STUDIES ON THE FEASIBILITY OF ISOLATING THE ACETYL CHOLINE RECEPTOR BY MEANS OF AFFINITY CHROMATOGRAPHY

Thesis by

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ABSTRACT

Feasibility studies were conducted on the isolation of the acetyl choline receptor by means of affinity chromatography. Sepharose resins bearing acetyl choline receptor binding materials of at various distances from the backbone of the resin and of varying degrees of substitution were treated with extracts from electric eel organ, washed free of non-binding materials and the material bound to the column extracted by various means. Extracts from the column were studied for purity, binding to decamethonium and α-bungarotoxin, and presence or absence of acetyl cholinesterase or choline acetylase activity. Although material possessing the proper characteristics was extracted from some of the resins, electrophoretic purity was not obtained.

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INTRODUCTION AND BACKGROUND

The existence of an "acetyl choline receptor" was first postulated by Nachmansohn[1]. According to his theory tne receptor is a protein existing in the post-synaptic membrane which changes conformation upon binding to acetyl choline. This change affects the membrane in some way which causes it to depolarize, thereby initiating some sequence of events which culminates in an action potential.

An isolated single electroplax preparation from electrophorus electricus was developed by Schoffeniels and Nachmansohn[2] to achieve a reliable system for studying the effects of various chemical agents on acetyl choline mediated neural transmission. Using this system they found that the electroplax post-synaptic membrane is depolarized by agents such as acetyl choline, carbamyl choline, decamethonium, and prostigmine; while d-tubocurarine and procaine are among those agents which block depolarization by the former. Such blocking is reversible.

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1 Nachmansohn, D., Chemical and Molecular Basis of Nerve Action (New York: Academic Press, 1959)

2 Schoffeniels, E., Nachmansohn, D., Biochim. Biophys. Acta, 26, 1 (1957)

Evidence indicating[2] that the receptor is probably at least partly proteinaceous has also been obtained using this preparation. Karlin and Bartels[3] found that the depolarization of the electroplax by activators was inhibited by prior treatment with p-chloromercuribenzoate or with 1,4-dithiothreitol. These reagents would be expected to react with protein sulfhydryl groups and disulfide bonds, respectively. Further studies by Karlin and Winnik[4] revealed that the dithiothreitol reduced receptor could be more or less specifically alkylated by 4-(N -maleimido)phenyltrimethylammonium iodide in the presence of N-ethylmaleimide. Another affinity label for the receptor, p-(trimethylammonium)benzenediazonium fluoroborate (Tdf), was developed by Changeux, et. al.[5] also using the single electroplax preparation. The success of this affinity label also lends support to the idea that the receptor is a protein, since the diazonium group tends to form covalent bonds with tyrosine, histidine, and lysine side-chains in proteins.

Studies such as these provide a basis for the

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[3] Karlin, A., Bartels, E., Biochim. Biophys. Acta, 126, 525. (1966)

[4] Karlin, A., Winnik, M., Proc. Natl. Acad. Sci. U.S., 60, 668, (1968)

[5] Changeus, J., Podleski, T., Wofsy, L., Proc. Natl. Acad. Sci. U.S., 58, 2063 (1967)

many of the attempts to isolate the receptor which will be described.

In 1958, before the evidence supporting the protein nature of the receptor had accumulated,[6] Chagas isolated a mucopolysaccharide which equilibrium dialysis studies revealed bound acetyl choline, succinylcholine and gallamine. His claim that this molecule was the receptor was later withdrawn when it was found that the execution of the equilibrium dialyses in distilled water had led to random binding of the positive charge on the quaternary ammonium groups of the ligands to negatively charged groups on the mucopolysaccharide.

A second claim to having isolated the receptor came from Ehrenpreis[7], who extracted the electric organ of Electrophorus electricus with .1 M. phosphate and found that a protein from the 30% ammonium sulfate fraction of the supernatant precipitated with d-tubocurarine. The complex redissolved at pH 9 and was reported to bind d-tubocurarine, procaine, prostigmine, atropine, and eserine in a manner which parallels the

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6 Chagas, C., Penna-Franea, E., Nishie, K., Garcia, E.J., Arch. Biochem. Biophys., 75, 251 (1958)

7 Ehrenpreis, Seymour, Biochim. Biophys. Acta, 44, 561 (1960)

binding of these compounds in electroplax. This claim to the receptor was withdrawn when Beychok[8] pointed out that the claims for parallel binding were questionable since no binding curves had been presented; that many materials precipitated with d-tubocurarine under Ehrenpreis' experimental conditions and the material which redissolved at pH 7.5 bound d-tubocurarine as well as the "receptor" and that the material which redissolved at pH 9, when subjected to electrophoresis yielded several subfractions.

The next entrant onto the stage of receptor isolation, O'Brian[9], was somewhat more modest in his claims, stating only that he had isolated membrane fragments from Torpedo electroplax which contained the receptor. He homogenized the electroplax in .05M. tris, .2 M. sodium chloride in the presence of ^{3}H-muscarone and centrifuged down the membrane fragments. He found most of the radioactivity in the pellet and also noted that the amount of radioactivity in the pellet decreased in the presence of d-tubocurarine, tetraethylammonium, atropine, paraoxon plus acetyl

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8 Beychok, S., Biochemical Pharmacology, 1963, Vol. 14, pp.1249-1255, Pergamon Press Ltd, Great Britain

9 O'Brian, R., Gilmour, L., Proc. Natl. Acad. Sci., 63, 496 (1969)

choline, and high concentrations of eserine. Although it is not unlikely that the observed binding might be due to the acetyl choline receptor, there has been no subsequent report of further purification of the binding material.

A claim that the receptor had been isolated and found to be a nucleoprotein came from Namba and Grob[10]. Their work was unusual in that it used human skeletal muscle rather than electroplax for a source of material. It was observed that a material from saline muscle extract could be precipitated with d-tubocurarine and that the amount of precipitate decreased if acetyl choline, choline, decamethonium, or urea was present in the extract. The material redissolved when the d-tubocurarine was dialysed out and was resolved into one major and two minor components by free boundary electrophoresis and chromatography on diethylaminoethyl-cellulose. The material was found to be a ribonucleoprotein which possessed acetyl cholinesterase activity. This casts doubt on the purity of the major component since acetyl cholinesterase has been purified and not found to be a nucleo-

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[10] Namba, T., Grob, D., Ann. N.Y. Acad. Sci., 144, 772 (1967)

protein[11]. Also Changeux[12] has shown that acetyl cholinesterase activity can be separated from receptor activity, indicating that the two forms of activity are not present in the same molecule. The nonspecificity of d-tubocurarine precipitation has already been discussed and there is no evidence to indicate that interference with such precipitation by choline or urea should be characteristic of the receptor.

Probably the most spectacular claim to receptor isolation came from De Robertis[13,14] who isolated a material from electroplax which decreases the resistance of a lipid bilayer artificial membrane when acetyl choline is added to the solution surrounding the bilayer. This resistance change is blocked by the addition of d-tubocurarine. The material was extracted from electroplax by chloroform-methanol and characterized as a proteolipid. Its purification

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11 Leuzinger, W., Baker, A.L., Proc. Nat. Acad. Sci. U.S. 57,446 (1967)

12 Meunier, J., Huchet, M., Boquet, P., Changeux, J., C. R. Acad. Sc. Paris, 272, 117 (1971)

13 La Torre, J., Lunt, G., De Robertis, E., Proc. Natl. Acad. Sci. U.S., 65, 716 (1970)

14 De Robertis, E., Science, 171, 963 (1971)

by chromatography on Sephadex LH-20 is, however, questionable in the absence of supporting electrophoretic evidence. Although binding of the material to acetyl choline, TDF, and hexamethonium was reported, only acetyl choline and d-tubocurarine were mentioned to affect the resistance of the lipid bilayer. A question which is worthy of study is whether or not TDF and hexamethonium inhibit the effects of acetyl choline as they do in the single cell studies of the receptor; also whether other activators such as carbamyl choline can also induce the observed resistance drop. Although the bilayer study is impressive, it is worth noting that a similar resistance drop upon addition of acetyl choline was observed by del Castillo, et. al.[15] upon addition of acetyl cholinesterase to a lipid bilayer. Also resistance drops in lipid bilayers containing antibodies are observed upon the addition of antigens;[16] thus it is not clear that the observed resistance drop in the bilayer is related to the depolarization of the electroplax by acetyl choline in vivo. It appears, therefore that

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15 del Castillo, J., Rodriguez, A.,Romero, C., Ann. N.Y. Acad. Sci., 144, 803 (1967)

16 del Castillo, J., Rodriguez, A., Romero, C., Sanchez, V., Science, 153, 185 (1966)

considerably more evidence is needed before the proteolipid can be accepted as the receptor.

The observation by Lee and Chang[17] that α-bungarotoxin, a polypeptide purified from the venom of Bungarus multicinctus, combines irreversibly with the cholinergic receptor at the motor endplate has stimulated attempts to isolate the material bearing a radioactive α-bungarotoxin label. The first of these attempts was reported by Changeux, et. al.[18,19] Material for these experiments was obtained by solublizing protein from the membrane fragments resulting from the homogenization of electric organ of Electrophorus electricus in Ringers, pH 8.0 with 1% deoxycholate. After centrifugation, the supernatant was found to bind decamethonium, carbamylcholine, phenyltrimethylammonium, d-tubocurarine, flaxedil, and hexamethonium in a manner which more or less parallels their binding to to an isolated electroplax. The number of receptor sites in the extract was estimated on the basis of binding to α-bungarotoxin to be roughly equivalent to the number of catalytic esterase sites.

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17 Lee, C.Y., Chang, C.C., Mem. Inst. Butantan, Simp. Internac., 33, 555 (1966)

18 Changeux, J., Kasai, M., Huchet, M., Meunier, J., C.R. Acad. Sc. Paris, 270, 2864 (1970)

19 Changeux, J., Kasai, M., Lee, C.Y., Proc. Natl. Acad. Sci. U.S., 67, 1241 (1970)

Although the characteristics of the extract correspond to those which one would expect to find in the receptor, such an extraction without a subsequent purification by chromatography and electrophoresis can hardly qualify as an isolation of the receptor.

The most recent report of receptor isolation is that of Miledi, et. al.[20] Using the electric tissue from Torpedo, homogenized, osmotically shocked membrane fragments were incubated with ^{131}I-α-bungarotoxin, and an ^{131}I labeled material extracted with triton X-100. The toxin-tagged protein was eluted in the void volumn of Sephadex G-200 in the absence of SDS and moved as two peaks having molecular weights of 88,000 and 180,000 in the presence of SDS. It was purified by ultracentrifugation and on the basis of the ratio of μg of toxin bound to μg of protein, the molecular weight of the protein subunit which binds the toxin was estimated to be 80,000. Although d-tubocurarine and carbamylcholine appeared to slow the binding of the toxin to the protein, more precise binding studies carried out on more cholinergic compounds are needed to solidify the protein's status as the receptor. Electrophoretic evidence of purification is also needed.

