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Affinity Chromatography and Characterization of the Acetylcholine Receptor from Torpedo californica*

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INTRODUCTION

Affinity chromatography has become one of the most important tools available to the biochemist for the selective isolation and purification of interesting biological macromolecules. The pioneering work of Porath [1,2] and subsequent developmental work by Porath [3] and Cuatrecasas [4] have served as practical guides for the successful application of affinity chromatography in many

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areas of biochemical research. Specific ligands can be covalently attached to an insoluble, stationary support such as agarose or polyacrylamide beads and used to "fish out" those components which show an appreciable affinity for the ligands.

The successful application of affinity chromatography to a particular problem is governed by many variables, some of which are not fully understood at present, although progress is being made along these lines. (See other papers in this symposium.) Some of the design variables which must be optimized for efficient use of affinity chromatography are (1) the type of ligand, (2) the length of the arm or "leash" by which the ligand is coupled to the insoluble support, (3) the chemical nature of the leash, either polar or nonpolar or some combination thereof, and (4) the concentration of the ligand on the support. Normally, ligands are chosen for use in affinity chromatography because of their known specific interaction with the active center of the macromolecule of interest. Occasionally, however, ligands which exploit other chemical characteristics of the molecule can be utilized; for example, simple long-chain alkyl groups can be used to separate molecules based on their hydrophobic characteristics [5].

The purification of membrane proteins presents a special problem. Many membrane-bound protein molecules which can be solubilized by mild detergents have similar molecular weights, sedimentation coefficients, isoelectric points, and other chemical properties. However, by exploiting differences in the chemical specificity of these proteins, the techniques of affinity chromatography have been especially useful for separating and purifying specific membrane proteins. This has been especially so for neurochemistry. Substantial interest has been focused on the molecular events which occur during the neuron-specific functions of synaptic transmission and axonal conduction. In order to understand the mechanisms which involve the release of neurotransmitter and the interaction of the transmitter with a receptor to produce ionic conductance changes in the postsynaptic membrane, it is

necessary to separate, purify, and characterize the various components of the synapse.

The neuromuscular junction utilizes acetylcholine (AcCh)* as its transmitter substance. Acetylcholine is synthesized in the nerve terminal by the enzyme choline acetyltransferase and stored in vesicles. When an impulse arrives at the nerve terminal, AcCh is released, diffuses across the synapse, and interacts with an integral membrane protein called the acetylcholine receptor (AcChR). Thereupon, the postsynaptic membrane displays an increase in cation permeability with concomitant depolarization of the membrane. Also located in the synaptic cleft is the powerful enzyme acetylcholinesterase (AcChE) which rapidly hydrolyzes acetylcholine and effectively controls the duration of the post-synaptic response.

Because of its central role in neuromuscular transmission, the AcChR has been the subject of numerous studies and reviews [6-8]. This protein can be removed from the membrane and purified by affinity chromatographic techniques. The function of this article is to describe in detail some of our experiences with affinity chromatography encountered during the purification of the acetylcholine receptor and to describe some of the structural characteristics of the purified protein molecule as they relate to the functioning of the molecule in the postsynaptic membrane.

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*Abbreviations: Acetylcholine receptor, AcChR; acetylcholine, AcCh; acetylcholinesterase, AcChE; diisopropylfluorophosphate, DFP; diethylaminoethyl, DEAE; (diethyl-2-hydroxypropyl)aminoethyl, QAE; ethylenediaminetetraacetate, EDTA; phenylmethanesulfonylfluoride, PMSF; sodium dodecyl sulfate, SDS; bis-(3-aminopyridine-1,10) decane diiodide, DAP; 5,5'-dithio-(2-nitrobenzoic acid), DTNB; α-bungarotoxin, α-BuTx; 4-(N-maleimido)-benzyltrimethylammonium iodide, MBTA: trimethyloxonium tetrafluoroborate, TMO.

CHEMICAL SPECIFICITY OF THE ACETYLCHOLINE RECEPTOR

The chemical specificity of the AcChR is best described in terms of the molecules which interact strongly with the receptor. Some of the cholinergic ligands which bind to the AcChR are depicted in Fig. 1. These can be grouped into three general classes [9]: (1) agonists - those molecules which cause an immediate depolarization of the postsynaptic membrane; (2) depolarizing antagonists - molecules which cause an immediate but prolonged depolarization and render the membrane insensitive to subsequent application of AcCh; and (3) antagonists and blocking agents - those ligands which act simply as competitive inhibitors. Included in this last class are small proteins known as α-toxins found in the venom of many poisonous snakes. These neurotoxins, purified from several species of snakes, have molecular weights around 7,000 to 8,000 daltons, have similar amino acid sequences, and bind specifically and with high affinity to the AcChR [10]. The agonists of the receptor all contain a single quaternary trimethylammonium moiety while the antagonists are generally bifunctional or bisonium ligands. The reactive group or groups on the neurotoxins have not yet been definitively identified.

The binding of AcCh or one of the other reversible ligands is the natural method to assay for the receptor. Such binding can easily be demonstrated by equilibrium dialysis employing radioactive ligands. However, all of the small ligands in Fig. 1 also bind to the acetylcholinesterase molecule with substantial affinities. The esterase has a very rapid turnover, and even trace amounts of the enzyme are sufficient to make reversible binding studies with AcCh very difficult. Equilibrium dialysis can be employed, however, if the esterase activity is inhibited by diisopropylfluorophosphate (DFP) [11] or other organophosphate molecules [12].

The high specificity of the α-toxins for AcChR and not for the AcChE is surprising, especially since the active sites of the two distinct molecules must be similar for each to bind the many chol-

