Investigations on the Acetylcholine Receptor

Thesis

by

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Abstract

Investigations directed toward isolation of the acetylcholine receptor demonstrated that low molecular weight cholinergic ligands lack sufficient specificity for use as receptor labels. However, α-bungarotoxin, a neurotoxin from B. multicinctus, proved to have the desired characteristics of specificity and irreversibility of binding. Chemical and physiological characterization of the purified toxin revealed that a homogeneous species of ~8000 MW was obtained, and that the iodinated derivative was physiologically and antigenically identical to the native protein.

The radioiodide-labeled toxin was used as a specific, irreversible label and showed that the membrane of E. electricus electric tissue contained a unique toxin-binding substance which could be extracted by Triton X-100. The toxin complex had a molecular weight of $\sim 2.5 \times 10^5$ by density gradient centrifugation and a pI ~5.1; uniqueness was suggested by the symmetry of the electrofocused peak. The toxin-binding component was enzymatically and chemically characterized as a protein with a membrane-supplied phospholipid requirement which could also be supplied by Triton X-100 when the latter was used to solubilize the protein. PCMB treatment of the Triton extract suggested involvement of a sulfhydryl group in toxin binding; this group was insensitive to mild oxidation.

A toxin-binding component in similar membranes of T. marmorata was characterized in the same fashion, yielding like results, except that a sensitivity to DTT instead of DTNB was noted, implicating an easily-reduced disulfide bridge.

The possible relationship of these toxin-binding components to the acetylcholine receptor is discussed.

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Abbreviations Used

ACh	acetylcholine
AChE	acetylcholine esterase
AChR	acetylcholine receptor
ATPase	adenosine triphosphatase
BAC	bromoacetylcholine
α-Bgt	α-bungarotoxin
Bgv	*B. multicinctus* venom
Carb	carbamylcholine
DEAE	diethylaminoethyl
Deca	decamethonium
DFP	diisopropylfluorophosphate
DOC	deoxycholate
DTNB	5, 5′-dithiobis(2-nitrobenzoic acid)
DTT	1, 4-dithiothreitol
Hexa	hexamethonium
IgG	immunoglobulin G
IgM	immunoglobulin M (macroglobulin)
K_S	enzyme-ligand dissociation constant
MPTA	4-(N′-maleimido)-phenyltrimethylammonium ion
MSF	methanesulfonyl fluoride
NEM	N-ethyl maleimide
PCMB	*p*-chloromercuribenzoate
PTA	phenyltrimethylammonium ion
d-TC	d-tubocurarine
Tdf	*p*-(trimethylammonium)benzenediazonium fluoroborate
TLC	tender loving care
TMA	trimethylammonium ion
TMB	trimethylbutylammonium ion

Introduction and Historical Background

The nature and mechanism of neuromuscular transmission, and indeed the whole area of neural impulse transmission, are problems which have at once intrigued and taxed the ingenuity of neurochemists since the awakening of modern day science.

Perhaps the first indication that impulse transmission was not a simple phenomenon came from the work of Bernard in 1857 (1); he found it was impossible to produce indirect muscle stimulation (i. e., through the attached nerve) after treatment with curare, although direct (electrical) stimulation remained possible. Curare is an alkaloid derived from the bark of certain South American plants and is used by some Indian tribes to poison the tips of spears and darts. The compound causes motor paralysis when introduced into the blood stream.

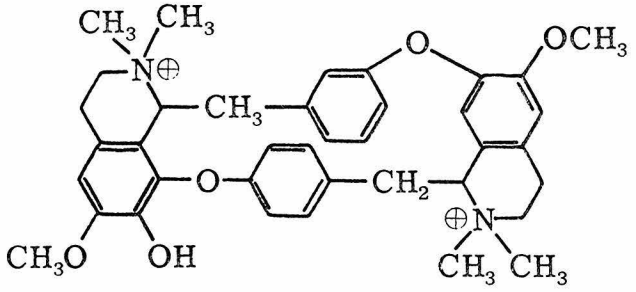
Langley, in 1906 (2), postulated a "receptive substance" as the entity within the synapse which was susceptible to curare-caused impairment of synaptic transmission. In 1919 Dole (3) associated the effect of curare with a "nicotine action" and emphasized the then-known curare-like action of quaternary ammonium bases. He also recognized the curare-like action of choline and its derivatives; of the known esters of choline, the acetyl derivative was the most active. This result led Loewi in 1921 (4) to propose that nervous impulses are transmitted by a chemical mechanism and to emphasize

(5) the importance of the site of action of choline esters and nicotinic compounds in nervous tissue, showing that this site should be identified with the "receptive substance" postulated by Langley.

