy to see that no nucleated cell fragments remained and that the nerve nets were detached from the membrane fragments. This homogenate was centrifuged at 6000 rpm and 20 min to remove nuclei, nerve nets, and connective tissue. The supernatant was centrifuged at 19000 rpm overnight (8–16 hr). The pellets plus 100 ml of 1 mM Na-EDTA (pH 7.0) were homogenized in a VirTis "45" at 80% full speed for 30 sec, and the fraction was stored at −20°.

The membrane fraction was extracted successively by 1 M NaCl–2 mM PO_4^{-3}–1 mM EDTA (pH 7), 50 mM NaCl–10 mM PO_4^{-3}–1 mM EDTA (pH 8), and 3% Triton–50 mM NaCl–10 mM PO_4^{-3}–1 mM EDTA–3 mM NaN_3 (pH 8). In each case the protein concentration was 5–10 mg/ml. Sedimentation was in a 60 Ti rotor (Beckman) at 60,000 rpm for 40 min (4°). Dispersion was in a VirTis at 80% full speed for 30–60 sec. The last extraction was at 30° for 2 hr with stirring. The final extract was stored at 0°–4°.

"Slow Labeling". Samples of extract or of fractions from the affinity column were reduced by 0.2 mM dithiothreitol in 0.2–1.5% Triton–100 mM NaCl–5 mM PO_4^{-3}–10 mM Tris·HCl–1 mM EDTA (pH 8.0), volume 1–2 ml, for 10 min at 25°. The reaction was slowed by addition of 0.1 volume of 0.53 M PO_4^{-3} (pH 6.7) and cooling to 0°. The mixture was applied to a Bio-Gel P6 column, 24 × 0.9 cm, 4°–6°, equilibrated with 0.2% Triton–150 mM NaCl–10 mM PO_4^{-3}–1 mM EDTA (pH 7.0), and eluted with the same buffer at 20 ml/hr. The protein was recovered after the void volume, well-separated from dithiothreitol. To the pooled protein fractions, [^{3}H]MBTA (2 Ci/mmol; ref. 3) was added to give a final concentration of 1–100 nM. After 2 min (25°), the reaction was stopped with a 100-fold excess of 2-mercaptoethanol; the mixture was chromatographed on a Bio-Gel column, separating labeled protein from small labeled products.

"Quick Assay". In 15-ml, screw-top, plastic tubes (Becton–Dickinson), quadruplicate 50-μl samples containing 1–50 μg of protein were mixed with 50 μl of 0.4 mM dithiothreitol, in a final buffer composition as in the "slow-labeling" reduction. After 20 min at 25°, 10 μl of 0.53 M PO_4^{-3} (pH 6.7) was added, bringing the pH to 7.0. To two samples, 0.4 ml of 0.2% Triton–150 mM NaCl–10 mM PO_4^{-3}–1 mM EDTA (pH 7.0) was added; to two others, 0.4 ml of 3 μg of *N. n. siamensis* toxin per ml of the same buffer. After 10–15 min, 0.5 ml of 2 μM [^{3}H]MBTA–200 μM *N*-ethylmaleimide (NEM)–0.2% Triton–150 mM NaCl was added; after 3 min (25°), 25 μl of 0.1 M 2-mercaptoethanol was added. Finally, about 100 μg of reduced, carboxamidomethylated, and succinylated lysozyme (20) was added as carrier and the tubes were capped and put in ice.

Protein was precipitated by addition of 14 ml of saturated ammonium sulfate solution to a tube, the contents of which were rapidly mixed and filtered by suction through a 24-mm glass filter (Whatman, GF/A) held in a polypropylene filter holder (Gelman). The tube, including the top, was washed twice with 15 ml of saturated ammonium sulfate solution. The filter was washed three more times with 25 ml of saturated ammonium sulfate, twice with 25 ml of ice-cold 1 N HCl, and twice with 15 ml of acetone. The dry filter was placed in a counting vial, 0.1 ml of H_2O and 0.5 ml of NCS solubilizer were added, and the filter was kept at 50° for 1 hr. Finally, 10 ml of PPO–dimethyl POPOP–toluene scintillant was added and the vials were counted. The wash procedure reduces the background retention to about 0.05% of applied ^{3}H activity. The specific labeling is the difference between incorporation in the absence and in the presence of *N. n. siamensis* toxin.