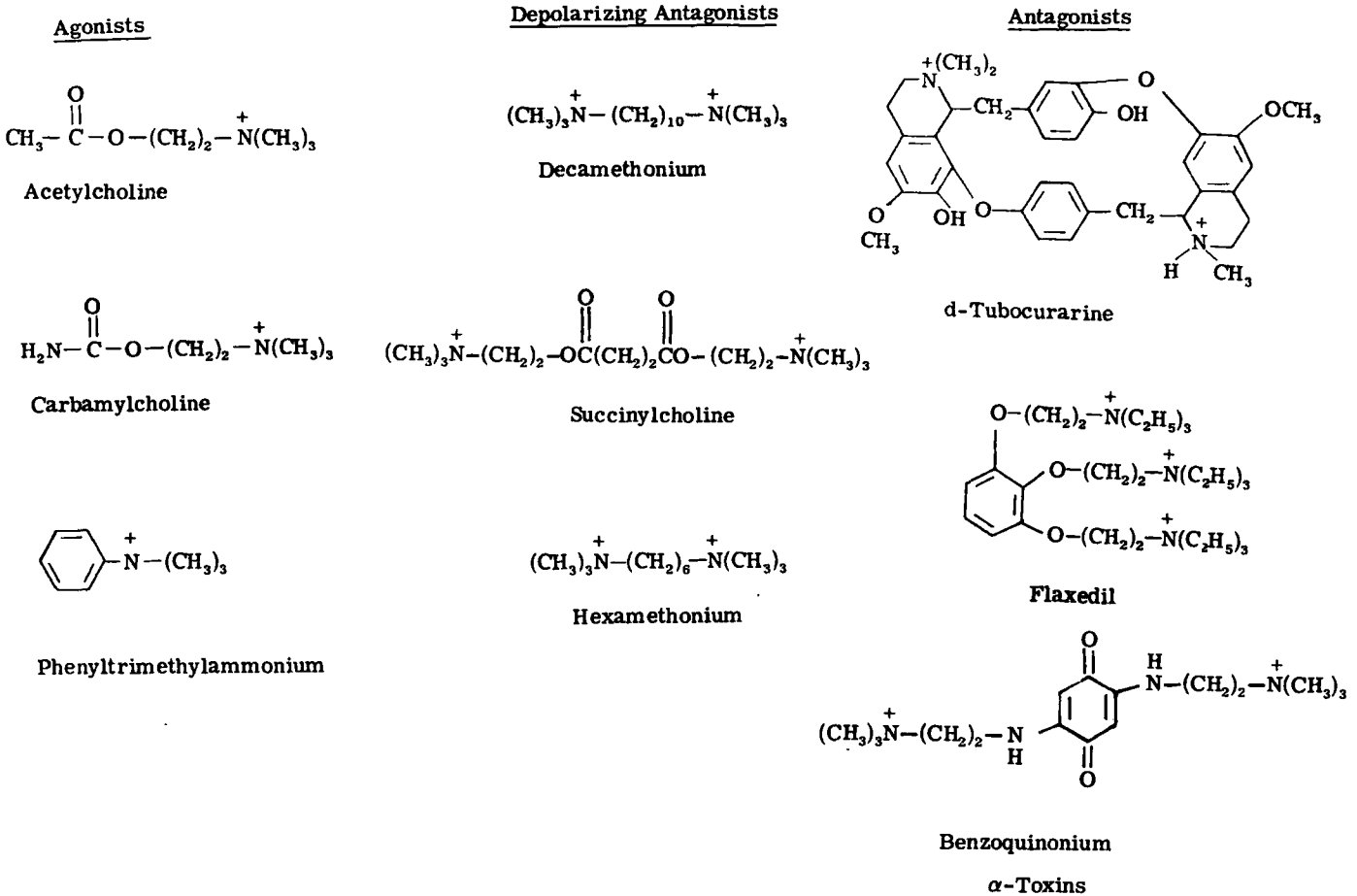


FIG. 1. Cholinergic ligands which bind to the acetylcholine receptor and to the acetylcholinesterase.

inergic ligands. Because these toxins bind with such specificity and high affinity, radioactively labeled derivatives of the toxins can be used as specific probes of the AcChR. One such toxin which has found wide use is α-bungarotoxin (α-BuTx), the major protein component of the Formosan krait Bungarus multicinctus [13]. The toxin can be purified from the crude venom [14], labeled with radioactive iodine [^{125}I], and used for a simple and extremely sensitive assay for the AcChR [15], since it binds essentially irreversibly. The receptor-toxin complex is acidic while the toxin is basic [10]. The complex can be adsorbed onto DEAE paper disks and unbound toxin washed away. The radioactivity trapped on the disks can be counted in a scintillation counter with the bound radioactivity proportional to the receptor concentration. The amount of toxin bound per unit mass of AcChR protein (n mole/mg) is defined as the specific activity of the receptor and is a measure of its purity. The specific activity can also be expressed as the amount of protein to which one molecule of toxin binds, or daltons AcChR/toxin molecule.

The quantity of AcChR in mammalian systems is extremely small due to the relatively few numbers of synapses. However, the electric organs of certain fish are composed of modified muscle cells containing large numbers of synapses. These synapses are typically cholinergic in nature and contain a large number of AcChR molecules [16]. Two common sources of electric organs are the electric eel from the Amazon River, Electrophorus electricus, and the marine electric ray of the Torpedo family. Torpedo organs comprise 20 to 25% of the total body weight (up to 200 lbs. for some species) and contain 10 to 20 times the concentration of acetylcholine receptor as does the eel electroplax.

RECEPTOR ISOLATION AND PURIFICATION

Membrane fragments enriched in AcChR from Torpedo californica are obtained by the procedure shown in Fig. 2 [17]. All electric organs are obtained fresh and used immediately or stored at -90°C. A marked deterioration of the organs is observed if the organs are

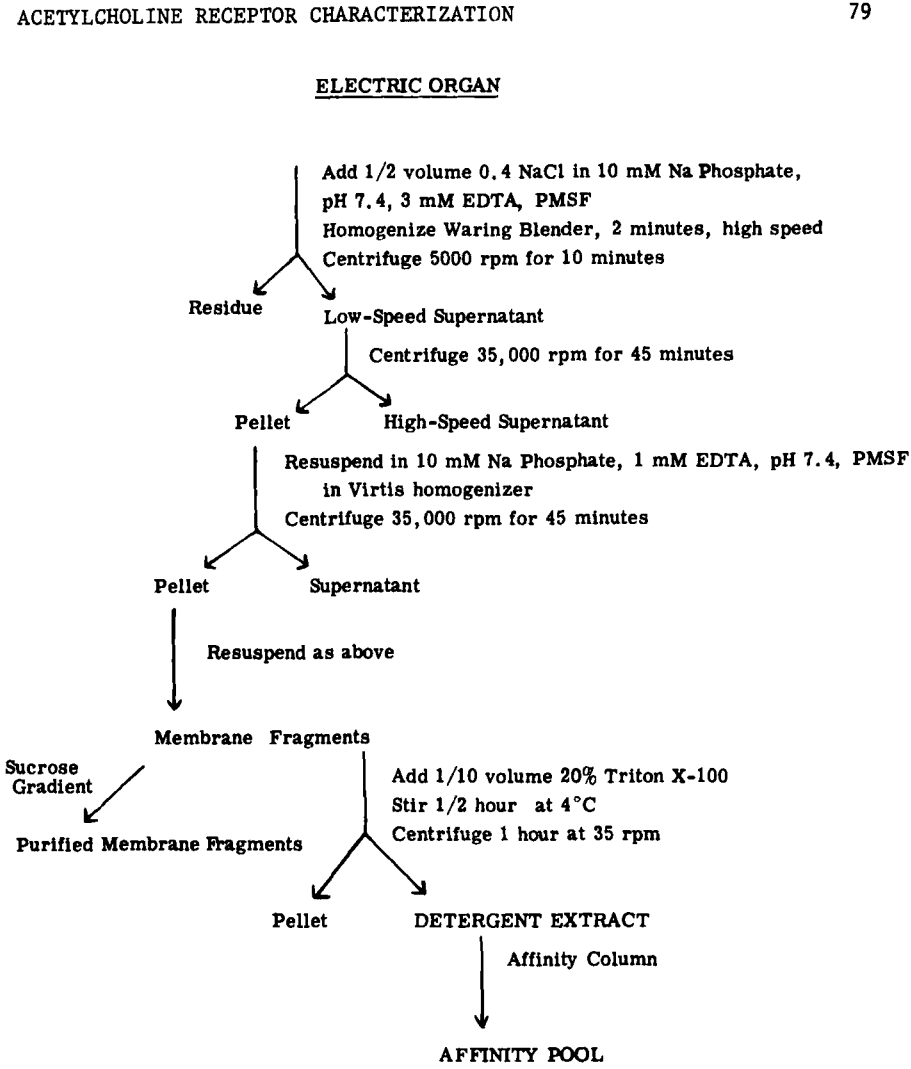


FIG. 2.

Extraction and purification procedure for the AcChR from Torpedo californica.

stored at higher temperatures for any length of time. The first low-speed centrifugation removes connective tissue and other large particles. The supernatant after the first high-speed spin contains all the water-soluble proteins and about 50% of the total

AcChE. A second wash with low ionic strength buffer further removes soluble proteins and reduces the ionic strength of the preparation. The membrane fragments can then be further enriched in AcChR by centrifugation on sucrose gradients or the AcChR can be solubilized from the membrane by use of the nonionic detergent Triton X-100. The receptor is quantitatively extracted from these fragments with 2% Triton in less than one-half hour at 4°C. After extraction, the solution is centrifuged once more to pellet the unextracted proteins. The solubilization and centrifugation step alone represents a 2-fold purification step.