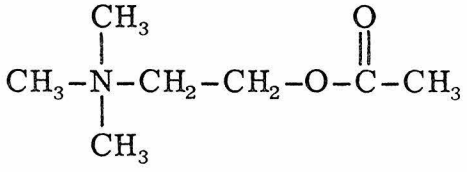
When this theory of synaptic transmission became accepted, Nachmansohn (6), Ahriens (7) and others began to picture the sequence of events occurring at the motor endplate as the result of the action of acetylcholine (ACh). The presently widely accepted theory is that there are three macromolecules functionally related to ACh: acetylcholine esterase (AChE), which hydrolyzes the ester to (relatively) inactive choline and acetate; choline o-acetyl-transferase, which synthesizes the ester; and the acetylcholine receptor (AChR), which is postulated as the entity which specifically recognizes ACh and other nicotinic molecules, resulting in a depolarization of the conductive tissue or a block of such depolarization, as with curare.

In 1942 Büchtal and Lindhard (8) showed that very small amounts of ACh applied to motor endplates produce a propagated contraction of the muscle fiber which, however, can be blocked by curare and by high concentrations of ACh. Their interpretation of these observations is that curare blocks the recognition site on the macromolecule specific for ACh.

The observed competition between ACh and curare has been attributed to their chemical similarity; for this reason curare and like compounds have been used in attempts to characterize the



d-Tubocurarine (curare)



Acetyl choline

"receptor". The compounds neither interfere with the release of ACh nor disturb the electrical properties of the endplate or muscle fibers, and are not destroyed by AChE. Competitive inhibition by curare of ACh action has two characteristics (9): 1) the blockade is antagonized by the drugs neostigmine and tensilon, both of which are also inhibitors of AChE; 2) a lasting tetanus effect (state of continuous contraction) is not obtained in muscles under curare action.

Many attempts have been made to isolate and/or characterize the postulated AChR; only the major ones will be outlined here. Noting the similarity of the specificity of the recognition site for ACh and other nicotinic substances on AChE and the postulated receptor, Župančič (10) and others have suggested that the two may in fact be the same; i.e., that the esterase serves the dual function of first recognizing the transmitter and then destroying it, in preparation for the next "message".

Nachmansohn et al. (11), on the other hand, made a distinction between the receptor and the enzymes of ACh metabolism,

describing the former as a protein which changes conformation under the influence of ACh, producing a change in ionic permeability. Ahriens (7) and Van Rossum et al. (12, 13) also described the receptor as a protein.

Chagas in 1956 (14) made the first attempt at isolation of the AChR from extracts of the electric tissue of Electrophorus electricus, the electric eel. He used radioactively labeled gallamine, which is a curare-like quaternary ammonium compound, to measure the binding capacity of fractionated extracts. Eventually an acidic mucopolysaccharide analogous to hyaluronic acid was isolated which bound gallamine to a greater extent than the crude extract (15). Further studies (16) showed, however, that neither equilibrium dialysis nor precipitating ability provide a reliable means of identifying the receptor, because the positively charged ammonium group will interact strongly with any acidic (and thus negatively charged) group, rendering the assay nonspecific. For example, both insulin and AChE can be readily precipitated by curare. Ehrenpreis (17) reported isolation of the receptor from the eel by curare precipitation of an ammonium sulfate fraction of the soluble portion of the homogenized tissue. However, no evidence for homogeneity of this precipitable fraction was given. He later reported nonspecificity in binding of ACh for his "receptor", which was confirmed by Beychock (18), who reported that a very heterogenous preparation was obtained by following Ehrenpreis' isolation scheme. Indeed, none of the fractions obtained from the heterogeneous preparation

bound curare any more tightly than other protein fractions from the eel regarded by Ehrenpreis as not containing the AChR.

Another attempt at isolation of the receptor by Trams et al. (19) resulted in the characterization of a sialo protein; however, comparative measurements of the binding capacity of this material for curare in equilibrium dialysis with that of Chagas' acidic mucopolysaccharide showed that the sialo protein interacted more weakly than the polysaccharide (20).

These early attempts at isolation of the receptor made clear that many pitfalls awaited investigators. In fact, as one reviewer pointed out, no concrete evidence is yet available which shows that the "receptor" is in fact a discrete, unique entity (20).