Acetylcholinesterase (AChE) was assayed spectrophotometrically (18) or in the pH-stat (21), with similar results. Particulate fractions were diluted in 1 M NaCl before assay (21). Protein was assayed according to Lowry *et al.* (22) or with "Fluram" (Roche Diagnostics; ref. 23).

The gel techniques, with minor modifications, were those of Fairbanks *et al.* (24). For sample preparation, Triton was removed by precipitation and wash of protein with acetone; ionic strength was lowered (25) by dialysis against 1% SDS–2 mM Tris·HCl (pH 8). Gels to be sliced contained 10% glycerol, which was added to the staining and destaining solutions as well. Gels were frozen and sliced (0.5 or 1 mm) with a slicer from MRA Corp. Slices from unfixed gels were gently shaken for 40 hr with 1 ml of 1% SDS–2 mM Tris·HCl (pH 8) and counted in 10 ml of "Scintisol Complete" (Isolab). Slices from fixed gels were dried in vials at 50° for 4–6 hr; 0.1 ml of H_2O and 0.5 ml of NCS were added. The vials were capped and kept at 45°–50° for 24 hr; 10 ml of toluene scintillant was added. Of the applied ^{3}H activity, 95% was recovered in slices of unfixed gels and 80% in slices of fixed gels.

RESULTS

Solubilization of Receptor from Membrane. Starting from a homogenate of about 600 g of main electric organ containing 26 ± 1 mg of protein per g of tissue and 41 ± 2 μg of AChE per g of tissue, a membrane fraction was prepared, which contained 13 ± 2% of the total protein and 36 ± 7% of the total AChE of the homogenate. Of the total particle-bound AChE, 76% is in this fraction, which thus contains most of the innervated membrane fragments rich in AChE. The quantity of receptor in this fraction estimated by saturation of the specific labeling by [^{3}H]MBTA is 3 pmol of sites per mg of protein or 10 pmol of sites per g of original tissue (1, 2). These are considerable underestimates, however, since much more receptor is assayed after detergent extraction of the membrane. By electron microscopy, the membrane fraction appears to be composed of vesicles.

Resuspension of the membrane in 1 M NaCl (21) solubilizes 9 ± 1% of the protein and 57 ± 4% of the AChE. Subsequent extraction of the membrane in 3% Triton X-100 solubilizes 21 ± 3% of the original membrane protein and 2 ± 0.2% of the original membrane AChE. The Triton extract contains 70–80 pmol of receptor sites per mg of protein and 60–70 pmol per g of original tissue, 6- to 7-fold more than estimated for the membrane directly.

Affinity Chromatography. The affinity gel is very efficient in adsorbing receptor, and to a lesser extent, AChE from the Triton extract (Fig. 1). All detectable receptor is bound on passage of about 200 ml of extract containing 425 mg of protein, 1.3 nmol of AChE catalytic sites, and 25 nmol of receptor