At this point, conventional biochemical techniques were initially used to further purify the receptor in detergent solution. Figure 3 shows the protein and activity profile for gel filtration chromatography on Sepharose 6B in 0.1% Triton X-100 with 10 mM Na phosphate buffer at pH 7.4. The peak of toxin binding activity is

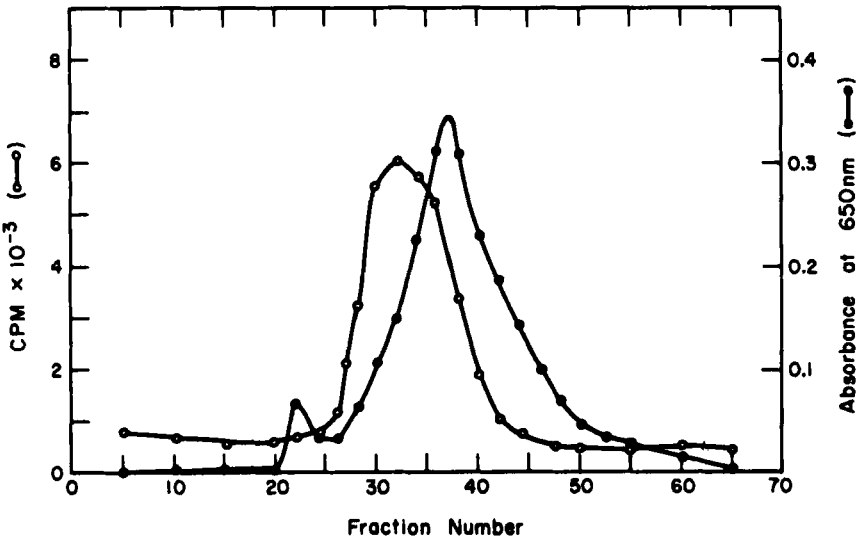


FIG. 3.

Sepharose 6B chromatography (5 X 80 cm column) of Triton X-100 extract. Eluting solvent was 10 mM Na phosphate pH 7.4, 0.1% Triton. Fractions of 20 ml were collected and analyzed for toxin-binding activity (o—o) and protein (●—●).

partially separated from the bulk of the detergent-solubilized proteins. The separation is not sufficient, however, to use gel filtration in a large-scale isolation procedure. Isoelectric focusing of detergent extracts was also performed with the results presented in Fig. 4 [18]. It was observed that the toxin-receptor complex has a pI of about 5 and that there was some separation of the toxin-binding material from the bulk of the other detergent solubilized protein. However, at its isoelectric point the AcChR tends to aggregate and denature, which limits the usefulness of this technique. Similarly, sucrose density gradient centrifugation does not provide a clean separation [18].

In addition to the structural information derived from these studies, the experiments with conventional biochemical techniques showed clearly that a different approach must be used if significant progress was to be made in the purification of the AcChR. Because many of the membrane bound proteins in the Torpedo electro-

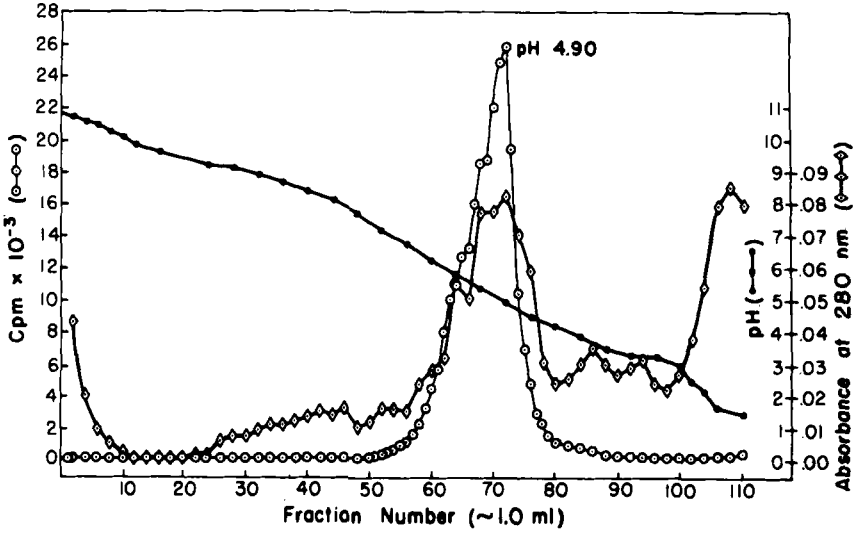


FIG. 4.

Isoelectric focusing of [^{125}I]-α-BuTx-receptor complex after Sepharose 6B chromatography (Fig. 3).

plax solubilized by Triton have similar molecular weights, isoelectric points, and hydrodynamic properties, one must use a technique which depends on some specific properties of the receptor molecule rather than its gross properties. Hence affinity chromatography was considered and because there were many different ligands which show affinity for the receptor, and which could be coupled to an insoluble support, there was a reasonable chance for success.

AFFINITY CHROMATOGRAPHY OF THE ACETYLCHOLINE RECEPTOR

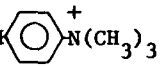
Since all cholinergic ligands have quaternary nitrogen groups, it was thought that DEAE-cellulose or QAE-Sephadex might be useful as affinity chromatography resins [19]. It became readily apparent that little significant prufication could be achieved due mainly to the ion-exchange properties possessed by these resins, the concentration of functional groups on these resins being about 10^{-1} M. At much lower ligand concentrations, around 10^{-2} M, other resins which contained as an affinity ligand the phenyltrimethylammonium moiety, similar to that used by Berman and Young [20] and Dudai et al. [21] for the purification of the AcChE, were tested. These showed considerably more promise but sill retained a substantial amount of nonspecific protein which was eluted with the receptor by a sodium chloride gradient.

The affinity resin which has been most useful in the purification of the AcChR is resin A (Table 1). The ligand was synthesized [19] and coupled to Sepharose according to Cuatrecasas' procedure for the coupling of amines to agarose [4]. A series of resins with varying concentrations of ligand were made and tested for their ability to selectively adsorb the AcChR from the detergent extract and, upon elution, yield purified material. At ligand concentrations above 4×10^{-4} M, the resins displayed typical ion-exchange properties; in addition, only a small amount of the receptor could be eluted with low concentrations of cholinergic ligands. At concentrations below 4×10^{-4} M the capacity of the resin was too low to allow use as a feasible purification step. The resin

Table 1. Affinity Resins

A. Sepharose 2B - $NH(CH_2)_5CONH(CH_2)_3\overset{+}{N}(CH_3)_3$

B. Sepharose 2B - $NH(CH_2)_2NHCO(CH_2)_2NH(CH_2)_2NHCO$  $NHCO(CH_2)_5NHCOOCH_2CH_2\overset{+}{N}(CH_3)_3$

C. Sepharose 2B - $NH(CH_2)_6NHCO(CH_2)_2\overset{O}{\overset{\|}{C}}NH(CH_2)_6NHCO(CH_2)_2CONH(CH_2)_5CONH$  $\overset{+}{N}(CH_3)_3$