More recently, much work has been done which sheds some light on the physical and chemical properties of the postulated receptor. Some of these studies have been directed toward establishing whether or not the receptor and esterase active sites were identical, nonidentical but on the same macromolecule, or located on distinct macromolecules. Most have been performed on single cell preparations from eel, using electrophysiological methods to assess the effects of various reagents on the response of the cell to chemical stimuli. The cell used for these studies comes from the organ of Sachs, a section of the posterior part of the electric organ. These cells are extraordinarily large, measuring 5-10 mm in length and approximately 1 mm^2 in exposed surface typically; thus they are relatively easy to manipulate intact, and can be mounted in such a

fashion that their anterior and posterior surfaces are bathed by separate solutions. This useful feature allows the experimenter to vary the composition of the medium bathing either side of the cell. The preparation was originally described by Schoffeniels (21). The cell is asymmetric; one face is highly innervated and is chemically and electrically excitable, while the other is not innervated and is not electrically excitable. The innervated side carries a large amount of AChE and is considered to be the site of the receptor as well. The noninnervated face appears to be responsible for the maintenance of the $Na^{\oplus}$, $K^{\oplus}$ balance within the cell, and contains an $Na^{\oplus}$, $K^{\oplus}$-dependent ATPase.

Karlin in 1966 published the first in a series of papers in which he reported the effects of various sulfhydryl reagents on the response of the cell to chemical stimuli. He found (22) that exposure of the innervated face of the cell to 5×10^{-4} M p-chloromercuribenzoate (PCMB) or 10^{-3} M 1, 4-dithiothreitol (DTT) at pH 7.8-8.0 resulted in inhibition of the response to 5×10^{-5} M carbamylcholine (Carb), 5×10^{-6} M ACh, and 2.5×10^{-5} M trimethylbutylammonium ion (TMB). This inhibition was characterized by an increase in the concentration of Carb needed to elicit a half-maximal depolarization; there was no significant decrease in the maximal response when a high enough activator concentration was reached. He noted that such inhibition blocks the postsynaptic potential and indirect spike (both of which are normally the result of the presence of an activator such as ACh), but had no effect on the resting potential of the cell,

the direct spike (one stimulated electrically from an external source), or repolarization following a depolarization.

If PCMB was allowed to react for more than 10 min, both the rate and extent of repolarization were adversely affected. The same effect was observed if PCMB was applied to the noninnervated face, suggesting that the reagent was affecting part of the cation transport system. DTT did not have this effect.

The inhibition cannot be relieved by washing, but the PCMB effect could be reversed by thiol reagents, while the DTT inhibition was reversible by oxidizing agents (such as ferricyanide or 5, 5′-dithiobis-(2-nitrobenzoic acid) (DTNB), neither of which alone had any effect). It becomes irreversible, in the case of DTT treatment, if N-ethyl maleimide (NEM) is added. NEM alone, at pH 8, seems to adversely affect the ion transport system in a manner analogous to PCMB.

The inhibition produced by DTT could be duplicated by 50×10^{-3} M cysteine or 2-mercaptoethanol, and was reversible by DTNB. Lower concentrations of these thiols were without effect.

Noting that AChE had been used as a model for the receptor, and that suggestions had been made that the two entities are on the same molecule with fully or partially coincident active sites, Karlin (23) pointed out some of the distinctions between the receptor and the esterase. For example, the dissociation constant (K_S) for ACh from the receptor in the eel cell is 5×10^{-6} M, while from the esterase in the same cell it is 1×10^{-4} M. Irreversible inhibitors of

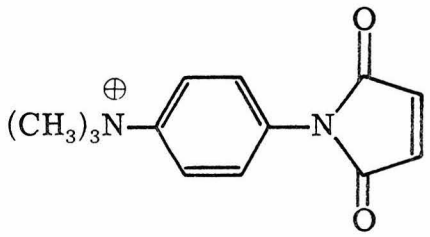
the esterase (such as organophosphates) potentiate the effect of ACh on the receptor and do not block the effects of nonhydrolyzable congeners of ACh. Sulfur and selenium analogs of ACh behave differently toward the esterase and receptor. Finally, as shown in the first paper (22), PCMB and DTT react with AChR to produce a 3-4 fold increase in K_S for ACh, while AChE reacted with these compounds under identical conditions, in both soluble and bound forms, suffers no change in catalytic properties. These results are taken as evidence that the two active sites cannot be fully coincident, and that it is unlikely they are even partially coincident.