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[20] Miledi, R., Molinoff, P., Potter, L., Nature, 229, 556, (1971)

In viewing these attempts to isolate the acetyl choline receptor it must be noted that little consideration has been given to the elimination of other molecules which might show binding characteristics similar to those of the receptor. Two other molecules which are almost certain to be present in the extracts used are acetyl cholinesterase and choline acetyl transferase (choline acetylase). Both of these molecules would be expected to bind quaternary ammonium compounds since they bind acetyl choline and choline respectively in vivo. Before any molecule can be believed to be the receptor, it must be purified and shown not two be one of these two.

In the attempts to isolate the receptor by means of affinity chromatography described in the following chapters, the presence or absence of these enzymes in the preparations was monitored and will be reported along with the presence or absence of binding to cholinergic ligands.

CHAPTER 2. APPROACH TO THE PROBLEM

The purpose of this research was to investigate the feasibility of isolating the acetyl choline receptor by means of affinity chromatography. Affinity resins were prepared using techniques described by Cuatrecasas.[21] Affinity chromatography utilizes the specific affinity enzymes or regulatory proteins have for their substrates and inhibitors by covalently attaching such a substrate or inhibitor to a resin such as Sepharose. If an extract containing the desired protein is passed over such a resin, it will adhere to the resin while the other elements of the extract are washed through the resin. It may then be removed by eluting the resin with a solution containing another inhibitor or substrate.

Given the availability of substances possessing the desired affinity for the sought after macromolecule, the success or failure of an affinity resin depends upon at least four factors: (1) the distance of the inhibitor from the backbone of the resin: If the inhibitor is too close to the resin backbone, steric factors may prevent binding between the macromolecule and the inhibitor. (2) The concentration of inhibitor

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[21] Cuatrecasas, P., Jour. Biol. Chem., 245, 3059, (1970)

must be high enough so that the macromolecule will adhere to the resin while other components are being washed off, but low enough so that it can be eluted off with reasonable concentrations of another inhibitor or substrate. (3) specificity of the inhibitor or substrate: The covalently bound molecule must not have an affinity for other molecules present in the extract. (4) Attachment of the inhibitor or substrate to the resin must not destroy its affinity for the macromolecule. The experiments to be reported represent attempts to prepare a resin which satisfies these conditions when the desired macromolecule is the acetyl choline receptor.

Inspiration for these experiments came from a single electrophoretic band obtained by Dr. Michael Raftery[22] upon elution of a sepharose resin bearing an acetyl choline analog with 1% deoxycholate. The resin had been previously eluted with extract of electric organ of Electrophorus electricus, Ringer's solution, and 10^{-2}M. decamethonium in Ringer's, respectively. Lowry assay indicated the presence of about 8 mg. of protein, and the molecular weight according to gel electrophoresis was roughly 60,000.

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[22] Raftery, M., unpublished data, this laboratory.

Before any molecule can be identified as the acetyl choline receptor at least four criteria must be established:

1) It is pure.

2) It is not acetyl cholinesterase.

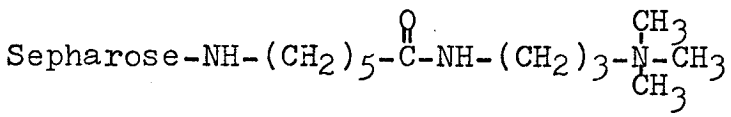
3) It is not choline acetyl transferase.

4) Its binding characteristics parallel those observed in the single electroplax studies. Even these characteristics are not conclusive, but the ultimate criterion, whether or not the molecule induces the appropriate depolarization in post synaptic membranes, is difficult to experimentally determine.

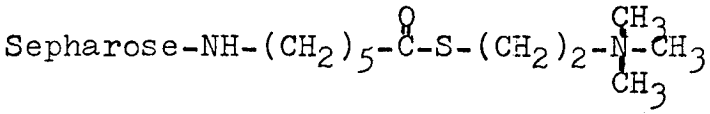
The protein isolated from the aforementioned affinity column had deteriorated before its behavior with respect to the last three criteria could be studied. Consequently the following affinity resins were synthesized to attempt to reisolate and characterize that material:

1) Sepharose-cobra toxin

2) Duplication of the original affinity column:



3) Same as (2) only 10% substitution of ligand

4) 

5) Sepharose-NH-$(CH_2)_6$-NH-C(=O)-$(CH_2)_2$-C(=O)-NH-$(CH_2)_6$-NH-

C(=O)-CH($NH-C(=O)-CH_3$)-$(CH_2)_2$-S-$(CH_2)_2$-C(=O)-O-$(CH_2)_2$-$N^+(CH_3)_3$

The experimental details regarding each resin will be presented in separate chapters.

Materials and Methods

Source of acetyl choline receptor material: The large electric organ of Electrophorus electricus is very rich in cholinergic neurons. Consequently electric eels were obtained from World Wide Aquariums, in Venice, California. For each experiment a fresh electric organ was dissected from an eel, weighed and sliced with a razor blade into approximately 1 cm^3 pieces. The pieces were homogenized at 0 degrees in a Virtis 45 in a volume of Ringer's equal to twice the weight of the pieces. The Ringer's consisted of: 9.35 gm. NaCl, 0.373 gm. KCl, 1.21 gm. tris, 2.03 gm. $MgCl_2 \cdot 6H_2O$, 1.11 gm. $CaCl_2$ anhydrous in 1 liter distilled water.[23] Homogenization was conducted at 75% maximum speed for 1 min. The homogenate was then centrifuged for 20 min.

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[23] Changeux, J., Kasai, M., Huchet, M., Meunier, J., C.R. Acad. Sc. Paris, 270, 2864, (1970)

in a Sorvall superspeed RC-2 centrifuge using a GSA head at 9,000 r.p.m. The supernatant was filtered through celite and washed with Ringers to prevent it from clogging the resin during chromatography, and was stored at 4^{o} if it was not used immediately. The pellet was resuspended in an equal volume of $5x10^{-2}$ M. tris, pH 8 + .1M. NaCl, and stirred at room temperature with the addition of enough 10% deoxycholate to bring the final concentration to 1%. Acetyl cholinesterase release was monitored and when it reached a plateau, the mixture was centrifuged again in the same manner. To the supernatant was added enough 1M. $MgCl_2$ to bring the final concentration to $7x10^{-2}$M. Mg. The precipitate was centrifuged down as before, and 2 mg. spermine per ml. supernatant added. After centrifugation, the supernatant was ready for use with the resins.

Materials used in synthesizing the resins: CNBr and 1,6-hexanediamine, 6-amino hexanoic acid, and N,N,dimethyl-1,3-propanediamine were obtained from Eastman Chemicals, 1-ethyl-3-(3-dimethylaminopropyl carbodiimide was obtained from Cyclo Chemical Co., succinic anhydride, and choline chloride were obtained from Matheson, Coleman, and Bell, N-acetyl-DL-homocysteine thiolactone from CalBiochem, acryloyl choline from Aldrich, and Sepharose 4B-200 from Sigma. The details of the synthesis of the individual resins will be presented in the appropriate chapter.

Assays: Materials and Methods

Protein determination was done by the Lowry Method[24], the Ninhydrin method[25], and by comparison of the absorbance of the samples at 280mμ and 260mμ[26]. Absorbance measurements were performed using a Gilford 240 Spectrophotometer with a model 410 digital absorbance indicator and a model 4008 data lister. Spectra were taken using the Cary 14.

Acetyl cholinesterase assays were performed using the method described by Ellman, et. al.[27] Absorbance changes were followed using the Gilford in conjunction with either the data lister or a model 242 chart recorder. Reagents for the assay, 5,5'-dithiobis-(2-nitrobenzoic acid, and S-acetylthiocholine were obtained from Aldrich.

Choline acetyl transferase activity was determined by the micro assay method of Fonnum using ^{3}H-acetyl-CoA[28]

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24 Lowry, O., Rosebrough, N., Farr, A., Randall, R., Jour. Biol. Chem. 193, 265 (1951)

25 Moore, S., Stein, W., Jour Biol. Chem. 176, 367 (1948)

26 Warburg & Christian, Biochem. Zeit. , 310, 384 (1942)

27 Ellman, G., Courtney, K., Andres, V. Featherstone, R., Biochem. Pharm. 7, 88 (1961)

28 Fonnum, F., Biochem. J., 115, 465 (1969)

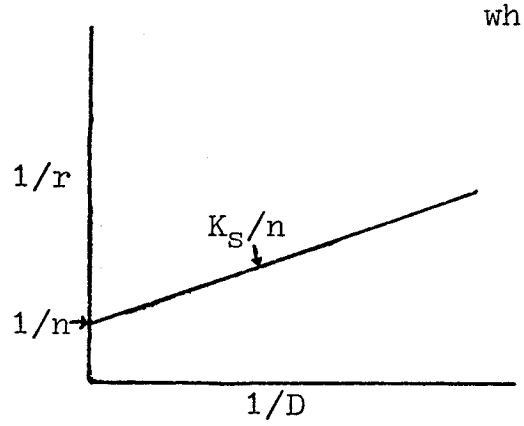
obtained from New England Nuclear; .05 mc./.0325 mg. Other reagents for the assay were obtained as follows: acetyl-CoA and eserine from Sigma Chemical, EDTA from Baker Chemical, albumin from Armour Pharmaceutical, sodium tetraphenyl borate from Alfa Inroganics, and 3-heptanone from Aldrich. Radioactivity was counted for 1 min. per sample using a Packard Tricarb Liquid Scintillation Counter Model 3375 using the ^{3}H setting.

Binding of the samples to decamethonium was determined by equilibrium dialysis using a large slowly rotating wheel onto which were clamped culture tubes containing 50 ml. ^{14}C-decamethonium in Ringer's and a small dialysis casing containing 1 ml. of sample. The tubes were mixed by the rotating wheel at 4^{o} until a control sample indicated that equilibrium had been reached. Each binding experiment utilized four different concentrations of decamethonium as follows:

concentration deca.	mc./ml. Ringer's
1×10^{-5} M.	5.00×10^{-5}
2×10^{-6} M.	2.50×10^{-5}
1×10^{-6} M.	1.25×10^{-5}
2×10^{-7} M.	2.50×10^{-6}

When equilibrium was reached the tubes were removed and a 100 ul. sample pipetted from each dialysis bag and from its surrounding medium into a scintillation vial containing 1 ml. of NCS solublizer. 15 ml. toluene based scintillator fluid was added and the samples were counted

for 10 min. each using the ^{14}C setting of the scintillation counter. Protein content of the dialyzed samples was determined by the Lowry method. The binding constant of the sample to decamethonium and the amount of binding species present in the sample were calculated from this data in the manner shown by the plot below.



where: $1/r = \frac{\text{mg. protein}}{\text{mmole bound ligand}}$

D = mmole free ligand

A = cpm inside bag

A' = A - background

B = cpm outside bag

B' = B- background

$a = 5.417 \times 10^9$ cpm/mmole

$C = \frac{\text{mg. protein}}{\text{100 µl. sample}}$

$1/r = C(a)/A'-B'$

$D = B'/a$

$K_S = \frac{(\text{mg. protein})(\text{mmole free ligand})}{(\text{mmole bound ligand})}$

$n = \frac{\text{mmole bound ligand}}{\text{mmole protein}}$

Due to difficulties in reaching a reproducible equilibrium as well as the error implicit in attempting to measure the radioactivity bound to a relatively small number of molecules, the error in these calculations

is often as high as 50%. Consequently these binding studies have primarily qualitative significance.