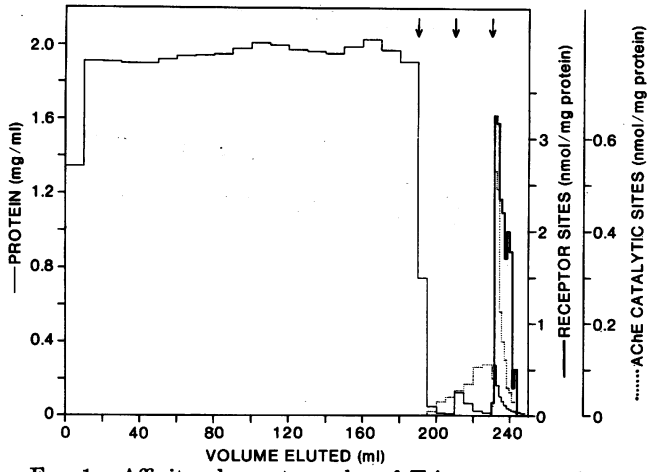

FIG. 1. Affinity chromatography of Triton extract of membrane. The column contained 1.8 ml of affinity gel to which 190 ml of membrane extract in 3% Triton–50 mM NaCl–10 mM PO_4^{-3}–1 mM EDTA–3 mM NaN_3 (pH 7.0) was applied at 0.9 ml/min. The column was washed at 0.5 ml/min first with 20 ml of buffer, containing 0.2% Triton–50 mM NaCl–10 mM PO_4^{-3}–1 mM EDTA–3 mM NaN_3 (pH 7.0) (*left arrow*), then with 20 ml of buffer containing an additional 50 mM NaCl (*middle arrow*), and finally with 20 ml of buffer containing 50 mM carbamylcholine (*right arrow*). Carbamylcholine was removed by dialysis, and protein (——), AChE (· · · ·), and receptor (——) were assayed. AChE catalytic sites are calculated on the basis of 10 mol of acetylcholine hydrolyzed per min per g of enzyme and 80,000 g of enzyme per mol of catalytic site (28).

sites through the 1.8-ml column. None is eluted in the buffer wash or in the buffer containing an additional 50 mM NaCl. About 11% of that applied is eluted in 50 mM carbamylcholine at a purification of 42-fold for the pooled fractions or of 55-fold for the two peak fractions, which contain 70% of

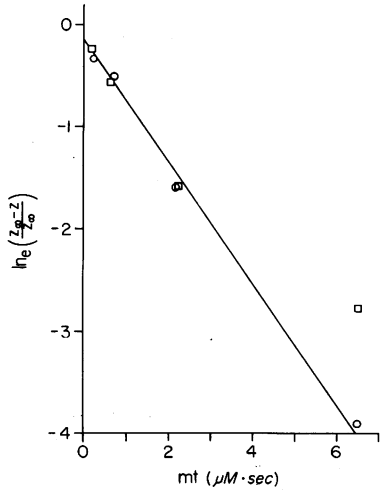

FIG. 2. Pseudo-first-order reaction kinetics of the reaction of [^{3}H]MBTA with solubilized (O) and with membrane-bound (□) receptor. Solubilized receptor was "slow labeled" at various concentrations of [^{3}H]MBTA for 2 min (25°) with and without pretreatment with dithiobischoline. Protein was precipitated and washed with acetone, redissolved in 1% SDS, and counted in Scintisol. Membrane fragments were labeled (1, 2, and material in preparation) at the same [^{3}H]MBTA concentrations as the solubilized receptor. z is the extent of specific reaction; z_∞, the maximum extent of reaction; m, the concentration of [^{3}H]-MBTA; and t, the duration of the reaction.

the total recovered. In four preparations, the peak fractions contained 2–4 nmol of receptor sites per mg of protein. AChE is less tightly bound by the gel; 30% is not bound; 28% is recovered in 50 mM carbamylcholine; and 66% is recovered overall. About 2% of the protein eluted in the carbamylcholine step is active AChE.

The Rate of Affinity-Labeling of Detergent-Solubilized Receptor. The specificity of the receptor for MBTA is unimpaired by solubilization in Triton X-100. The reaction is blocked by dithiobischoline, *siamensis* toxin, and *d*-tubocurarine. In a typical experiment, solubilized receptor was "slow labeled" at various concentrations of [^{3}H]MBTA, with and without prior treatment with dithiobischoline. The concentration of [^{3}H]MBTA does not change during the 2-min reaction under the conditions used, and the extent of specific reaction, z, should obey the pseudo-first-order kinetic equation, $z = z_\infty (1 - e^{-kmt})$, where k is the second-order rate constant, m is the concentration of [^{3}H]MBTA, and t is the duration of the reaction. The maximum extent of reaction, z_∞ is obtained by extrapolation of the saturation curve, using the usual inverse plot. Data from such an experiment and from the specific labeling of membrane vesicles are plotted so that the slope of the fitted line yields the rate constant, k (Fig. 2; Table 1).