At about the same time Podleski (24) reported that he too was able to distinguish between the active sites of the receptor and esterase. He found that AChR was inhibited by 1-methyl-7-hydroxyquinolinium ion, and that similar inhibition of the esterase could be achieved with the structurally analogous 3-hydroxyphenyltrimethylammonium ion (3-OH-TMA). When methanesulfonyl fluoride (MSF) was used to inhibit the esterase, he found no reduction in AChR response to either trimethylammonium ion (TMA) or 3-OH-TMA. If the esteratic site were coincident with the receptor site, then one would expect the presence of the covalently linked methane sulfonyl moiety to interfere with activator binding. Since it did not, the results were taken to be incompatible with the "same site" theory.

Changeux et al. (25) similarly showed that the two sites could not be the same using p-(trimethylammonium)benzenediazonium fluoroborate (Tdf). The reagent was chosen because it is a structural

analog of phenyltrimethylammonium ion (PTA), a potent depolarizer of the cell, and because it has a diazonium group capable of reacting with nucleophilic side chains. Tdf treatment renders cells irreversibly insensitive to receptor activators. It antagonizes the action of PTA, reacting with the receptor, but d-tubocurarine (d-TC) and flaxedil (gallaminetriethiodide) will protect the cell against Tdf. These investigators found that the rate of inactivation of the receptor is unaffected by di-isopropyl fluorophosphate (DFP) labeling of the AChE active site.

Karlin and Winnik in 1968 (26) reported a "specific" affinity labeling of the receptor. Their reagent was 4-(N′-maleimidophenyl)-trimethylammonium iodide (MPTA). This compound, like



MPTA

N-ethylmaleimide (NEM), reacts with sulfhydryl groups to produce a stable thioether.

When added to the native receptor in the single cell preparation, MPTA acts as a reversible competitive inhibitor, even at 5000 times the concentration at which, in the presence of DTT, it causes 50% irreversible inhibition. If the cell is pretreated with

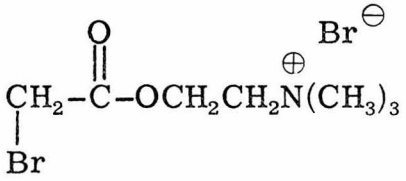
10^{-3} M DTT at pH 8, the DTT washed out, and then MPTA added at 10^{-8} M or higher, subsequent response of the cell to Carb is inhibited irreversibly. NEM reacted in the same way produces the same effect, only the necessary concentration of NEM is much higher. The relative enhancement of the rate of reaction of MPTA with the reduced receptor compared to NEM was calculated to be about 300-fold, after correction for intrinsic rate differences of the two maleimides reacted with L-cysteine.

Experiments involving reoxidation of DTT-treated AChR with DTNB showed that, in the absence of alkylating agent, the reduction and reoxidation were completely reversible. DTNB treatment of alkylated receptor did not restore any lost response. Hexamethonium (Hexa: $(CH_3)_3\overset{\oplus}{N}(CH_2)_6\overset{\oplus}{N}(CH_3)_3$) which is normally a reversible competitive inhibitor of AChR, becomes an activator after DTT reduction of the receptor, causing a depolarization. Hexa will partially protect the receptor active site from MPTA labeling, whereas there is no protection from NEM. This indicates that Hexa occupies the same anionic site as MPTA, competing with it at that location rather than at the reduced disulfide.

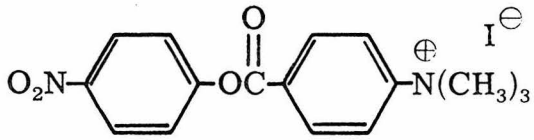
The investigators found further that DTT treatment decreases the apparent affinity constant of AChR for activators and that the esterase is not inactivated by DTT under the conditions used. They calculate that the receptor anionic site is 8-10 Å from the reduced disulfide on the basis of the distance between the quaternary nitrogen and the reactive double bond in MPTA.

The claim of specificity in labeling the receptor is made in the paper, but the authors show no evidence that this is in fact the case; indeed, all that can be concluded from the evidence presented is that the receptor is rendered inactive by MPTA under certain conditions, and that this inactivation proceeds 300-fold faster than alkylation by NEM under like conditions. There is no way to ascertain the extent of nonspecific labeling of other proteins present, since the only assay used is the physiological response of the cell.