If deoxycholate was present in the sample, it was necessary to dialyse it out first to prevent its precipitation with the magnesium present in the Ringers. Also if a sample was obtained by eluting an affinity resin with decamethonium solution, the decamethonium was dialysed out prior to the binding studies.

Reagents for these experiments were obtained as follows: ^{14}C-decamethonium chloride from Amersham/Searle, and decamethonium chloride from K & K Laboratory.

Binding to α-bungarotoxin was studied chromatographically in the following manner. The sample was incubated with ^{125}I-α-bungarotoxin which was purified from bungaro venom by David MacMurchie and iodinated by Aurora Landel[29]. It was then applied to a .9 x 27 cm. Pharmacia chromatography column containing Sephadex G-75 equilibrated with 1% Triton in Ringer's, and eluted with the same. 350 µl. samples from 400 µl fractions were pipetted into scintillation vials containing 2 ml. NCS solublizer. 10 ml. toluene based scintillator fluid was added and the samples were counted for 5 min. on the scintillation counter. The radioactivity emerging at the exclusion volumn of the column was compared with

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[29] to be published from this laboratory

that emerging at the position of free α-bungarotoxin. Bungaro venom was obtained from Miami Serpantarium Labs.

Electrophoreses of the samples were conducted by John Racs on 7.5% SDS acrylamide gels according to the method of Laemmli, et. al.[30]; and gels were stained with Comassi Blue as described by Weber and Osborn[31].

Before any studies were undertaken on the samples eluted from the affinity resins, the samples were concentrated by ultrafiltration through PM-10 Permicon membranes until their final volume was between 1 and 6 ml.

Since the chromatographic methods vary in detail for the different affinity resins, they will be described in the appropriate chapters.

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[30] Laemmli, Uli, Maizel, Jake, Nature, 227, 680 (1970)

[31] Weber, K., Osborn, M., Jour. Biol. Chem. 244, 4406 (1969)

CHAPTER 3: THE COBRA TOXIN RESIN

The cobra toxin resin was synthesized by Michael Raftery using the cyanogen bromide activation of Sepharose 4B-200 described by Cuatrecasas.[32] The activated Sepharose was then stirred with purified cobra toxin in the proportion 50 mg. toxin/25 ml. resin. 25 mg. of the toxin was successfully bound to the Sepharose.

Three separate chromatographic experiments were conducted using this resin and each experiment also employed a control chromatography conducted on unsubstituted Sepharose. All three experiments used the initially extracted supernatant from electric eel organ described in chapter 2. The resins were equilibrated with Ringer's and poured into Pharmacia chromatography columns of the dimensions listed in table 1. Columns were eluted first with Ringer's, pH 8; then with 10^{-2}M. hexamethonium in Ringer's, pH 8; then with Ringer's, pH 2, and finally again with Ringer's, pH 8 following application of the eel organ extract.

The acetyl choline receptor was expected to bind reversibly to the cobra toxin[33], thereby being retained on the column while other components of the extract were

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32 Cuatrecasas, P., Jour. Biol. Chem., 245, 3059, (1970)

33 Chang & Lee, Brit J. Pharmac. Chemother. 28, 172 (1966)

being washed off with Ringer's. Hopefully the affinity of the receptor for hexamethonium[34] would allow the hexamethonium containing eluent to compete effectively with the resin for the receptor, thereby eluting it off the column. If that failed it was hoped that the receptor could be eluted from the column by dissociating the receptor-toxin complex at pH 2. The experimental details and results of these three experiments are summarized in Table 1, and Figs. 1-6.

TABLE 1

description	expt. 1	expt. 2	expt. 3
column size (cm.)	.9x10	.9x10	1.5x30
resin volume	8 ml.	8 ml.	29 ml.
fraction size	2 ml.	2 ml.	5 ml.
sample volume	7 ml.	10 ml.	47 ml.
sample protein (Lowry)	126 mg.	180 mg.	762 mg.
volume Ringers wash #1	30 ml.	20 ml.	135 ml.
volume hexamethonium wash	22 ml.	20 ml.	50 ml.
volume pH 2 wash	20 ml.	22 ml.	50 ml.
volume Ringers wash #2	20 ml.	20 ml.	50 ml.
A_{280} eluted after wash #1	.466	.170	.870
A_{280} eluted/mg. protein applied	.0037	.00094	.0012
esterase activity	none	none	none

An observation of interest was that attempts to perform a Lowry assay on the peaks from these columns resulted in a yellow precipitate which began to appear in the same fractions where the A_{280} began to rise.

[34] Karlin, A., Winnik, M., Proc. Natl. Acad. Sci. U.S. 60, 668, (1968)

The amount of precipitate appeared to rise along with the A_{280} and remained at a plateau for several fractions after the absorbance began to decrease. A similar precipitate resulted upon performing a Lowry on a blank containing hexamethonium. It would appear then that the hexamethonium does not begin to appear at the same point after its application in the different experiments, however its emergence is always accompanied by the emergence of material which absorbs at 280 mμ. The hexamethonium solution itself does not absorb at this wavelength. It is also worth noting that the column which yielded the highest A_{280}/mg. protein applied retarded the emergence of the hexamethonium to the greatest extent. A speculative explanation for this might be the following: If the receptor contains more than one binding subunit, elution with hexamethonium could result in the binding of one subunit to the hexamethonium, while another was still bound to the cobra toxin on the resin. This would temporarily bind the hexamethonium to the resin until another hexamethonium molecule could replace the toxin, thereby having the net effect of retarding the emergence of the hexamethonium from the column. This is, however, purely speculative, since the apparent inconsistancy of the three chromatographies led to the abandonment of this series of experiments and consequently

nothing is known about the purity or other binding characteristics of the material obtained from these columns. This explanation of the inconsistancies is somewhat unlikely, however, since such a high concentration of decamethonium was used. Nonetheless, further studies using the cobra toxin resin might be of future interest.