The rate of specific reaction of [^{3}H]MBTA with the reduced receptor is not changed significantly by solubilization. Furthermore, the rate remains three to four orders of magnitude greater than the rate of reaction of NEM with the reduced receptor, as inferred for receptor *in situ* (5). A purified preparation containing 3 nmol of receptor sites per mg of protein was reduced and reacted for 2 min (25°) with a mixture of 0.1 μM [^{3}H]MBTA and 130 μM [^{14}C]NEM (9.8 Ci/mol; New England Nuclear Corp.). The extents of alkylation were 2.5 nmole of [^{3}H]MBTA per mg of protein and 26 nmol of [^{14}C]NEM per mg of protein. The extent of specific reaction of [^{3}H]MBTA was 2.3 nmol/mg, so that at most 0.7 nmol of specific receptor sites per mg reacted with [^{14}C]NEM. The ratio of the rates of reaction of [^{3}H]MBTA and [^{14}C]NEM with the specific receptor site is thus at least (2.3 nmol ÷ 0.1 μM) ÷ (0.7 nmol ÷ 130 μM) = 4300. In this preparation, one specific SH group is alkylated per 330,000 g of protein and one nonspecific SH group (or other nucleophile) per 40,000 g of protein.

TABLE 1. *Rate constants for reaction of maleimidobenzyltrimethylammonium (MBTA) with reduced receptor*

Preparation	Method	Rate constant [10^5·liter·(mole·sec)$^{-1}$]
Intact electroplax	Inhibition of response to carbamycholine (5)	3 ± 0.5
	Protectible reaction with [^{3}H]MBTA (3)	0.7 ± 0.2
Membrane fragments	Protectible reaction with [^{3}H]MBTA: specificity by gel electrophoresis (in preparation)	6 ± 0.3
Detergent extract of membrane fragments, partially purified	Reaction with [^{3}H]-MBTA	6 ± 1

Gel Electrophoresis of Labeled Receptor. Purified receptor preparations yield three major bands and several minor bands after SDS–acrylamide gel electrophoresis and staining for protein. Receptor labeled with [^{3}H]MBTA gives a similar pattern when stained for protein (Fig. 3*a*). The distribution of ^{3}H activity in slices of the fixed and stained gel shows one major (48% of total) and two minor (10 and 16%) peaks (Fig. 3*b*). The relative mobility of the major peak corresponds exactly to that of stained band G (Fig. 3*c*) with no overlap on bands E and F. The major stained bands are readily identified visually in the slices; by this means also it was determined that most of the ^{3}H activity is associated with band G and negligible ^{3}H activity with bands E and F. One minor peak of ^{3}H activity corresponds in mobility to stained band H, and the other, diffuse minor peak overlaps stained band C. The relative mobilities of the stained bands and ^{3}H peaks, when compared with those of protein standards on parallel gels, yield estimates of 53,000, 47,000, and 41,000 daltons for E, F, and G (Fig. 3*c*). Gels run in parallel to the illustrated ones which had ^{14}C-labeled methylated protein standards (4) added to the sample of labeled receptor and which were not stained, yielded a distribution of ^{3}H activity identical to that of the stained gels and molecular weight estimates of 40,000 for the major peak and 103,000 and 32,000 for the two minor peaks of ^{3}H activity.

DISCUSSION

The affinity labeling procedure used here and previously used to label intact cells and subcellular membrane fragments has the virtue that a small, readily synthesized molecule is covalently attached to the receptor molecule apparently by way of a cysteinyl residue adjacent to the acetylcholine-binding site. The relative success of this procedure is partly due to the fortuitous, but seemingly general (1, 2, 26), presence of a readily reduced disulfide in the region of the binding site of "nicotinic" acetylcholine receptors at synapses in electric tissue and in striated muscle. The requirement for reduction before affinity-alkylation, however, has been a drawback in the use of this procedure as a practical assay for receptor, since it is necessary to remove excess reducing agent before the addition of low concentrations of affinity-label. The "quick assay" method obviates this problem. Its basis is that MBTA reacts 5000-fold faster than NEM with specific SH groups of reduced receptor but only 4-fold faster with all other SH groups. Thus, after reduction with dithiothreitol alone, the concentrations of dithiothreitol, [^{3}H]MBTA, and NEM can be selected so that nearly all the specific receptor sites, but very few nonspecific sites, react with [^{3}H]MBTA before most of the [^{3}H]MBTA reacts with dithiothreitol. The NEM reacts almost entirely with the dithiothreitol, quickly reducing its concentration and slowing its reaction with [^{3}H]MBTA. The reaction of [^{3}H]MBTA with the receptor is blocked by *siamensis* toxin, which is used to determine the extent of nonspecific reaction. The assay is applicable to mixtures containing as little as 1 pmol of receptor sites in 25 μg of protein. The results of the "quick assay" agree with the results of the "slow labeling" procedure, in which the dithiothreitol is removed by gel filtration before alkylation.