D. Sepharose 2B - $NH(CH_2)_2NHCO(CH_2)_2CONH(CH_2)_2NHCO$  N=N-Tubocurarine

E. Sepharose 2B-Cobrotoxin

with 4×10^{-4} M ligand concentration was chosen as the best compromise between the observed ion-exchange effects at high concentration and the low capacity at low concentrations. The experimental conditions were optimized so as to yield a preparation with reasonable specific activities. Elution of the receptor was achieved by a sodium chloride gradient from 0 to 0.1 M NaCl in a buffer of 10 mM Na phosphate, 0.1% Triton, pH 7.4. This affinity chromatography step represented 10- to 20-fold purification over the detergent extracts. A large column, 5 X 80 cm, containing 2 liters of resin was prepared and has been routinely used for 2 years with no noticeable deterioration or change of its chromatographic properties. This column has been incorporated into a semiautomatic system for large-scale purification of the AcChR [17] employing an LKB Ultragrad gradient mixer (model 1300). The gradient mixer automatically controls the application of the detergent extract to the affinity column, washes the column with starting buffer (10 mM Na phosphate, 0.1% Triton, pH 7.4) until the nonspecific proteins are eluted, elutes the AcChR with a salt gradient, elutes the AcChE and other tight-binding proteins with a 1 M NaCl buffer wash, and reequilibrates the column with starting buffer. One complete cycle requires 24 hr. In addition, because of the highly reproducible performance of the affinity column when controlled by the gradient maker system, only the column effluent which contains the AcChR, and sometimes the AcChE, is routinely collected, the rest being shunted into a waste container. The total elapsed time from the homogenization of the organ to the elution of up to 100mg of purified receptor from the column is less than 30 hr. A typical profile of a chromatographic run with the automatic system is shown in Fig. 5.

A clean separation of receptor activity, as judged by toxin binding, from the bulk of the nonspecific protein, as well as from the AcChE is achieved. The purification procedure is summarized in Table 2. Several modifications to the originally published procedure [19], namely the inclusion of EDTA and the proteolytic enzyme inhibitor PMSF, and shorter extraction times, resulted in a substantial in-

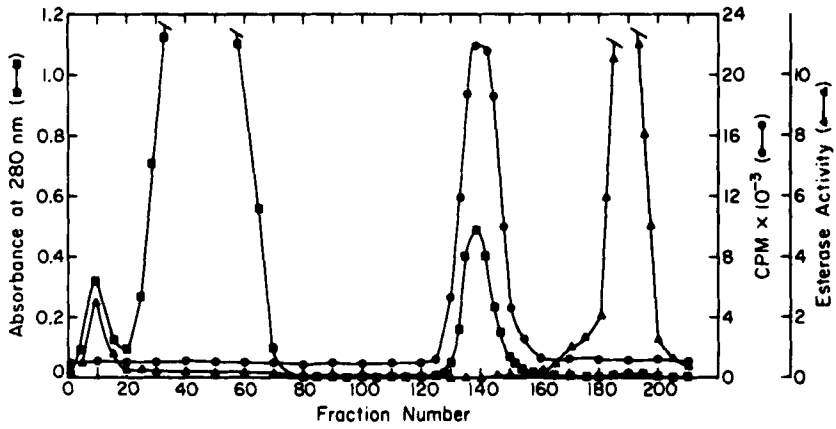


FIG. 5.

Large-scale purification of AcChR. The Triton X-100 extract was applied to the column and elution controlled by LKB Ultragrad gradient mixer. Fractions of 25 ml were collected and analyzed for toxin-binding activity (o—o), protein (●—●), and AcChE activity (▲—▲).

crease in the specific activity at all stages of the purification procedure. The yields in Table 2 would be even greater after affinity chromatography if the whole of the activity peak were pooled.

In practice, elution of AcChR from an affinity column should be possible with low concentrations of cholinergic ligands. It was somewhat surprising, then, that low concentrations of NaCl (about 50 mM) would elute the receptor in high purity. This observation led to the eventual discovery that ligand binding to AcChR was sensitive to the concentration and charge of cations [22], the inhibitory effect being greater than that due to ionic strength or electrostatic interactions between oppositely charged species. The inhibition constant for monovalent ions has been estimated to be about 6 to 8 mM and for divalent ions about 0.4 to 0.6 mM.

Ligand elution experiments with carbamylcholine, decamethonium, and hexamethonium with this affinity resin have been attempted. It was found that AcChR elution by cholinergic ligand could only be achieved at high ligand concentrations, those concentrations at which the ionic strength was roughly equivalent to 30 mM NaCl.

Table 2. Purification Table - AcChR

	Protein (mg)	Activity (nmole)	Specific activity (nmole/mg)	Purification fold	Recovery (%)
Homogenate	18,600	1,300	0.07	-	100
Membrane suspension	3,250	1,300	0.40	5.7	100
Triton extract	1,350	1,300	0.96	13.7	100
Affinity pool resin A	95	820	8.63	125	63
Affinity pool resin B	73	730	10.00	143	56

Thus it was suspected that instead of an affinity column in the strict sense of the word, this column was acting as a highly efficient and selective ion-exchange column. To test this hypothesis several additional experiments were conducted on a small scale and their results are shown in Fig. 6. A detergent extract was heated for a period of time known to destroy all toxin and small ligand-binding to the AcChR. The extract was applied to the column and the receptor protein eluted (Fig. 6b) in the same way as the control (Fig. 6a). The protein profile was nearly identical for the two runs, but the heat-treated sample had no binding activity. An additional experiment was conducted with an extract which had been saturated with radioactive toxin. Some of the activity was not retained by the column but some was eluted at the usual salt concentration as above. This experiment was not as definitive as the former, since the toxin (pI of 9.5) would tend

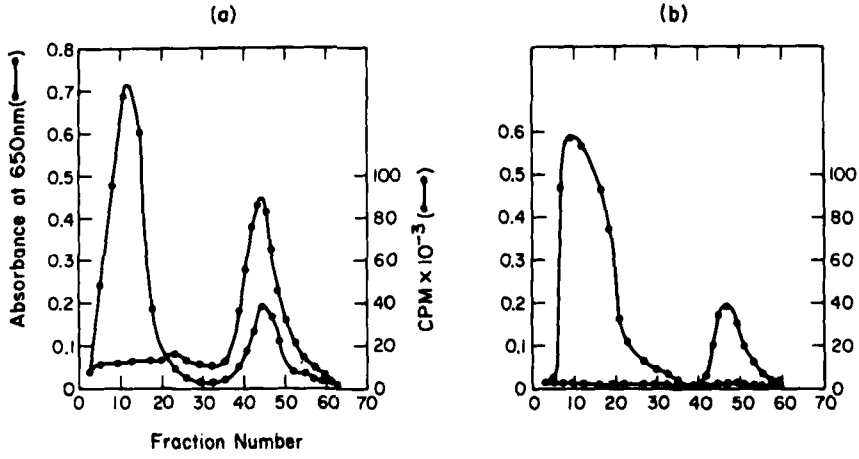


FIG. 6.

(a) Affinity chromatography of detergent extracts on Resin A. After application of the extract to the column equilibrated in 10 mM Na phosphate, 0.1% Triton, pH 7.4, the column was washed with the same buffer before a linear gradient of 0 to 0.1 M NaCl in buffer was begun. Fractions of 8 ml were collected and analyzed for toxin-binding activity (o—o) and protein (●—●). (b) Affinity chromatography of detergent extracts after heat treatment.

to change the ionic characteristics of the receptor (pI of 4.9). However, these experiments showed that the interaction of the affinity ligand at the active site of the receptor is not the only variable which is important in this affinity chromatographic procedure.