Figure 1

Chromatography of Eel Supernatant on Cobra toxin Resin; Experiment #1

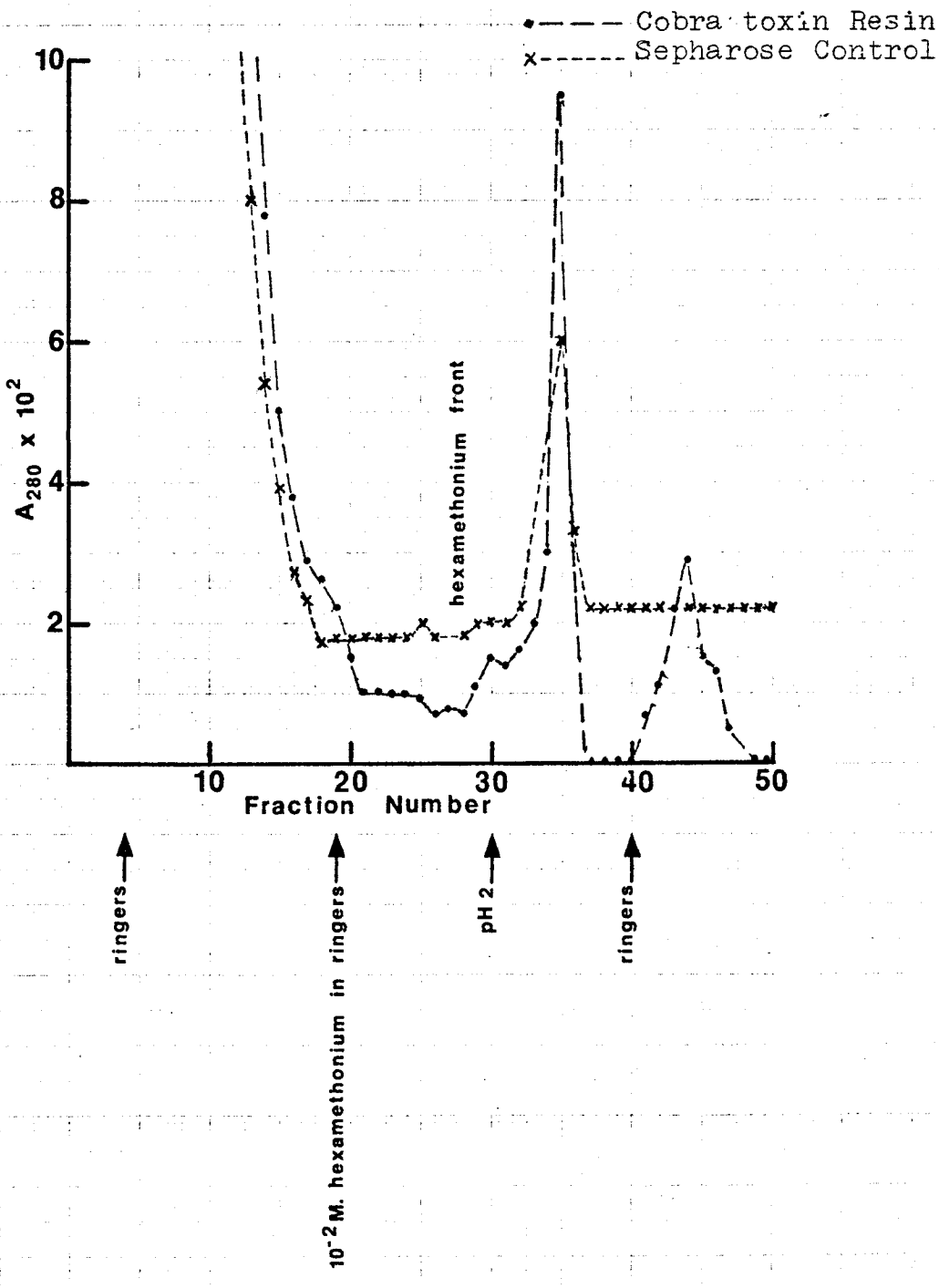


Figure 2

Chromatography of Eel Supernatant on Cobra toxin Resin; Experiment #2

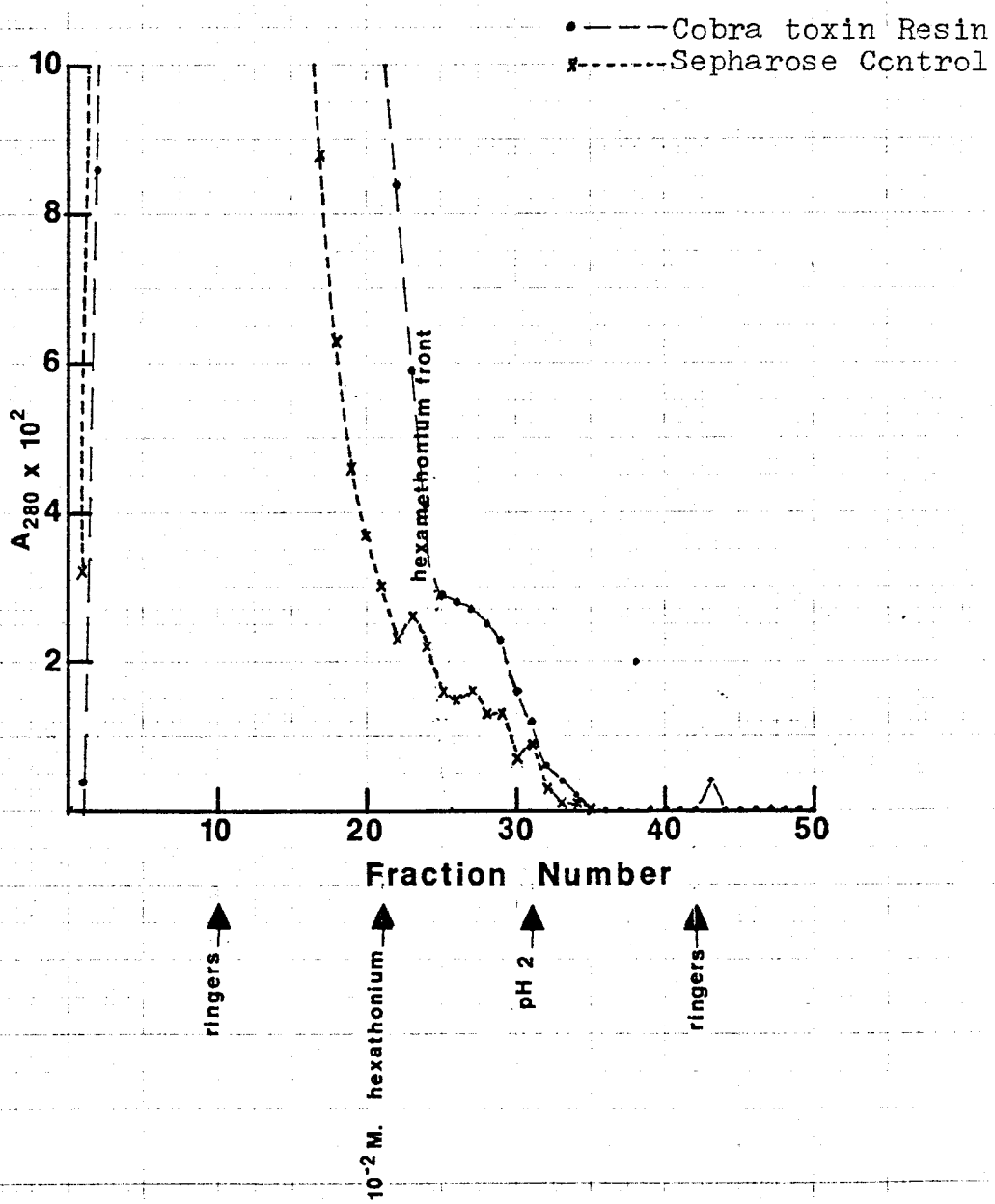
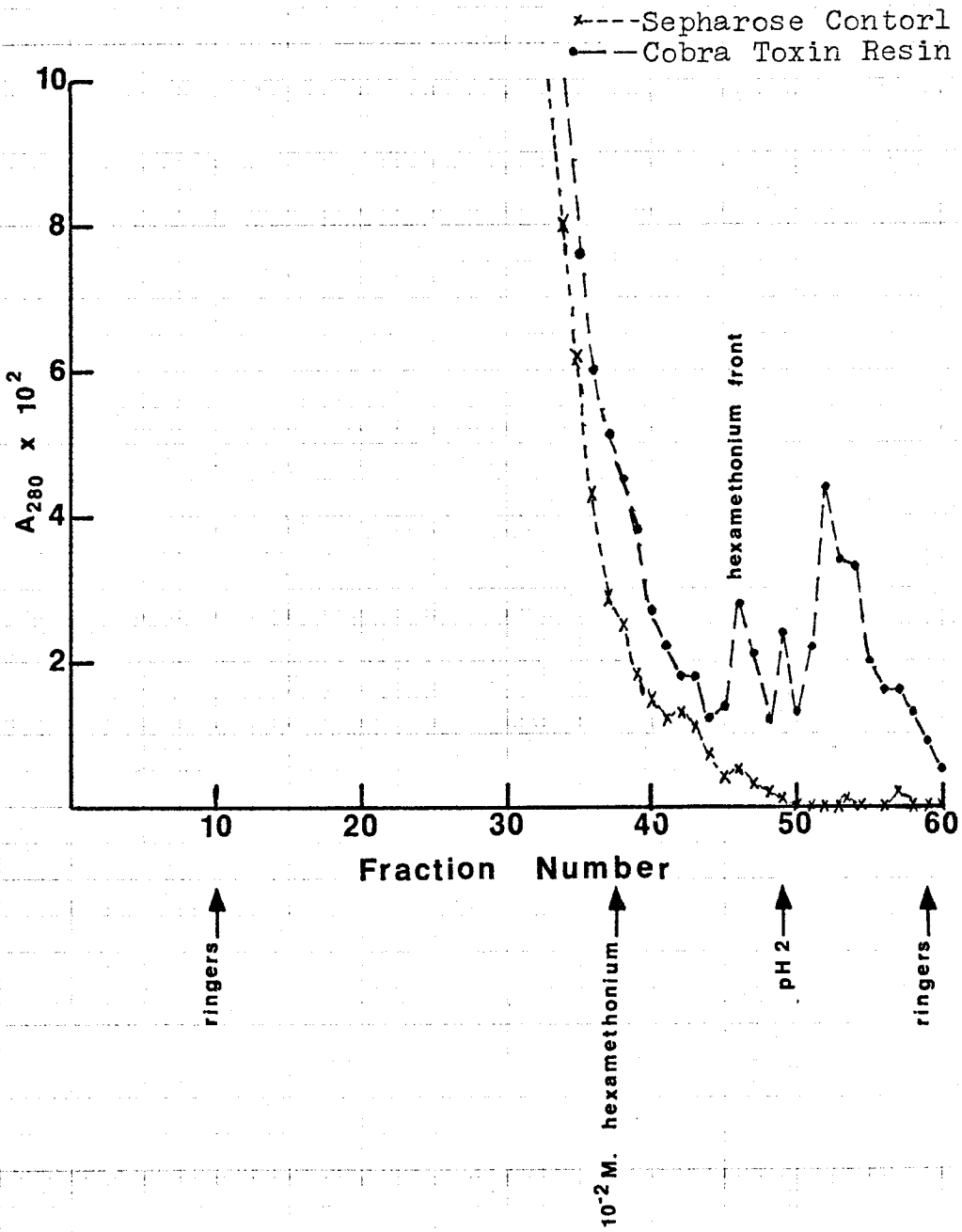


Figure 3

Chromatography of Eel Supernatant on Cobra toxin Resin: Experiment #3



CHAPTER 4: THE THIOCHOLINE RESIN

The thiocholine resin was designed to be as similar to the original affinity resin as possible while eliminating the necessity to resort to such extreme measures as incubation with deoxycholate in order to remove the bound material from the column. Since a thio-ester can be cleaved by hydroxylamine, bound material could be removed from the column simply by cleaving the binding moiety with hydroxylamine if an elution with 5×10^{-2}M. decamethonium in Ringer's failed to bring it off the column.

Synthesis was carried out using the previously mentioned methods of Cuatrecasas by the following synthetic pathway:

(1) Sepharose 4B-200 (100 ml.) + $NH_2-(CH_2)_5-\overset{O}{\overset{\|}{C}}-OH$ (12 gm.) + CNBr (10 gm.)

$\xrightarrow[\text{pH 10}]{}$ Sepharose-$NH-(CH_2)_5-\overset{O}{\overset{\|}{C}}-OH$

(2) Sepharose-$NH-(CH_2)_5-\overset{O}{\overset{\|}{C}}-OH$ + $HS-(CH_2)_2-\overset{CH_3}{\overset{|}{N}}-CH_3$ (10 gm.) $\overset{|}{CH_3}$

$\xrightarrow[\text{carbodiimide}]{\text{pH 4.7}}$ Sepharose-$NH-(CH_2)_5-\overset{O}{\overset{\|}{C}}-S-(CH_2)_2-\overset{CH_3}{\overset{|}{N}}-CH_3$ $\overset{|}{CH_3}$

In spite of an extremely low degree of substitution due to inefficiency of the diimide coupling, the

resin was used to chromatograph both the initial supernatant extract and the membrane fragments extract of electric eel organ. The organ used in these experiments weighed 268.5 gm. Experimental details and results are presented in Table 2 and Figures 4 & 5.

TABLE 2.

Description	Supt. ext.	Memb. ext.
column size (cm.)	1.5 x 30	1.5 x 30
resin volume	50 ml.	50 ml.
fraction size	5 ml.	5 ml.
sample volume	750 ml.	800 ml.
vol. Ringer's, pH 6.5	1 liter	1 liter
vol. $5x10^{-2}$decamethonium	200 ml.	200 ml.
time in 2M NH_2OH	1 hr.	1 hr.
A_{280} eluted with deca.	.2	8.75
A_{280}/A_{260} of peak tube	?	.758
esterase activity	none	?
K_s (by A. Landel)	3.9×10^{-7}	1.0×10^{-7}
n (mmole rec. sites/mg.	4.1×10^{-6}	2.6×10^{-6}
electrophoresis (Fig.5)	1 band	8 bands

Although the electrophoresis revealed the relatively large amount of material from the column eluted with membrane extract to be multicomponent, it did demonstrate binding to decamethonium with a K_s that is reasonably close to that of the receptor. Of even greater interest, however is the single electrophoretic component obtained from the column eluted with eel supernatant. It also has the correct binding constant to decamethonium. As can be seen from figure 5, this

single band occupies the same position as the one from the original affinity column, and is also present among the electrophoretic bands observed from the membrane extract treated column. The binding of this component to both the thiocholine resin and decamethonium as well as the absence of esterase activity are encouraging, however, there was not enough material isolated to conduct further binding studies or acetylase assay.

The ability of the material to be eluted with decamethonium was attributed to the extremely low substitution of the resin, as was the low yield of material obtained (only 2.5% of the yield of the same material from the original column). It therefore seemed advisable to return to the original affinity resin and attempt to obtain a degree of substitution intermediate to that of the thiocholine resin and the original resin. Hopefully this might permit the binding of more material while still allowing it to be eluted off with decamethonium. This attempt will be discussed in the following chapter.