The "slow labeling" procedure is readily applicable to the preparation of a large quantity of labeled receptor or to the determination of rate constants through the variation of the concentrations of alkylating agent or of the duration of the reaction. By this procedure, it is possible to demonstrate that the rate of reaction of [^{3}H]MBTA with the reduced receptor changes little if at all in the transitions from cells to membrane to Triton-solubilized receptor (Table 1). The similarity

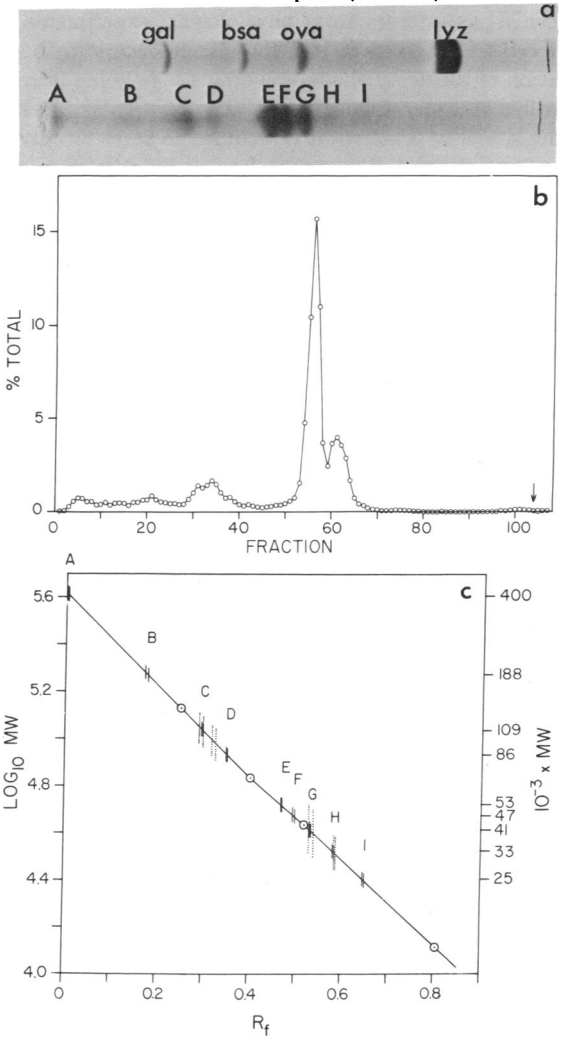

FIG. 3. SDS–acrylamide gel electrophoresis of labeled receptor. After "slow labeling" at 0.1 μM [^{3}H]MBTA of 100 μg of protein containing 3.2 nmol of receptor sites per mg of protein, the protein was precipitated with 9 volumes of acetone and washed with another 9 volumes to remove residual Triton. The precipitate was then dried under reduced pressure. The precipitate was dissolved in 0.1 ml of 2% SDS–20 mM Tris·HCl–1 mM EDTA–20 mM dithiothreitol (pH 8.0) and kept at 50° for 1 hr. Gels were 5.6% acrylamide–0.21% methylenebisacrylamide, containing 1% SDS–100 mM Tris·HCl–2 mM EDTA–10% glycerol (pH 8.0), and were prerun with the same buffer containing 1 mM dithiothreitol. 50-μl samples containing 22 μg of protein and 51,000 cpm of ^{3}H were applied and run at 8 ma/gel until the Pyronin Y tracking dye had migrated 9 cm. Parallel gels were run with about 5 μg each of β-galactosidase (*gal*), bovine-serum albumin (*bsa*), and ovalbumin (*ova*) and 25 μg of lysozyme (*lyz*), treated as above. The gels were stained with Coomassie Brilliant Blue and photographed on Polaroid P/N 55 film with a no. 16 filter. The labeled gels were then frozen, sliced, and counted. (*a*) Sample and standard gels. (*b*) Percent total ^{3}H activity against slice number. *Arrow* indicates front. (*c*) Log of molecular weight against relative mobility of protein standards (⊙). Relative mobilities of stained (——) and radioactive (· · · ·) bands are compared for duplicate sample gels. The letters refer to the stained bands in (*a*). Numbers in the *right-hand ordinate* have been multiplied by 10^{-3}.