One possible rationale for the above behavior concerns the length of the "leash" or spacer arm which separates the affinity ligand from the agarose support. Cuatrecasas et al. [23] have demonstrated the relative ineffectiveness of Sepharose-bound D-tryptophan methyl ester as compared to the ε-aminocaproyl-D-tryptophan ligand for the purification of α-chymotrypsin. The importance of the arm extension is even more dramatic for systems which involve ligand-protein interactions of low affinity [4]. Hopff et al. [24] found that their spacer had to be at least the equivalent length of 30 carbon atoms for adequate affinity chromatography of the AcChE on a trimethylammonium ligand. When the spacer was shorter than this, the enzyme could be eluted with buffers of sufficient ionic strength. The importance of an adequate spacer length cannot be overemphasized. It would logically follow that for resin A, the ε-aminocaproic spacer is insufficient for real affinity chromatography of AcChR to occur. However, in other experiments to be described presently, the AcChR can still be eluted with NaCl even with very long spacer arms. Hence the observed effect of cations on the affinity of cholinergic ligands [22, 25] is still an important consideration in this system.

After affinity chromatography on resin A, further purification can sometimes be achieved. The AcChR protein obtained from resin A usually has the highest specific activity that has been routinely obtained (9 to 10 nmole α-BuTx/mg AcChR) and further purification steps are unnecessary. Occasionally, however, the specific activity of the pool is lower, about 8 nmoles α-BuTx/mg. Chromatography on Sepharose 6B, DEAE-cellulose, or hydroxylapatite will yield AcChR protein of higher specific activity. The difference in specific activities is apparently due to inactivated receptor molecules rather than to other nonspecific proteins. This conclusion

was reached after numerous studies of the SDS gel electrophoresis patterns of preparations of high and low specific activities. (A more complete description of the SDS electrophoresis studies follows.) This is consistent with the observation that resin A retains inactive receptor produced artificially by heat treatment. This occasional variability in quality is apparently related to the condition or state of the organ when used, since the homogenation and purification procedures are constant from preparation to preparation.

It was desired to have an affinity resin which would separate active from inactive receptor molecules; resins B and C were synthesized and found to do just that. Resin B is a carbamylcholine analog linked to Sepharose by a long spacer arm at a concentration of 0.3 μ mole ligand/ml of packed resin. The pooled material from resin A is filtered through resin B followed by a short wash with buffer. The AcChR can be eluted with a buffer containing 1 mM succinylcholine. After dialysis to remove the succinylcholine, the specific activity of the receptor protein is consistently the highest we have ever obtained, about 10 nmole α-BuTx/mg protein. This corresponds to about one toxin molecule per 100,000 daltons of receptor protein.

Resin B has been used successfully to purify receptor from detergent extracts also. During initial experiments with the resin it was observed that the capacity of the resin was decreasing as a function of use. The cause of the diminishing capacity was, apparently, due to the hydrolysis of the carbamylcholine analog off the spacer arm by the acetylcholine esterase, which is known to hydrolyze carbamylcholine slowly [26] and is present in significant amounts in the detergent extracts. This problem could be effectively circumvented by inhibiting the esterase with DFP or other organophosphate inhibitors.

Resin C is composed of a phenyltrimethylammonium ligand used by Dudai et al. [21] for AcChE purification attached to a long arm at a concentration of 0.1 μmole/ml resin. This resin has a sub-

stantially lower capacity than resin B, but is not subject to the difficulties discussed above for resin B. Elution of the receptor is accomplished by a shallow gradient from 0 to 5 mM of succinylcholine with an equivalent purification as with resin B. The separation of esterase from receptor under these conditions is not as complete as observed with resin A; this resin was therefore not routinely used for receptor purification from detergent extracts but only for rechromatography after the esterase had been removed by resin A.

Even though resins B and C display desired affinity chromatography characteristics, such as elution of the receptor by cholinergic ligands at low concentrations and essentially quantitative recovery of toxin activity, sufficient concentrations of NaCl will also elute the receptor with the same yield and purity as succinylcholine, especially when the resins are used in rechromatography of the affinity pool from resin A. In contrast to the observed behavior of the esterase on affinity resins with different spacer arm lengths [24], the use of long spacer arms did not prevent the elution of the receptor with salt.

During the course of these studies, other affinity resins were prepared and tested. d-Tubocurarine was attached to a spacer arm by a diazo linkage [27] at a concentration of 0.2 μmole/ml resin. This resin was highly specific and showed great affinity for the receptor. The capacity of the resin was limited; only about 10% of the total amount of d-tubocurarine coupled to the resin was accessible to bind receptor. Only small amounts of active receptor (10 to 20% of the total amount bound) could be eluted with molar concentrations of cholinergic ligands, NaCl, or with millimolar concentrations of α-BuTx. Additional receptor in higher yields was obtained by reductive cleavage of the diazo bond with 0.1 M sodium dithionite solutions in a 0.2 M borate buffer (pH 9.0). Extensive dialysis was required to remove the d-tubocurarine before further studies were conducted on this material. An obvious drawback to the use of this ligand is the requirement to synthesize a new affinity resin before each use and the additional steps involved.

Because of the great specificity of the neurotoxins for the AcChR, one of the first attempts at affinity chromatography of the receptor utilized a resin (Resin E, Table 1) with the α-toxin from the cobra snake Naja naja atra coupled directly to Sepharose [28]. Initial experiments using α-BuTx to desorb the receptor resulted in the isolation and characterization of α-BuTx-receptor complexes. A high degree of purification with modest yields of 20 to 40% of the total adsorbed receptor activity can be obtained with this resin. Elution of the receptor could also be achieved by high concentrations of cholinergic ligands but not by sodium chloride. As observed with resin D, only about 10% of the total cobrotoxin coupled to the Sepharose is available to bind receptor.

Affinity resins containing other species of toxins have been used to isolate AcChR from the eel Electrophorus electricus as well [29-34]. In each case, high concentrations of ligands and/or elaborate procedures were used to desorb the receptor, with yields ranging from 10 to 50% of the adsorbed activity. The use of high concentrations of ligands to elute the toxin resins is accompanied by several possible disadvantages:

1. Extensive dialysis or other methods are required to remove the ligands from the receptor solutions.

2. High concentrations of acetylcholine and other ligands cause a phenomenon termed desensitization or receptor inactivation [35], a conversion of AcChR to an inactive form. The structural modifications of the receptor which accompany desensitization are unknown. This might be the origin of the observation by Eldefrawi and Eldefrawi [33] that AcChR desorbed by benzoquinonium will bind toxins but not small ligands.

3. Lindstrom observed that high concentrations of decamethonium (greater than 10^{-2} M) irreversibly denatured the receptor due to the high ionic strength [31]. Also, toxin-receptor complexes were eluted from the affinity column using benzoquinonium as elutant [36]. Apparently the toxin or a toxin-agarose piece is removed from the affinity resin during elution with high concentration of

ligands. Such a resin would have to be regenerated after usage and does not yield a satisfactory product.

Although affinity chromatography utilizing toxin resins would theoretically yield the purest receptor material because of the high specificity of toxin for receptor, in practice such chromatographic procedures are relatively less satisfying than affinity resins utilizing readily reversible ligands. Not only are the yields generally greater and the conditions milder with reversible ligands, but the ease with which large quantities of receptor can be obtained for subsequent biochemical studies is an important design variable to be optimized for affinity chromatography of the AcChR.