Silman and Karlin (27) continued this line of investigation with two other active site-directed alkylating agents, bromoacetylcholine bromide (BAC) and the p-nitrophenyl ester of (p-carboxyphenyl)TMA



BAC



(p-carboxyphenyl)TMA p-nitrophenyl ester

iodide. BAC applied to the cell without prior reduction is a reversible receptor activator, causing depolarization. It is also a substrate for AChE. If it is reacted with a DTT-treated cell, some of the ensuing depolarization cannot be washed out. It was found that d-TC is capable of reversing this residual depolarization, which returns when d-TC is removed. The TMA derivative gave the same results. These data are interpreted in the following way: in the absence of prior reduction, the implicated disulfide bond is in the oxidized state,

and the sulfhydryls composing it cannot be alkylated. In this situation the compounds described act as ordinary reversible receptor activators. If the disulfide is reduced, two sulfhydryl groups become available to react, by nucleophilic displacement, with the quaternary ammonium derivatives, resulting in the charged ammonium function being covalently attached to one of the sulfhydryls of the receptor disulfide, which itself is estimated to be about 8-10Å from the anionic recognition site. At least in the case of BAC, the hydrocarbon chain is flexible and would allow the positive nitrogen some freedom of movement. If a ligand which had a strong enough binding constant were present, then one might expect the BAC ammonium group to be temporarily displaced from the anionic site. This is what is observed when d-TC is added to the BAC-treated cell. Since d-TC is an inhibitor, the residual depolarization is reversed as long as it is present.

Karlin's group, continuing their study of the chemistry of the disulfide near the anionic site, found that the reduced receptor could be reoxidized in the presence of certain thiols, among them cysteine, and more importantly, choline thiol and homologs (28). These latter compounds were capable of catalyzing reoxidation at concentrations less than 10^{-3} of the required concentration of cysteine; they were effective in the micromolar range. They are depolarizers of the cell, but the "affinity oxidation" described takes place at 1/10 the concentration required for depolarization. The effect is retarded in the presence of Hexa. The disulfides of choline thiol and homocholine

thiol were found to be ten times as effective in reversing the reduction as the thiols themselves. In addition, these bis-quaternary compounds are found to be competitive inhibitors of the native receptor and activators of the reduced receptor, analogous to the previously observed change in effect of Hexa under similar conditions (26).

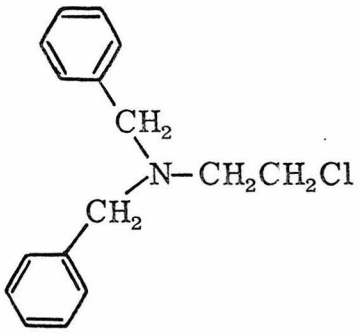
A variation on the above alkylation experiments was reported by Waser et al. (29). They used diazoacetylcholine, which is a depolarizer at concentrations in excess of 10^{-5} M. Like other receptor activators, it blocks indirect stimulation while leaving direct stimulation unaffected. The compound can be washed out slowly; however, if the preparation is strongly illuminated, a photo-decomposition of the diazo portion of the molecule occurs, producing a carbene which will insert into the protein. When this is done, the inhibition of the indirect response becomes irreversible.

In another approach, Bartels and Nachmansohn (30) studied the effects of organophosphate inhibitors on the electroplax. Normally compounds like DFP are considered rather specific reagents for active serine proteins; however, these authors found that at high concentrations and short time periods, organophosphates act as reversible inhibitors of the receptor. The rapid reversibility at high concentrations and short time periods, contrasted with an irreversible block observed using low concentrations over a long (30-60 min) exposure lent support to the contention that the interaction with the receptor is noncovalent, as opposed to the covalent linkage which

causes AChE inactivation. Further, the high concentrations required suggested that the interactions were weak. The authors postulated an interaction of the partial positive charge on the phosphorus atom with a nucleophilic group in the vicinity of the active site which would sterically hinder receptor activators. They also postulated an allosteric interaction as an alternative explanation; such an interaction could be thought to alter the protein's conformation and hence its affinity for cholinergic compounds.

Evidence for possible allostery was presented by Changeux and Podleski (31), who reported a sigmoid dose-response curve using Carb, PTA and decamethonium (Deca). They suggested there might be at least two sites on the AChR capable of binding ligands. The binding of one ligand might then influence the binding of a different one by changing the conformation of the AChR molecule ("protomer"). Such an influence was observed using Carb and Deca; the presence of a second activator shifts the dose-response curve for the first to lower concentrations and abolishes the sigmoid shape. This loss of "cooperativity" is not observed when receptor inhibitors (antagonists) are used.