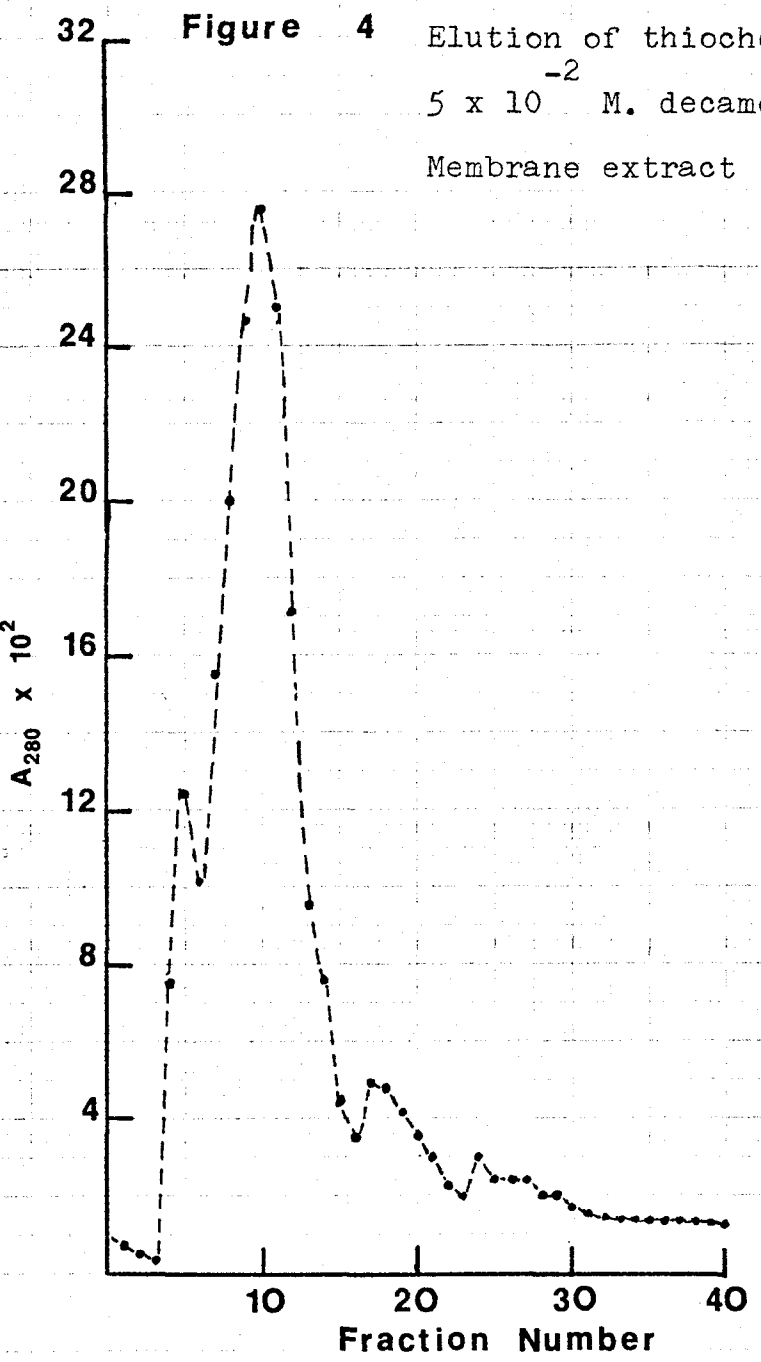
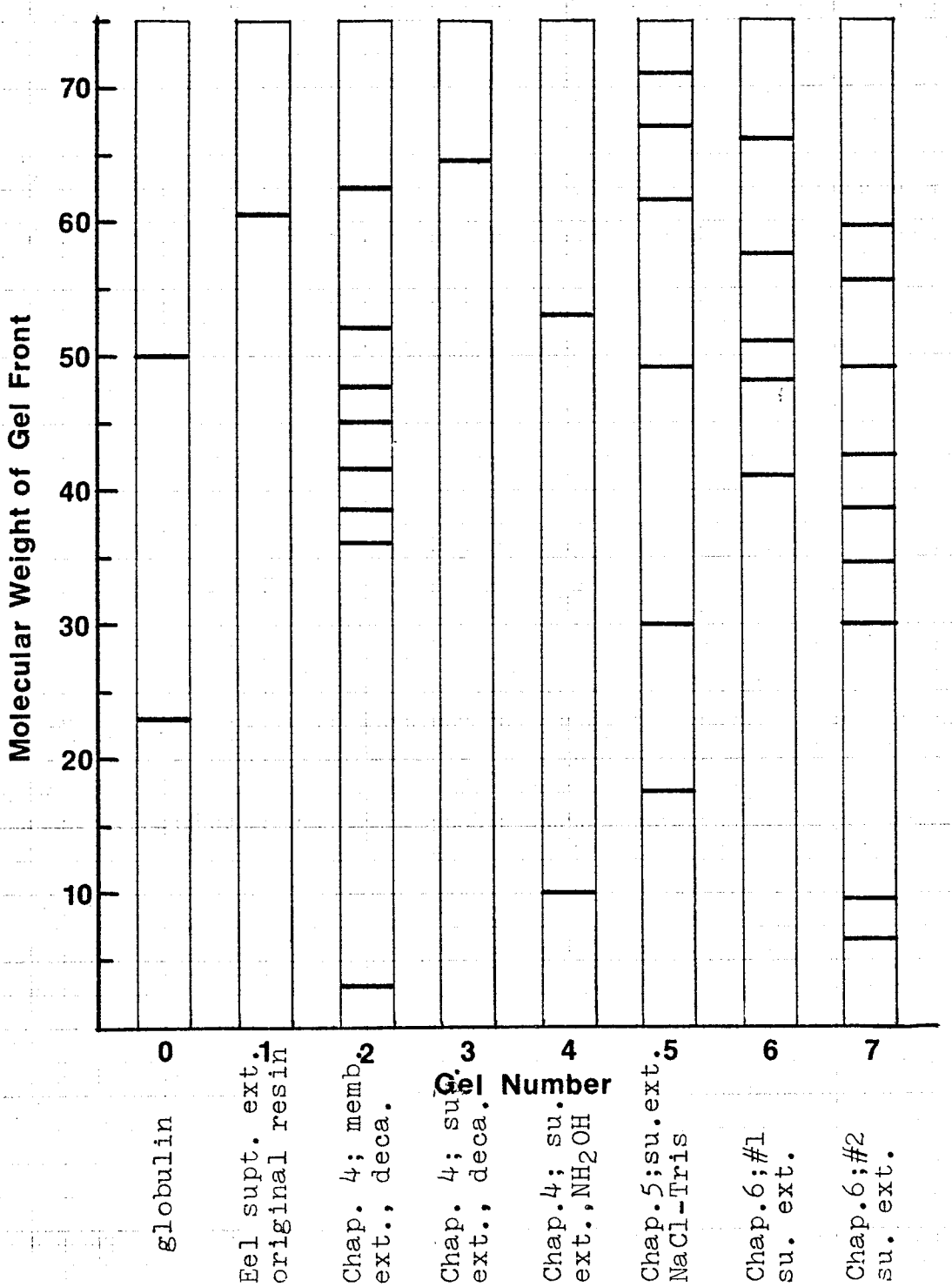


Figure 4 Elution of thiocholine resin with 5×10^{-2} M. decamethonium.

Membrane extract

Figure 5 7.5% acrylamide-SDS gel electrophoresis



CHAPTER: 5 THE ORIGINAL RESIN WITH 10% SUBSTITUTION

A resin having only 10% as much acetyl choline analog as the original seemed like a reasonable compromise between total binding capacity and ability to be eluted off with decamethonium. Synthesis of this resin was straightforward, since the group to be attached to the resin had been previously synthesized by Aviva Ashery, all that was needed was to allow it to react at pH 10 with cynaogen bromide activated Sepharose. Since the degree of substitution is controlled by the amount of cyanogen bromide, 1 gm. rather than 10 gm. per 100 ml. Sepharose was used. The structure of the resin was as follows:

$$\text{Sepharose-NH-(CH}_2\text{)}_5\text{-}\overset{\overset{\text{O}}{\|}}{\text{C}}\text{-NH-(CH}_2\text{)}_3\text{-}\overset{\overset{\text{CH}_3}{|}}{\underset{\underset{\text{CH}_3}{|}}{\text{N}}}\text{-CH}_3$$

Chromatography of both the supernatant and membrane fragment extracts of a 225 gm. eel electric organ was conducted on this resin. The procedure and results are presented in Table 3 and Figures 5 - 9 . The elution with 5×10^{-2}M. Tris, pH 8 + .1M. NaCl was intended to remove the magnesium present in the Ringer's from the resin so that subsequent treatment with 1% deoxycholate would not precipitate the deoxycholate. The results of that elution, however, gave a peak containing enough ma-

terial so that it could be shown to bind decamethonium, α-bungarotoxin, and the acetyl choline analog bound to the resin. Also assays revealed no esterase activity. Although an assay for choline acetyl transferase was not performed on this material, it can be inferred from a later experiment indicating an absence of inhibition of acetylase activity by bungaro venom (Fig. 13) that the binding characteristics of the material are not due to the presence of acetylase. Unfortunately, electrophoresis reveals many components in the material, although it appears to be especially rich in a component having a molecular weight somewhere around 30,000 (see Fig. 5).

TABLE 3

Description	Supt. ext.	Memb. ext.
column size (cm.)	1.5 x 30	2.5 x 30
resin volume	50 ml.	50 ml.
fraction size	5 ml.	5 ml.
sample volume	550 ml.	150 ml.
vol. Ringer's pH. 8	400 ml.	400 ml.
vol. .05M. deca.	200 ml.	160 ml.
vol. .05M. Tris, .1M. NaCl	200 ml.	100 ml.
mg. protein from deca.	0.8 mg.	0.8 mg.
mg. protein from Tris	7.2 mg.	1.4 mg.
mg. protein from DOC	0.02mg.	1.1 mg.
%nuc. acid/% protein Tris.	25%/75%	?
esterase activity	none	none
K_s decamethonium, Tris peak	$2.5\text{-}5.3\times10^{-7}$	$2.2\text{-}4.1\times10^{-7}$
n, Tris peak	$4.0\text{-}7.6\times10^{-7}$	$1.3\text{-}1.6\times10^{-6}$
K_s deca., Tris peak with α-Btx.	0	?
K_s deca, DOC extract	?	$3.1\times10^{-7}\text{-}1.0\times10^{-6}$