CHARACTERIZATION OF THE ACETYLCHOLINE RECEPTOR

Structural Characteristics

The conventional biochemical techniques gel filtration, isoelectric focusing, and sucrose density gradient centrifugation, while not being useful as purification steps, did nevertheless yield important information about the structural properties of the receptor. On gel filtration, the receptor-toxin complex migrates with an apparent Stokes radius somewhat less than that of β-galactosidase (MW ≈ 540,000) [18]. On sucrose density gradients, the S value obtained for the complex suggests a molecular weight somewhat less than catalase (MW = 250,000). This dichotomy between molecular weights determined by two different methods has also been observed for the AcChR solubilized by detergents from the electric eel (Refs. 37 and 38, and for AcChE, Ref. 39). Such differences can be due to a variety of causes: differential amounts of detergent bound to the receptor and to the standard proteins (all of which are water-soluble), a shape radically different from a globular shape assumed for the standard proteins, unusual frictional coefficients, or other artifacts inherent in these experimental methods. There is preliminary evidence from electron microscopy

and x-ray diffraction studies that the AcChR molecule is somewhat rod-shaped, but the deviation from globular shape would not be sufficient to account for the substantial differences between the apparent molecular weights determined by gel filtration and sucrose density gradients [40]. Another possible explanation is that there is a large amount of detergent bound to the receptor as compared to the standard proteins. It is known that about 100 molecules of Triton X-100 are bound for every 40,000 daltons of AcChR (D. Michaelson and M. A. Raftery, unpublished observations), while bovine serum albumin, a typical hydrophilic protein frequently used as a molecular weight standard, has four high affinity sites for Triton per molecule of 68,000 daltons [41]. The large amount of detergent bound to the receptor could drastically alter the apparent density and possibly the overall shape of the receptor molecule and give rise to anomalous molecular weights.

The question of purity of the AcChR eluted from affinity columns is still under investigation. Detergent-solubilized receptor of the highest specific activity exhibits a single sharp protein band on acrylamide gels run under nondenaturing conditions. A single band is also observed on gel filtration columns or on sucrose density gradients. There is, however, considerable discussion as to the subunit composition of the AcChR. When acrylamide gels are run in the presence of SDS [42] the most highly purified material exhibits a complex gel pattern (Fig. 7). There is a major component with an apparent molecular weight of about 40,000 daltons and other components with approximate molecular weights of 50,000, 60,000 and 65,000 daltons which cannot be eliminated by further purification steps. These subunits are in an approximate ratio of 4:1:1:1, respectively, which suggests a molecular weight for the AcChR complex of around 350,000 daltons. The relation of the minor subunits to the major subunit is unknown at present. The AcChR has two functions which might require a complex subunit structure, first, to bind acetylcholine and then, second, to open a gate or ionophore to allow ions to traverse the postsynaptic membrane and

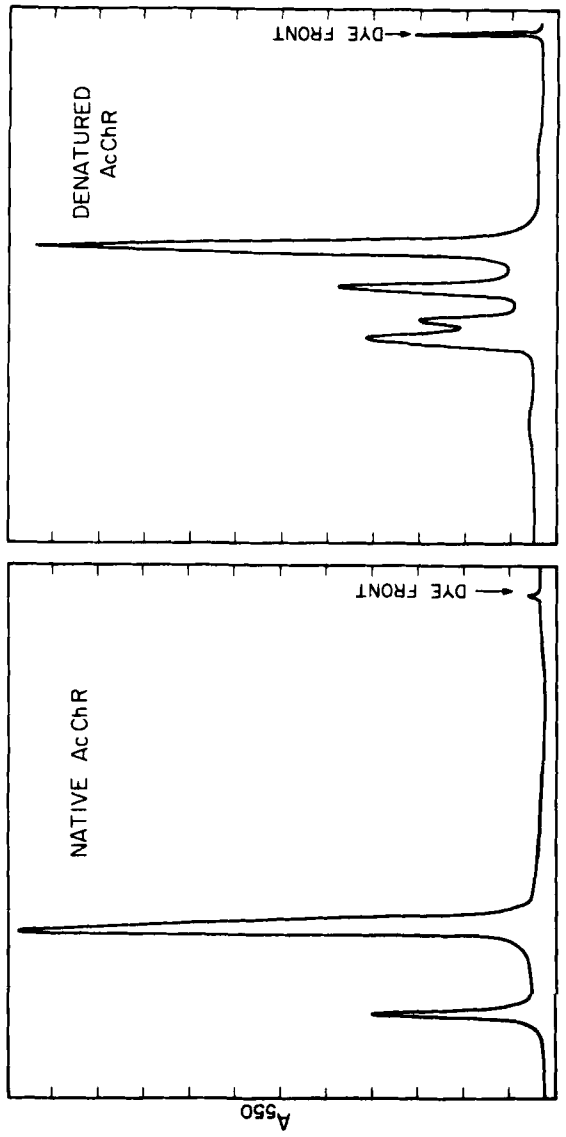


FIG. 7.

Gel scan analysis of purified AcChR. Left: Protein (10 μg) was electrophoresed in 4% acrylamide gels with 0.1% Na cholate and stained for protein [42]. The smaller peak to the left of the major peak corresponds to a dimer of the AcChR. Right: Same protein material (50 μg) was electrophoresed in 1% SDS acrylamide gels using the method of Fairbanks et al. [42]. The molecular weights of the major protein bands are indicated.

cause depolarization. Different subunits might be required for the binding of acetylcholine and for the opening of the ionophore. Toxin binding is a convenient assay for the binding component but there is no assay for the ionophore, if it even exists as a separate entity. It is known that the 40,000 subunit carries the ligand binding site [43]; the other subunits might be necessary for the organization of receptor molecules in the postsynaptic membrane.

Alternatively, the complex structure may be an artifact of detergent extraction. It is possible that during solubilization discrete membrane proteins may associate together to shield hydrophobic areas on the proteins from the aqueous solvent. Hence such aggregates would migrate as a single species under nondissociating conditions but would show a complex SDS gel electrophoresis pattern. While there is no direct evidence that would support this possibility, it should not be dismissed until more is known about the structure and function of membrane proteins.

One other factor which must be reconciled when a description of the subunit composition of the AcChR is made is the observation that even in the purest preparations to date, the ratio of the toxin sites to receptor is about one toxin per 100,000 daltons of receptor protein. Whether this means that more than one subunit is required to bind toxin or that the receptor is still only partially purified is open to debate and is under extensive study at the current time.

In spite of the open questions concerning receptor homogeneity and the subunit composition of the receptor, meaningful physical and chemical studies have been performed. The AcChR from Torpedo californica has been shown to carry carbohydrate moieties. When both SDS gels and nondenaturing gels of the purified receptor are stained for carbohydrate by the periodic acid-Schiff base procedure [42], all of the detectable polypeptides are found to contain sugar moieties [44]. Proteins which are known not to have carbohydrate do not stain by this procedure. The neutral sugar content of the receptor, as measured by the phenolsulfuric method

[45], with appropriate controls for detergent interference, is about 3%. The major neutral sugars are mannose, galactose and glucose in the approximate ratio of 7:2:1, respectively. In addition, 3 to 4 moles of N-acetylglucosamine per 40,000 daltons of protein have been detected in amino acid hydrolysates of the purified receptor. Purified AcChR can be completely adsorbed to affinity resins of Sepharose concavalin A or can be precipitated by concavalin A as observed by Meunier et al. [46]. The distribution of neutral sugars and the positive staining for carbohydrate on SDS gels rules out the possibility that the carbohydrate arises from a degradation of the Sepharose columns used during affinity chromatography [33, 36].