of the receptor in the cell and in solution is further supported by the finding that the rate of reaction of [^{3}H]MBTA with the reduced purified receptor is about 5000-fold that of [^{14}C]NEM, as inferred from physiological experiments with intact cells. It appears that little change occurs in the conformation of the acetylcholine-binding site in going from cells to purified receptor. Since [^{3}H]MBTA apparently blocks the binding site after reaction, it is assumed to react rapidly with only one of the two SH groups formed by reduction of the receptor.

The quantity of receptor assayed in the Triton extract of the membrane fraction is considerably greater than that determined directly by saturation of the specific labeling of the membrane fraction. Two factors could act to lower the extent of labeling in the membrane fragments. These fragments appear by electron microscopy after osmium fixation to be closed vesicles. If a considerable fraction of the vesicles were inside out (27), there would be a permeability barrier preventing access of dithiothreitol and of [^{3}H]MBTA to the receptor-binding site. A second factor could be the partial reoxidation of the reduced receptor during the repeated resuspension and sedimentation used to remove dithiothreitol from the membrane fragments before addition of [^{3}H]MBTA.

The simple purification presented here results in a specific activity within the range of published values for the purified neurotoxin-binding component and about half of the highest specific activities reported (9–14). About 1 mg of protein is obtained from 500 g of fresh tissue. Assuming 40,000 g/mol of affinity-labeled subunit, this subunit comprises 16% of the protein in the best preparations with 4 nmol of labeled sites per mg of protein. Significant quantities of active AChE are present, comprising about 2% of the protein. The correspondence between affinity-labeled sites and toxin-binding sites has not been determined; however, in this case the latter include the former since the specific labeling is taken as that blocked by toxin (4, 7). Whether or not the two assays are differentially sensitive to receptor denaturation, as might occur to some extent during isolation, is also undetermined.

The principal, specifically affinity-labeled component in the purified receptor preparation behaves as a polypeptide of molecular weight 40,000, as also found in intact cells, subcellular membrane fragments, and unfractionated detergent extracts of membrane. Since the same size component is obtained with intact cells labeled and then dissolved in 2% SDS containing EDTA at 50°, it is unlikely to be the product of proteolytic degradation of an originally larger component. On the other hand, the smaller labeled components, in the band centered at 32,000 daltons (Fig. 3c), could be degradation products of the 40,000-dalton species formed during extraction of the membrane, purification, storage, or labeling. Such minor components, even if present, would be difficult to detect in gels from labeled cells or membrane because of considerable nonspecific labeling. The minor labeled components around 100,000 daltons are likely to be the residue of the major nonspecifically labeled components found in electroplax, in membrane and in crude extract.

One major stained band (G) corresponds exactly to the 40,000-dalton peak of ^{3}H activity. The other two major bands, E and F, are not labeled. Whether they are receptor subunits is not known; however, either they themselves bind to the affinity column and are eluted by carbamylcholine, or they are associated with a component that so binds. The quantity of active AChE present (0.04 μg in a 20-μg sample) is not sufficient to account for either of these bands, but it is possible that proteolyzed and inactive AChE is present. The molecular weight of the native AChE subunit is about 80,000 and that of proteolyzed but still fully active AChE is 60,000 or lower (28; Chen, Rosenberry & Chang, in preparation). It seems possible that lower-molecular-weight, inactive forms could be obtained, perhaps accounting for either E or F. On the other hand, if all three bands, E, F, and G, contained unique components of the molecular weights indicated and all were subunits of the receptor, the minimum possible weight of protein per acetylcholine-binding site would be about 140,000 daltons, which is more than adequate to span a phospholipid bilayer and provide a controlled path for permeation of ions (1, 2).