Figure 6 Elution of 10% resin with 5×10^{-2} decamethonium

•-------Supernatant extract
x- - - - -Membrane extract

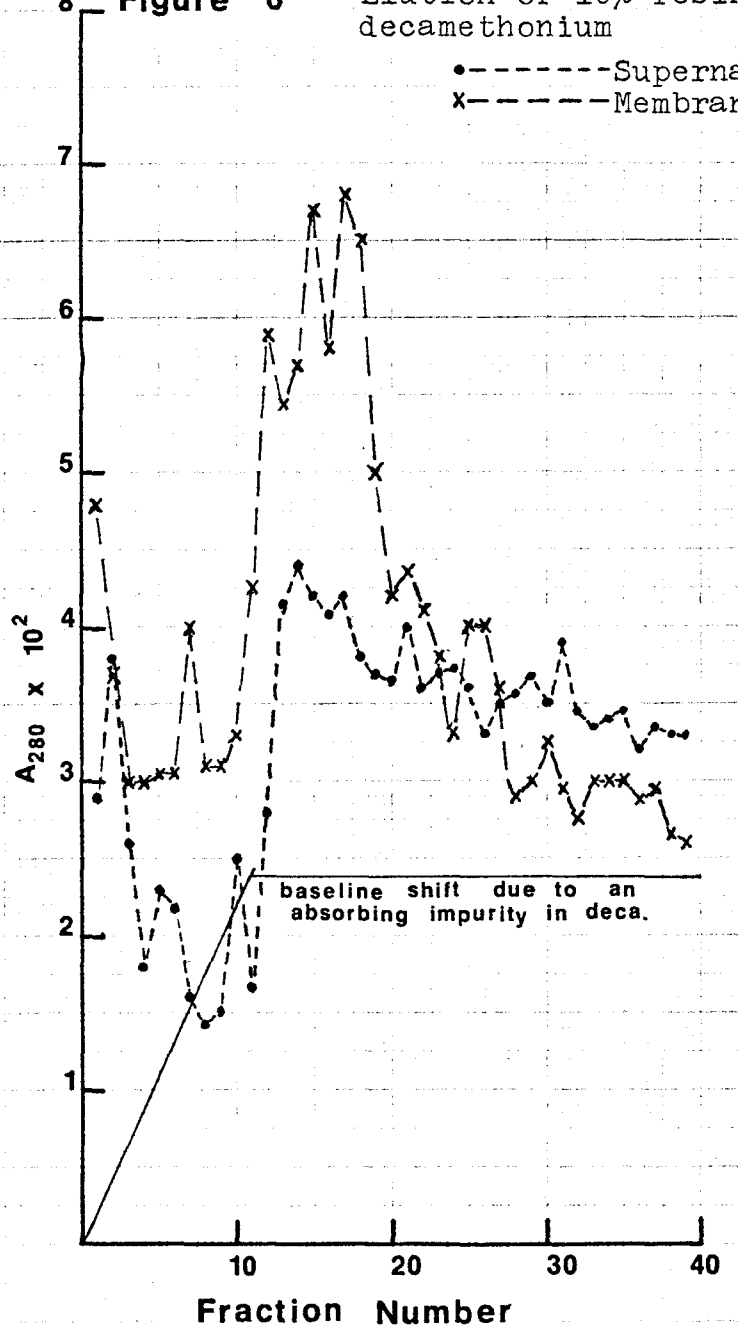


Figure 7 Elution of 10% resin with .05 M. Tris-.1 M. NaCl

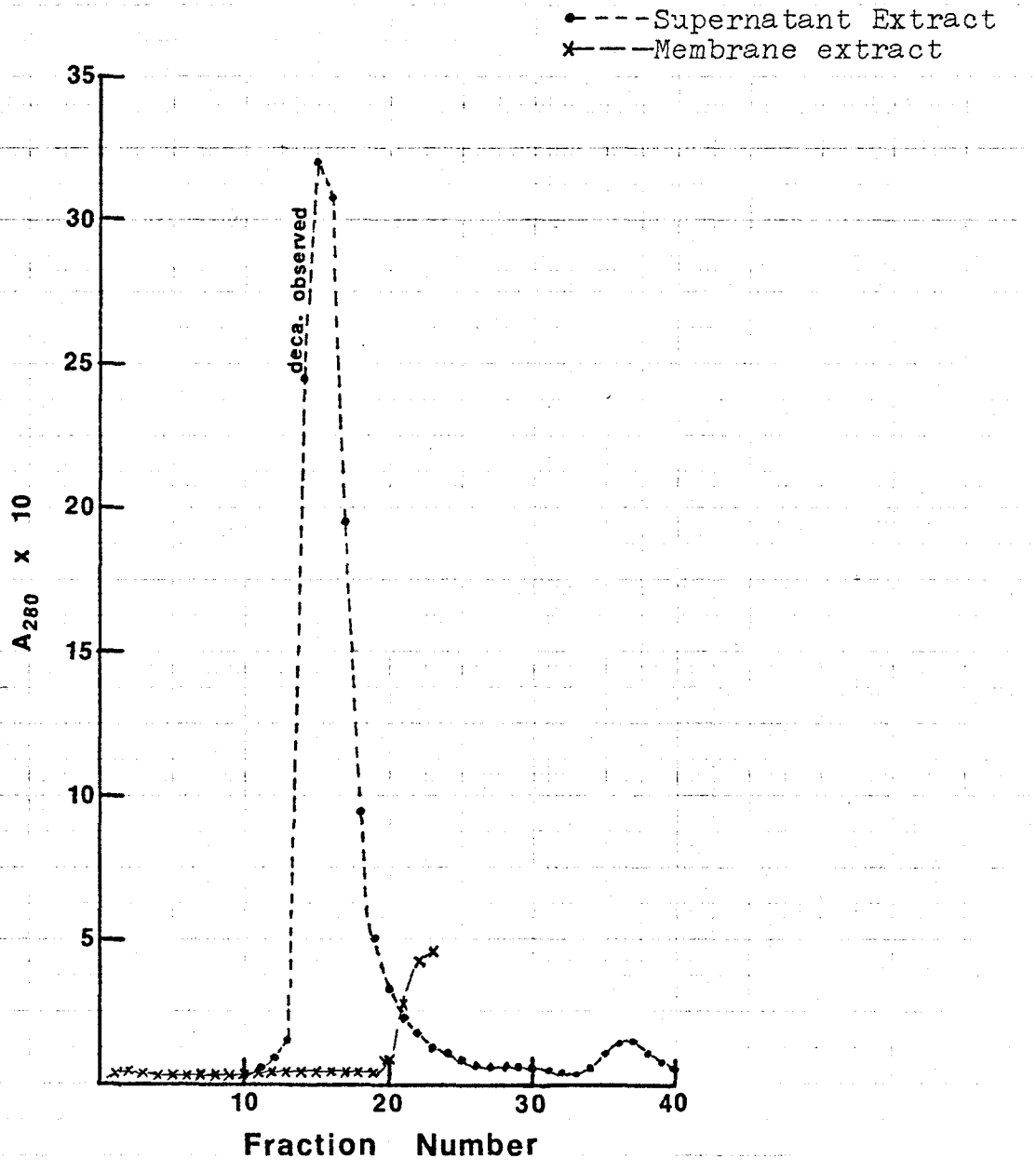
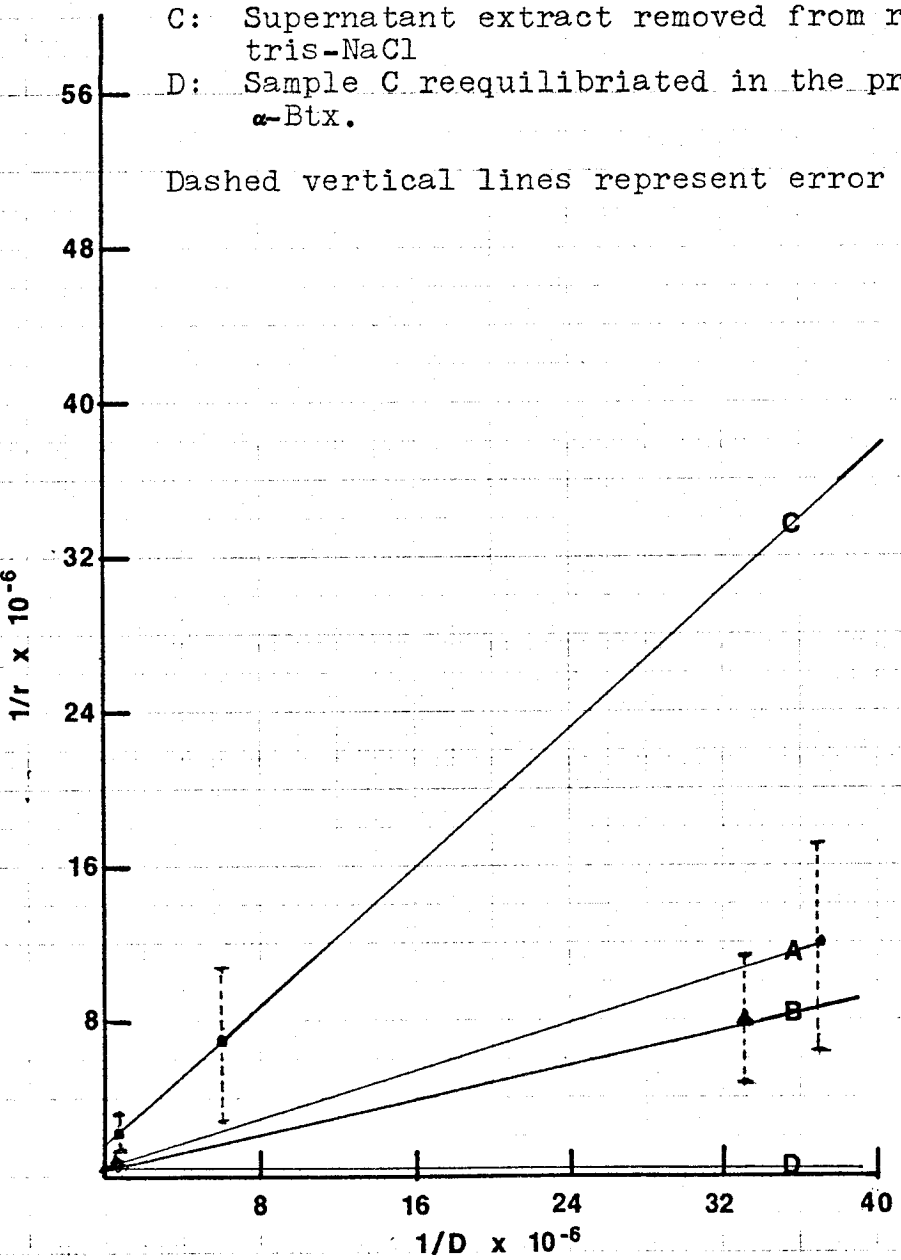


Figure 8

Equilibrium Dialysis on 10% resin elutions
A: membrane extract removed from resin by DOC
B: membrane extract removed from resin by tris-NaCl
C: Supernatant extract removed from resin by tris-NaCl
D: Sample C reequilibriated in the presence of α-Btx.