Functional Characteristics of AcChR

The functionality of the purified receptor material with regards to its binding of cholinergic ligands has been probed by direct binding methods employing radioactive ligands and equilibrium dialysis [47], by the inhibition of the rate of formation of toxin receptor complexes, and by the fluorescent probe DAP [25]. These studies have shown that the toxin-binding material purified by affinity chromatography binds cholinergic ligands with high affinity in a noncooperative fashion. Table 3 lists the ligand binding parameters obtained by these different techniques. Equilibrium dialysis and the fluorescence studies allow quantitation of the number of ligand binding sites; it was observed that there are nearly twice as many sites for toxin as for small ligands, or one small ligand per 200,000 daltons of AcChR. Similar findings have been reported for the receptor from eel [48] and for purified membrane fragments from Torpedo californica [49]. Hence the "half-site" reactivity is not an artifact due to solubilization of the receptor with detergents. Recent experiments have shown that there are two classes of ligand binding sites on the receptor molecule. One half of the toxin sites bind ligands with higher affinity, while the other half bind ligands with lower affinity, usually

TABLE 3. Ligand Binding Parameters of AcChR

Ligand	Equilibrium dialysis, a K_d (M)	Fluorescence, b K_d (M)
Acetylcholine	2.3×10^{-6}	5×10^{-6}
d-Tubocurarine	6.4×10^{-6}	5.2×10^{-7}
Decamethonium	55×10^{-6}	3.5×10^{-6}
Carbamylcholine		10.3×10^{-5}
DAP		1.3×10^{-7}
Na^+		11×10^{-3}
Ca^{2+}		2×10^{-4}

a Measured by direct binding of radioactive ligands in 200 mM NaCl, 10 mM Na phosphate, pH 7.4, 0.1% Triton X-100 [47].

b Measured by competitive inhibition of DAP binding in 15 mM NaCl, 10 mM Na phosphate, pH 7.4, 0.1% Triton X-100 [25].

by a factor of 5 to 25 fold. Toxin-inhibition studies have shown that cholinergic ligands slow the rate of complex formation; that concentration of ligand which slows the initial rate of complex formation by 50% is termed its protection constant, Kp. These protection constants are not true dissociation constants but do agree reasonably well with corresponding dissociation constants (Table 4).

TABLE 4. Protection Constants Determined by Toxin Inhibition

Ligand	K_p
Acetylcholine	2.5×10^{-6}
Carbamylcholine	7.7×10^{-5}
Nicotine	8.0×10^{-5}
Decamethonium	2.2×10^{-6}
Hexamethonium	2×10^{-6}
d-Tubocurarine	1.2×10^{-7}
Flaxedil	2×10^{-7}
Tetramethylammonium	9×10^{-4}
Tetraethylammonium	6.5×10^{-4}
Phenyltrimethylammonium	8×10^{-5}
Acetylthiocholine	1.6×10^{-4}
Choline	1.7×10^{-3}
α-Bungarotoxin	$<10^{-9}$

A comparison of the data from equilibrium dialysis obtained at a NaCl concentration of 200 mM and that from the fluorescence studies conducted at 15 mM NaCl shows a large difference for the apparent dissociation constants for decamethonium and d-tubocurarine. These discrepancies are due to the inhibitory effect of salt on the affinity of some ligands. The affinities of other ligands, especially acetylcholine and carbamylcholine, are not appreciably affected by salt. In general, the salt inhibition is greatest for those cholinergic ligands with two quaternary nitrogen functions and least for the monofunctional ligands. The apparent inhibition constants for sodium and calcium ions are given in Table 3 and are similar for both the inhibition of DAP and for the inhibition of

the rate of toxin binding. These effects are also observed with the purified membrane fragments [49].

The inhibition of the rate of formation of the toxin-receptor complex provides a simple, specific method for estimating apparent inhibition constants of ligands for the AcChR. The method employs the disk assay [50] and measures the amount of radioactive toxin-receptor complex formed as a function of the ligand concentration. Table 4 lists the measured protection constants for a variety of cholinergic and noncholinergic ligands.

CHEMICAL MODIFICATION STUDIES

In an attempt to determine some of the functional characteristics of the purified AcChR, various chemical modification studies have been conducted. The effect of a change in pH on the binding of DAP by fluorescence measurements [25] revealed that the dissociation constant for DAP decreased from pH 5.9 to pH 7.0 and remained constant at values above pH 7.0. Conversely, equilibrium dialysis measurements tend to indicate that the number of sites for small ligands changes with pH [51]. At pH 5 or lower there is essentially no ligand binding with a gradual increase in the number of sites relative to toxin. At pH 7.4, there are an equal number of high and low affinity sites for small ligands. As the pH was increased, there was a conversion of low affinity sites into high affinity sites so that at pH 9 there were an equal number of high affinity sites as toxin sites. From these studies it appears that there may be a group with a pK about 6 to 7 which affects small ligand binding. The rate and extent of binding of radioactive toxin to AcChR was also investigated as a function of pH [22] (see Fig. 8). The results indicate that protonation of an ionizable group with a pK of about 6 inhibits binding. Inhibition is also seen at higher pH values and may be due to denaturation of the receptor or to effects on the toxin.

Amino acid composition studies on purified material indicated a small number of sulfur-containing amino acids. Quantitation of sulfhydryl content with DTNB revealed the presence of one or two

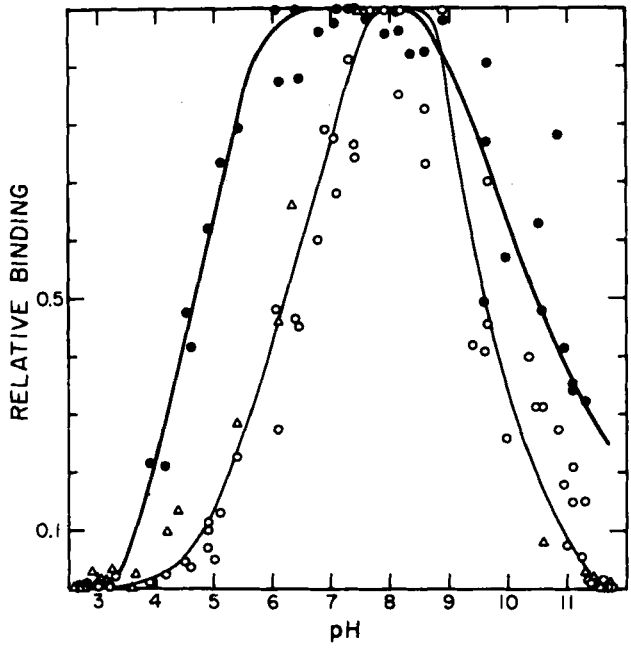


FIG. 8.

Effect of the pH on the rate and extent of binding of α-BuTx to purified AcChR. Receptor was incubated with a mixed citrate-acetate-phosphate buffer of constant sodium concentration and complex formation was started by the addition of toxin. Aliquots of 0.1 ml were withdrawn after 0.2 min (o) and 30 min (•) as described elsewhere [22].

sulfhydryl groups per 40,000 daltons of AcChR protein [52]. Treatment of the protein with β-mercaptoethanol or dithiothreitol to reduce any disulfide bonds, with subsequent removal of these reagents under nonoxidizing conditions, revealed two additional sulfhydryl groups. No additional sulfhydryl groups are found when the reactions are performed under protein denaturing conditions. Reaction of the free sulfhydryl group with ρ-mercuribenzoate or iodoacetamide has no effect on toxin or small ligand binding to the receptor.

Likewise, reduction of the disulfide bond does not affect toxin binding appreciably. Subsequent modification of the result-

ing sulfhydryl groups with the specific affinity label, 4-(N-maleimido-benzyltrimethylammonium iodide, first used by Karlin et al. [53], partially reduces toxin binding. Additionally, the specific incorporation of ^{3}H-MBTA at the reduced disulfide can be prevented by preincubation with toxin. These results generally agree with those published by Karlin and Cowburn for the AcChR from the eel [54]. Physiologically this disulfide bond seems to play an important structural role; reduction of the disulfide causes substantial changes in the electrophysiological response of the receptor to cholinergic ligands [55]. No effect is seen on the SDS gel patterns whether this disulfide bridge is reduced or not.