We thank Dr. L. Kremzner for purifying neurotoxin, Ms. R. Ornstein for electron micrographs, and Dr. I. Silman for his comments on the manuscript. The work was supported by USPHS Grant NS 07065, NSF Grant GB 15906, and The New York Heart Association, Inc. D.A.C was supported in part by USPHS Training Grant MH 10315.

1. Karlin, A. (1973) *Fed. Proc.* **32**, 1847–1853.
2. Karlin, A., Cowburn, D. A. & Reiter, M. J. (1973) in *Drug Receptors*, a Biological Council Symposium, ed. Rang, H. P. (MacMillan, London), in press.
3. Karlin, A., Prives, J., Deal W. & Winnik, M. (1971) *J. Mol. Biol.* **61**, 175–188.
4. Reiter, M. J., Cowburn, D. A., Prives, J. M. & Karlin, A. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 1168–1172.
5. Karlin, A. (1969) *J. Gen. Physiol.* **54**, 245s–264s.
6. Bartels, E., Deal, W., Karlin, A. & Mautner, H. (1970) *Biochim. Biophys. Acta* **203**, 568–571.
7. Prives, J. M., Reiter, M. J., Cowburn, D. A. & Karlin, A. (1972) *Mol. Pharmacol.* **8**, 786–789.
8. Lee, C. Y. (1970) *Clin. Toxicol.* **3**, 457–472.
9. Olsen, R. W., Meunier, J.-C. & Changeux, J.-P. (1972) *FEBS Lett.* **28**, 96–100.
10. Fulpius, B., Cha S., Klett, R. & Reich E. (1972) *FEBS Lett.* **24**, 323–326.
11. Lindstrom, J. & Patrick, J. (1973) in *Synaptic Transmission and Neuronal Interaction*, ed. Bennett, M. V. L. (Raven, New York), in press.
12. Franklin, G. I. & Potter, L. T. (1972) *FEBS. Lett.* **28**, 101–106.
13. Karlsson, E., Heilbronn, E. & Widlund, L. (1972) *FEBS. Lett.* **28**, 107.
14. Schmidt, J. & Raftery, M. A. (1973) *Biochemistry* **12**, 852–856.
15. Meunier, J.-C. & Changeux, J.-P. (1973) *FEBS Lett.*, in press.
16. Karlsson, E., Arnberg, H. & Eaker, D. (1971) *Eur. J. Biochem.* **21**, 1–16.
17. Silman, I. & Karlin, A. (1969) *Science* **164**, 1420–1421.
18. Ellman, G. L., Courtney, K. D., Andres, G. V. & Featherstone, R. M. (1961) *Biochem. Pharmacol.* **7**, 88–95.
19. Karlin, A. (1965) *J. Cell Biol.* **25**, 159–169.
20. Wiener, A. M., Platt, T. & Weber, K. (1972) *J. Biol. Chem.* **247**, 3242–3251.
21. Silman, H. I. & Karlin, A. (1967) *Proc. Nat. Acad. Sci. USA* **58**, 1664–1668.
22. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
23. Udenfriend, S., Stein, S., Bohlen, P., Dairman, W., Leingruber, W. & Weigele, M. (1972) *Science* **178**, 871–872.
24. Fairbanks, G., Steck, T. L. & Wallach, D. F. M. (1971) *Biochemistry* **10**, 2606–2617.
25. Reynolds, J. A. & Tanford, C., (1970) *J. Biol. Chem.* **245**, 5161–5165.
26. Ben-Haim, D., Landau, E. M. & Silman, I. (1973) *J. Physiol. (London)*, in press.
27. Kant, J. A. & Steck, T. L. (1972) *Nature New Biol.* **240**, 26–27.
28. Dudai, Y., Silman, I., Shinitzky, M. & Blumberg, S. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 2400–2403.