Dashed vertical lines represent error bars



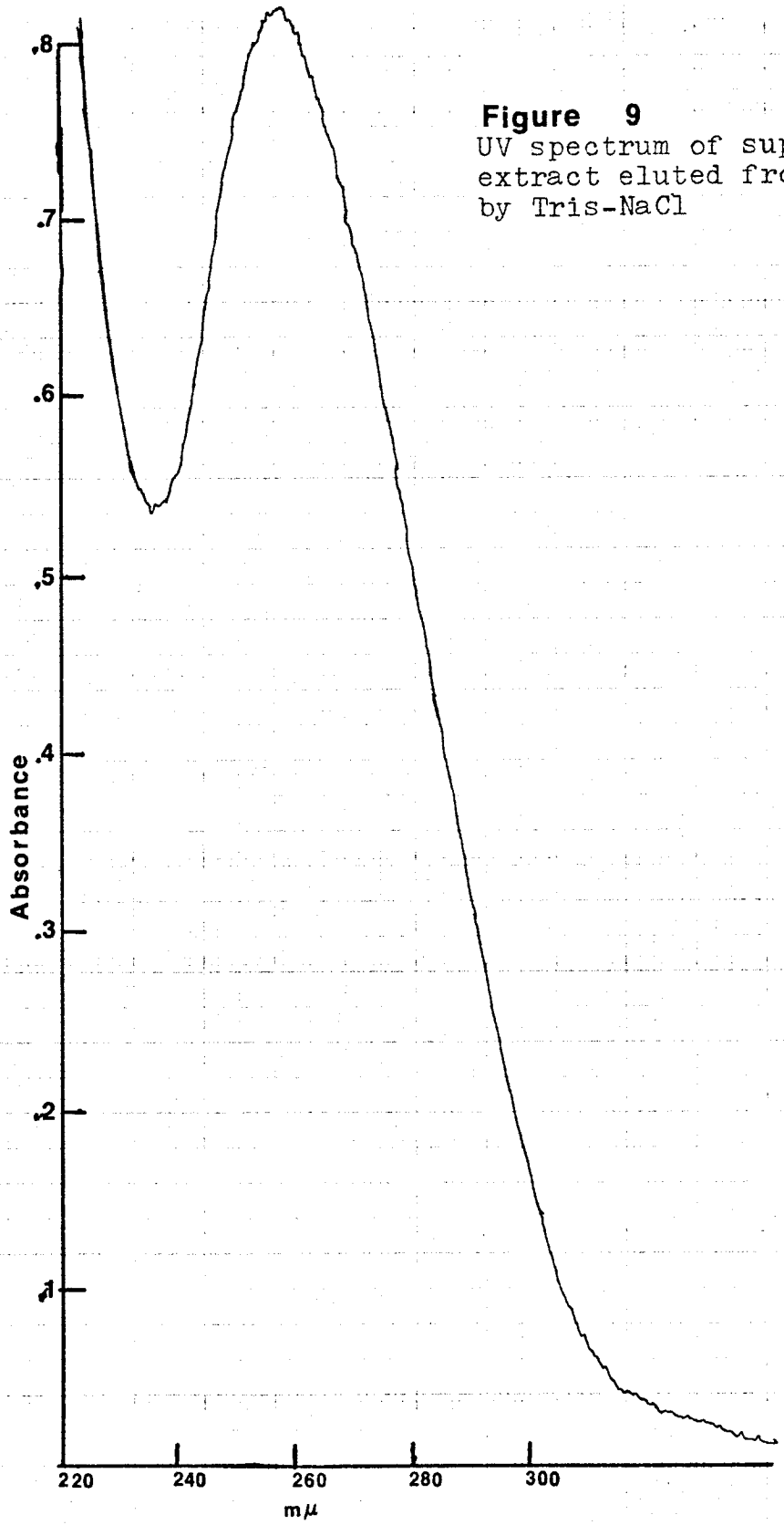


Figure 9
UV spectrum of supernatant extract eluted from resin by Tris-NaCl

CHAPTER 6: BATCH PROCESSES ON THE ORIGINAL RESIN

Since the only procedure which had thus far resulted in a reasonable yield of a single electrophoretic component was the incubation of the original resin with 1% deoxycholate in water; 50 ml. of that resin was stirred in a beaker at 4° for 4 hrs. with fresh supernatant extract from Electrophorus electricus organ. It was then washed on a scintered glass funnel with Ringer's until the A_{280} and the esterase activity of the wash was zero. It was then stirred for 3 hrs. with Ringer's at 4° and the wash procedure repeated. The resin was then stirred for two days at 4° with 1% deoxycholate, filtered and the filtrate dialyzed 4 times against the Tris-NaCl, pH 8 to remove the deoxycholate, then ultrafiltered through a PM-10 membrane down to a volume of 2 ml. The material extracted from the resin had the characteristics listed in table 4.

TABLE 4

Description	Expt. #1	Expt. #2
protein (Lowry)	.74 mg.	.77 mg.
electrophoresis (Fig. 5)	5 bands	9 bands
cholinesterase activity (moles/liter/hr.)	3.74×10^{-5}	none
acetylase activity	none	none
bind to α-Btx. (Fig. 17-18)	slight?	1.71×10^{-4} mg.
organ weight	93.5 gm.	350 gm.
mmole binding sites	?	2.14×10^{-8}
activity of α-Btx. (cpm./mg.)	5×10^{6}	5×10^{6}
cpm. α-Btx. used	8×10^{4}	1.7×10^{4}

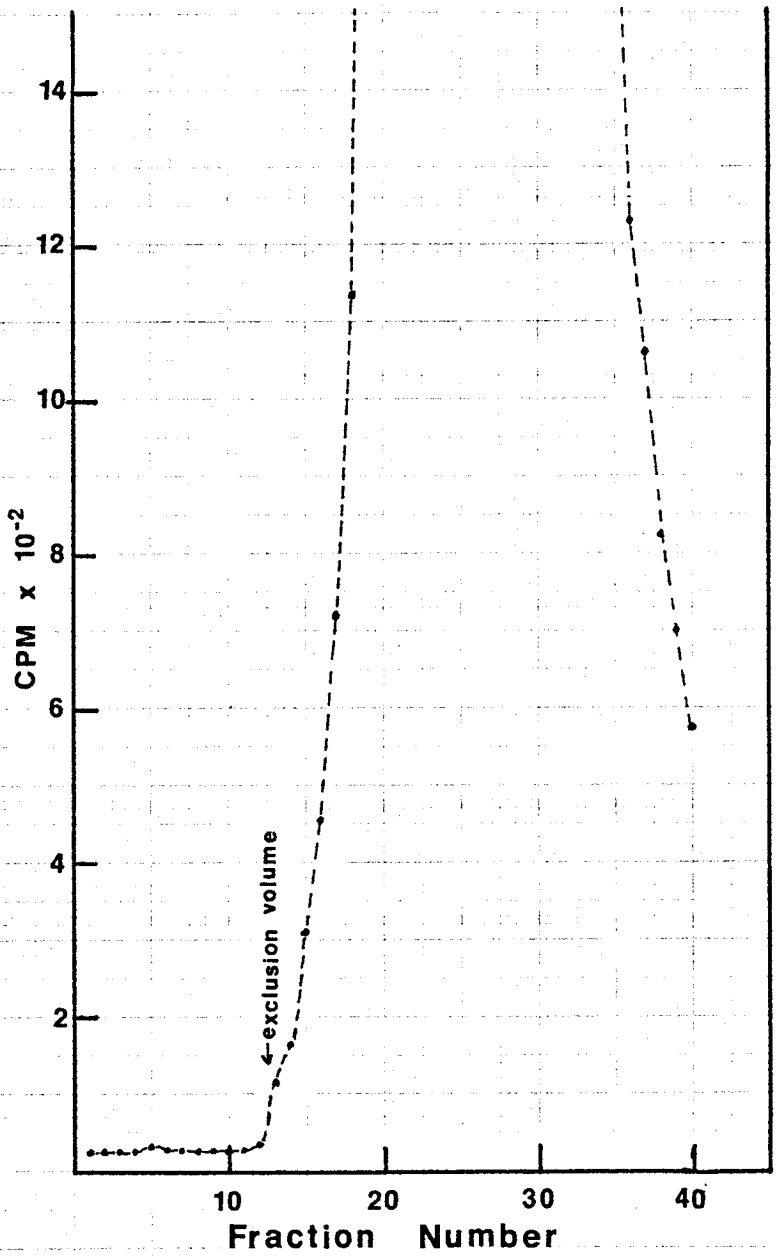
Fig. 6 indicates an extremely gradual release of the bound material from the column. This probably indicates that the decamethonium is barely competing with the resin for the binding material. This would support the advisability of a 1% substituted resin, although a 1% resin might not be capable of binding an optimum amount of material.

It is interesting to note that although there was no decamethonium (as observed by precipitation of the Lowry reagent) present in the tubes prior to the emergence of the Tris-NaCl peak, there was decamethonium accompanying the emerging protein. This again suggests a receptor with multiple binding sites, at least one of which is bound to the resin enough of the time to hold the material on the column even though others are often bound to decamethonium. It is conceivable that the peak emerged with Tris-NaCl because the absence of the divalent cations present in Ringer's shifted the equilibrium in favor of the decamethonium.

G-75 Chromatography of DOC extract from origional resin with ^{125}I -α-Btx.

Figure 10

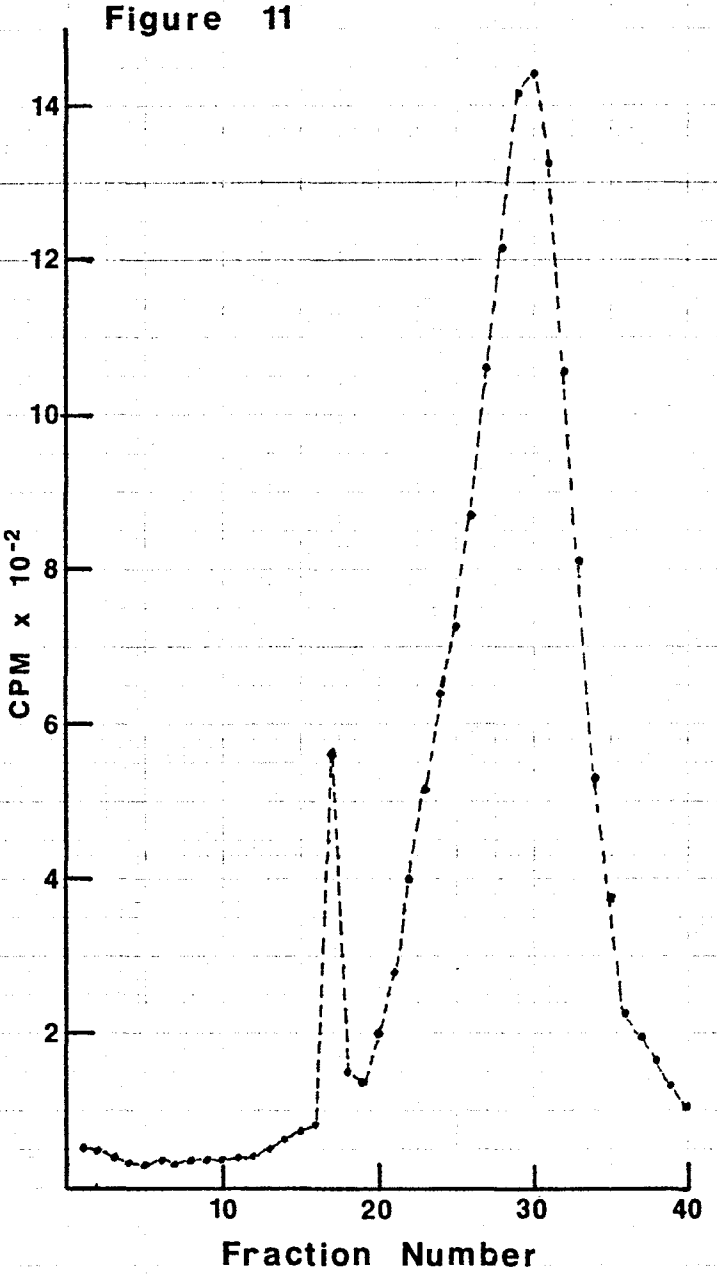
Experiment # 1



G-75 Chromatography of DOC extract from origional resin with ^{125}I-α-Btx.