Other chemical modification studies have centered around the use of the highly reactive methylating agent, trimethyloxonium ion ($(CH_3)_3O^+$). This reagent, or the triethyl derivative, has been used to selectively label specific carboxyl groups in lysozyme [56] and has been shown to inactivate AcChE [57] (Fig. 9, top). Structurally similar to the trimethylammonium group common to all cholinergic ligands, trimethyloxonium ion (TMO) has been used to probe the active site of the AcChR [58].

Incubation of AcChR solutions with millimolar concentrations of trimethyloxonium tetrafluoroborate produces little or no change in the binding of toxin to the receptor. However, the effect on the binding of decamethonium is substantial. Reaction of the receptor with increasing amounts of TMO, as depicted in Fig. 9 (bottom), results in a progressive decrease in the amount of activity as determined by decamethonium or DAP binding. Complete inhibition of the esterase occurs at substantially lower concentrations of TMO than for the receptor. No appreciable effect on the binding of acetylcholine is seen at concentrations of TMO at which decamethonium binding is substantially decreased. Furthermore, the inhibition of decamethonium binding by TMO is antagonized by high concentrations of cations or by decamethonium. If it is assumed that there are two negative subsites in the active site, one each to bind the positive quaternary nitrogen groups on bisonium ligands,

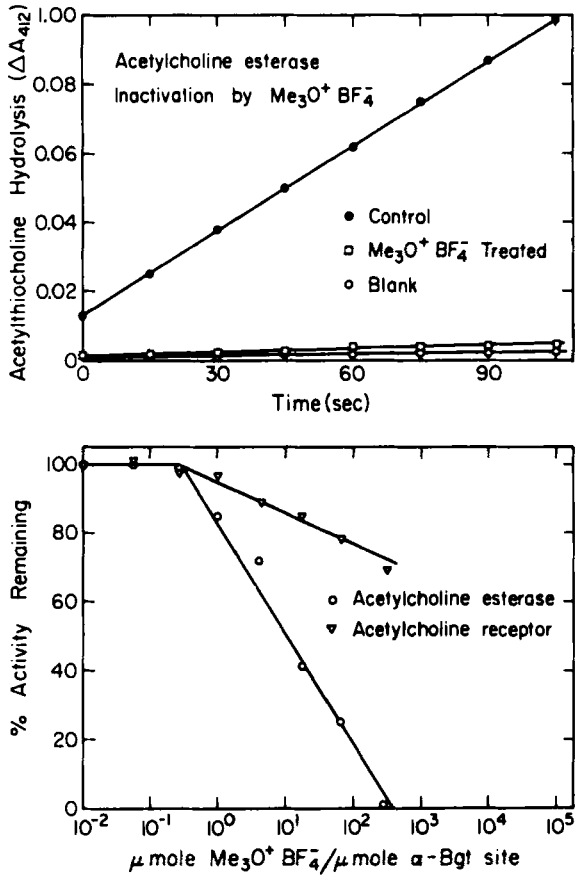


FIG. 9.

Inhibition of AcChR and AcChE by trimethyloxonium ion. Top: Inactivation of AcChE by TMO [58]. Esterase activity measured by method of Ellman et al. [62]. Bottom: Inactivation of decamethonium binding to AcChR and of acetylthiocholine hydrolysis by AcChE as a function of TMO concentration.

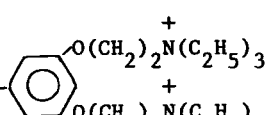
it appears that TMO reacts with a group (probably a carboxyl group) near the second negative subsite in a manner similar to that observed for cations and not at the subsite which binds the acetylcholine quaternary nitrogen atom. Those ligands whose affinities are affected by cations (except toxin) are especially affected by TMO.

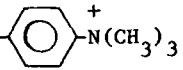
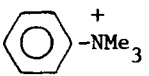
Other studies are underway which should further characterize the active site of the AcChR.

OTHER USEFUL AFFINITY LIGANDS FOR AcChR PURIFICATION

An article on the affinity chromatography of the acetylcholine receptor would be incomplete without a description of some of the other affinity ligands which have been used in the purification of AcChR from various sources. A summary of the various ligands used, eluting conditions, the specific activity of eluted material, and subunit molecular weights as determined by SDS gel electrophoresis is given in Table 5. (See also Refs. 8 and 43.) Immobilization of cholinergic ligand analogs containing quaternary ammonium functions have been used for routine AcChR purification by Raftery et al. [17], Changeux et al. [61], Karlin and Cowburn [54], and Biesecker [34], while neurotoxin affinity ligands have been exploited by Changeux et al. [47], Reich et al. [32], Eldefrawi and Eldefrawi [33], Lindstrom and Patrick [31], and Heilbronn et al. [30, 59]. The best preparations have specific activities corresponding to one toxin per 100,000 to 150,000 daltons of AcChR protein. Regardless of the apparent purity of the preparation, two or more bands are usually observed with SDS gel electrophoresis studies except as noted in Table 5. While affinity chromatography techniques have resulted in substantial purification of the receptor from detergent extracts and give rise to similar molecular species, the rather substantial differences in specific activities among the various groups indicate that there are other parameters which must be considered. The complex SDS patterns have prevented a rigorous definition of purity or even a description of the subunit composition although it is apparent that one main component has a molecular weight somewhere around 40,000 to 45,000 daltons. Even though many molecular properties of the AcChR are still to be conclusively established, important and substantial information has been gained concerning the function of the acetylcholine receptor in synaptic transmission.

Table 5. Affinity Chromatography of AcChR

Source	Affinity Ligand	Eluant	Activity[a]	Subunits[b]	Ref.
T. californica	|-spacer-NH$(CH_2)_3\overset{+}{N}(CH_3)_3$	0 to 0.1 M NaCl gradient	100 c	40,50 60,65	
T. marmorata	|-acetylated naja naja siamensis toxin	0 to 1 M carbamylcholine gradient	430	2 bands 45 to 50	59
	|-naja naja siamensis toxin	1 M carbamylcholine	83 d	-	33
	|-spacer-NH- 	0 to 0.1 M flaxedil gradient	165	41.5	60
E. electricus	"		145	45,54	46,61

|-naja naja siamensis toxin	50 mM hexamethonium	90	50[e]	32
|-spacer-NH-  -$N(CH_3)_3$	1 mM decamethonium	220	44,50	34
|-naja naja toxin	1 mM benzoquinonium	133	42,54	31
|-OC-  -NMe_3	50 mM carbamylcholine	250[f]	40,47 53	54

[a]Units are 10^3 daltons of AcChR/toxin site.

[b]Subunit molecular weight (10^3 daltons).

[c]The same specific activity can be obtained from affinity resins B or C (Table 1) with elution by succinylcholine gradients.

[d]Reported as 10^3 daltons/AcCh site.

[e]Suggested by "the integral ratios of amino acid residues derived from the histidine content" [32].

[f]Assay was by affinity labeling with [^{3}H]-MBTA.

The wealth of information about two components in the synapse, AcChR and AcChE, has been especially advanced because of the relative ease by which these components are separated from other membrane proteins by affinity chromatography. The inherent advantages of affinity chromatography will be extremely useful for future advances in neurochemical research and membrane biochemistry. There are many other components involved in synaptic transmission and axonal conduction, which because of their low concentrations would be difficult to purify by ordinary methods. The structure and function of membrane-bound proteins is not yet well understood. Affinity chromatographic techniques applied to these problems should aid future research on these systems.

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