Experiment # 2

Figure 11



As can be seen from Fig. 10 there is a slight indication that there might be a macromolecular species which binds α-bungarotoxin. However, the extensiveness of the free toxin peak obscures the area around fraction 14 where macromolecules would be excluded from the column. The quantity of free toxin was therefore decreased in experiment 2, revealing a small amount of toxin binding to a macromolecular component (see Fig. 11). The yield of that component calculated from the specific activity of the α-bungarotoxin is only 2.14×10^{-8} mmoles.

This quantity of binding material is considerably less than that which has been obtained by Clark, Schmidt, and Wolcott[35] using a resin bearing an antibody to α-bungarotoxin. In view of the observation by Berman and Young[36] that the yield of acetyl cholinesterase obtained by affinity chromatography was greatest when the terminal atom of the ligand was 31 residues removed from the backbone of the resin, It was thought that the higher yield from the antibody column might be because the antibody's larger size positioned its binding site further from the backbone of the resin. Consequently the resin to be described in the next chapter was prepared.

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35 Unpublished data from this laboratory

36 Berman, J., Young, M., Proc. Natl. Acad. Sci. U.S., 68, 395 (1971)

CHAPTER 7: A RESIN BEARING A 31 RESIDUE LIGAND

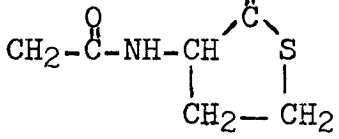
The resin pictured on page 14 represents an attempt to simultaneously overcome two possible reasons for the comparatively low yield of binding material: (1) incomplete removal of the material from the resin by the deoxycholate treatment, and (2) Inefficient birding of the material to the resin because of the proximity of the acetyl choline analog to the resin backbone.

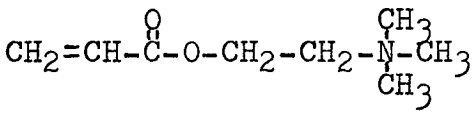
The resin was synthesized by previously referenced methods using the following sequence of reactions:

(1) Sepharose + $NH_2-(CH_2)_6-NH_2$ yields A

(2) A + succinic anhydride yields B

(3) B + $NH_2-(CH_2)_6-NH_2$ yields C

(4) C +  yields D

(5) D +  yields resin (P. 14)

The progress of the synthesis is shown in Table 5

TABLE: 5

reaction #	mole subs. /ml. resin	Assay method
1	1.90	Ninhydrin
2	1.75	Ninhydrin
3	0.87	Ninhydrin
4	0.72	DTNB
5	0.72	assumed 100%

The resin was treated with eel extract using the batch process described in Chapter 6 except that cleavage of the ester bond at pH 9 was used to remove the material from the column rather than extraction with deoxycholate. Experimental details and results are summarized in Table 6.

TABLE: 6

Description	Supt. Ext.	Memb. Ext.
eel organ weight	256 gm.	same organ
quantity of resin used	25 ml.	25 ml.
protein (Lowry) in pH 9 ext.	2.8 mg.	2.2 mg.
electrophoresis	no bands	no bands
esterase activity (mmole/min)	4.2×10^{-4}	9.9×10^{-5}
acetylase activity	none	none
α-Btx. binding	none	none

While the absence of electrophoretic bands probably reflects a problem with the electrophoretic method, the absence of binding to α-bungarotoxin is disconcerting. Since the protein yield is fairly high, it seems more likely that receptor activity and binding to the toxin were destroyed by the incubation at pH 9 than it does that the binding material failed to bind to the column. This is, however, purely speculative. It might be informative to use the remaining 25 ml. of resin in the original fashion, incubating with deoxycholate rather than pH 9 to remove the material from the resin.

CHAPTER 8: ACETYLASE ASSAYS

Assays for choline acetyl transferase activity were performed on a number of different samples under various conditions in order to determine if any critical characteristics were shared by the receptor and the acetylase. The results of these experiments are shown in Table 7 and Figure 13. In the cases of acetylase assay on cobra venom and bungaro venom, the activity appears somewhat lower than it actually is due to destruction of the synthesized acetyl choline by a small amount of acetyl cholinesterase activity which persisted even in the presence of eserine.

TABLE 7

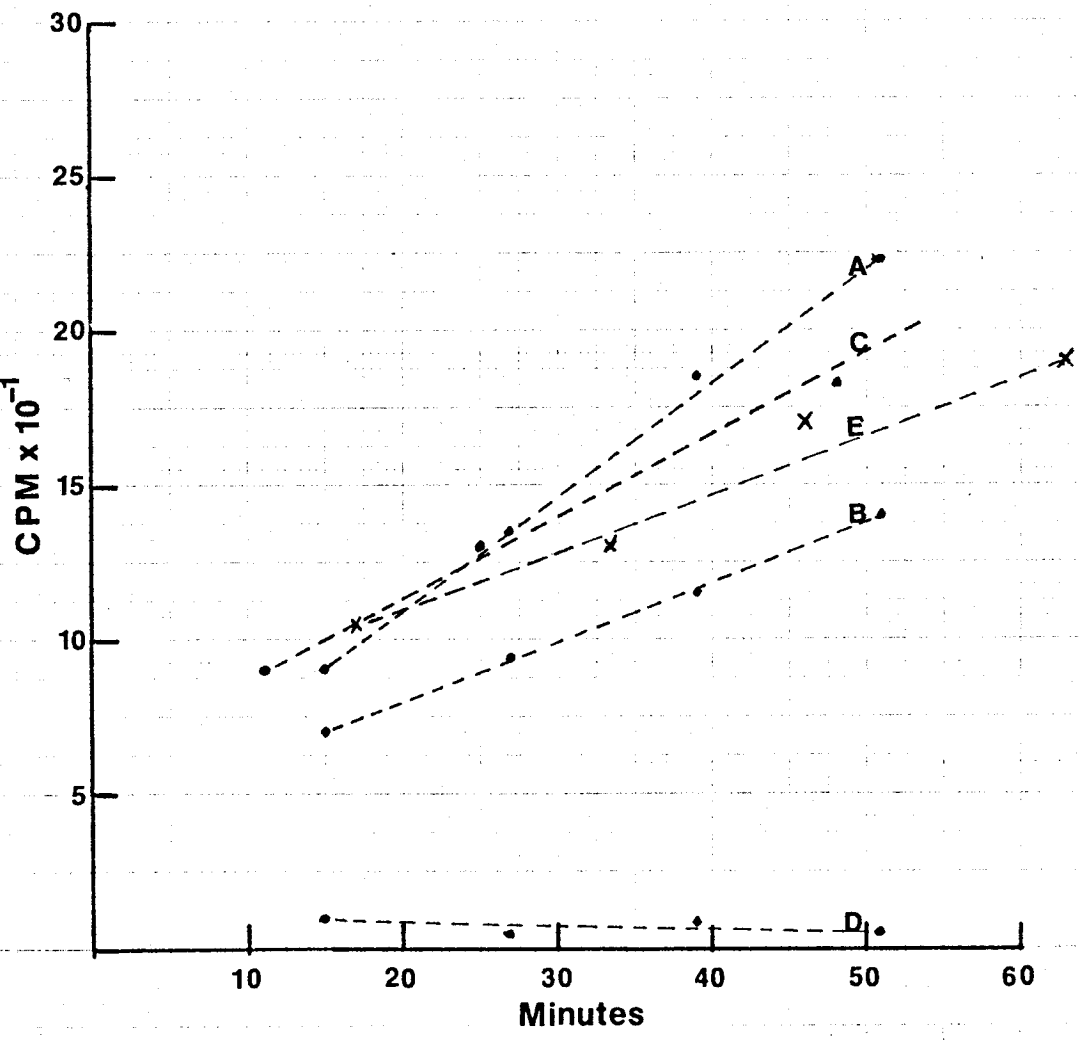
Characteristic	In receptor	In acetylase
present in supt. ext.	yes	yes
present in memb. extract	yes	yes
activity affected by triton	no	yes
activity affected by bung. ven.	yes	no
activity affected by cob. ven.	yes	no
active at pH 9	no (?)	yes, but less
present in Aurora's electro-focused α-Btx. binding band	yes(?)	no
present in affinity column ext.	yes(?)	no

The above table indicates that there is probably not too much danger of isolating the acetylase by techniques based upon the binding characteristics of the receptor.

Figure 13

Acetylase Assays:

A: eel supt. extract; slope = 3.6
B: eel Supt. + bung. ven.; slope = 1.9
C: eel supt. + cob. ven.; slope = 2.1
D: eel supt. + triton
heat denatured eel supt.
all affinity column extracts
Aurora's samples
} slope = 0
E: eel supt. extract, pH 9; slope = 2



CHAPTER 9: SUMMARY AND SPECULATIONS

Although the foregoing experiments did not succeed in isolating the pure acetyl choline receptor, the following observations seem significant:

(1) One or more components in both the supernatant Ringer's extract and the membrane fragments deoxycholate extract from eel electric organ are held up on resins bearing substituents resembling acetyl choline.

(2) Removal of the material from the resins with deoxycholate does not appear to impair its binding properties.

(3) For a resin bearing an acetyl choline analog which would be expected to exhibit a binding constant of roughly 10^{-6}, which had been activated by 1 gm. CNBr/100 ml., The binding material can be removed from the resin after eluting with .05 M. decamethonium in Ringer's by eluting with .05 M. Tris-.1 M. NaCl.

(4) The material obtained by this means contained a component which not only exhibted the proper binding constant to decamethonium, but failed to bind decamethonium under identical conditions in the presence of α-bungaro toxin. Such behavior would be expected of the receptor.

(5) In all the experiments which yielded a component which binds to α-bungaro toxin, there appears to be an electrophoretic band of molecular weight 47,000 - 49,000.

A band once isolated by Wolcott using the α-Btx. antibody column also appeared to have this molecular weight and exhibited binding to α-bungaro toxin.

(6) Affinity resins of the type herein described do not appear to be reproducibly specific enough to offer a reliable method for isolation of the pure receptor.

(7) Choline acetyl transferase is not likely to be confused with the receptor because of their different binding characteristics.

(8) In the presence of Ringer's very little if any acetyl cholinesterase is retained on a resin bearing an acetyl choline analog unless the chain between the analog and the backbone becomes reasonably long (31 residues). Even then not too much is retained.

(9) Retardation of hexamethonium and decamethonium by material bound to the column suggests the possibility of a receptor having 2 or more subunits which bind acetyl choline analogs. Based on the observations with the 10% resin the following calculation would tend to support the idea of 2 binding subunits:

$K_{ACh} = 10^{-6}$

With steric hindrance from the resin this could easily become 10^{-5}

For two subunits the effective $K = 10^{-10}$

Resin substitution = 10^{-5} M.

$$K_{deca.} = 10^{-7}.$$

An eluent can compete effectively with the resin when

$$\frac{K_{eluent}}{K_{resin}/\text{conc. ligand}} \leq \text{conc. eluent.}$$

Substituting for the case of the 10% resin

$$\frac{10^{-7}}{10^{-10}/10^{-5}} = 10^{-2}$$

This is in agreement with the concentration of decamethonium which began to elute the material from the 10% resin. While this tends to support the idea of 2 binding subunits, the matter is still highly speculative.