Some of the more recent attempts at isolation will now be discussed. The first recent attempt at specific labeling was reported by Takagi et al. (32), who found that a component of smooth muscle from the small intestine of the dog could be irreversibly labeled with dibenamine. If the labeling was performed in the presence of atropine sulfate, a compound which competes with ACh for its



Dibenamine

receptor, then muscle contractions elicited by histamine or 5-hydroxytryptamine (serotonin) were irreversibly abolished. If then the atropine was washed out, a contraction could be elicited by application of ACh. This result was taken as evidence that the specific receptor for ACh could be protected (by atropine) while other receptors were vulnerable to inactivation.

These investigators treated cold-labeled, protected preparations with ^{3}H-dibenamine after washing out the atropine and found incorporation of label. This bound label could be digested by pronase, which suggested it was attached to protein. In a simple sedimentation experiment, they found that labeled material in a homogenate sedimented at 50, 000 xg, 60 min, but remained in the supernatant of a 10, 000 xg, 30 min spin. This would suggest that it was in the form of fairly large particles (larger than ribosomes, for example). This approach appears not to have been further pursued.

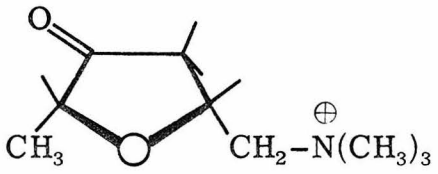
Another approach, followed by the group led by E. DeRobertis, involved extraction of material soluble in chloroform/methanol 2:1 followed by labeling with a radioactive cholinergic drug. Typically, these workers (33) used lyophilized whole tissue from the electric organs of either Torpedo marmorata (a marine skate) or E. electricus and extracted 0.16 or 1.0 g (equivalent to 2 or 12.5 g wet weight) with 15 ml of chloroform/methanol 2:1. Eel organs generally gave about 1/6 the yield of an equal amount of Torpedo tissue. The lipid extract was concentrated to about 1/3 its original volume under a stream of N_2. Then the radioactive drug was added and the mixture incubated at room temperature for about 20 min. The mixture was subsequently chromatographed on Sephadex LH-20 in a series of solvents. A separation of lipids and phospholipids from proteolipids was achieved, and cholinergic drugs such as methylhexamethonium, acetylcholine and p-trimethylammonium benzenediazonium fluoroborate were recovered mostly bound to the proteolipid fraction. The concentrations used were of the order of 10^{-6} M. If any excess free drug was present, it did not interfere with the results because experiments with free drugs showed they were irreversibly bound to the column. The investigators concluded that the only radioactivity observed must be bound to proteolipid. On the basis of known specific activities, they estimated about 3 molecules bound per 20,000 molecular weight, and about 65 mg of "receptor proteolipid" per Kg of fresh Torpedo tissue, or 33 mg per Kg of fresh Electrophorus tissue.

In a review (34), DeRobertis points out that previous approaches to isolation of the receptor have been essentially non-structural; that is, they have used whole tissue as a source rather than the synaptic region alone. Radioactive d-TC was found to bind in fractions of cerebral cortex which were rich in AChE; however, on isolation of "junctional complexes" he found that, after treatment with low concentrations of Triton X-100, binding of d-TC and AChE activity were separable; the enzyme was found in the supernatant while binding capacity remained in the sediment. Such binding could be interfered with by atropine and ACh.

Similar experiments performed with electric tissue from the eel gave the same results; binding of cholinergic agents was found in isolated membrane fractions which were also rich in AChE; in this case it was found that the enzyme could be completely solubilized by 1 M NaCl, while 85% of the binding capacity remained in the membranes.

DeRobertis also reported (34) studies on black lipid membranes using a proteolipid purified from electric tissue by LH-20 chromatography. He found that such membranes lost considerable electrical resistance when either "receptor" proteolipid or other proteolipid was added, but only the "receptor proteolipid" reacted to added ACh with a rapid and transient increase in conductivity. This was blocked by d-TC.

A third approach to the study and isolation of the ACh-receptor has been described by O'Brien's group. They used muscarone, a derivative of muscarine, which unlike the latter is effective at both



Muscarone

muscarinic and nicotinic receptors; this compound is structurally similar to ACh, but is a much more potent against at the receptor, and has a relatively low affinity for AChE, which does not hydrolyze it. Using extracts of the heads of the common house fly, they found high binding capacity in the supernatant from a 100,000 xg × 1 hr spin by equilibrium dialysis (35). The binding is reversible to at least 90%, and the apparent dissociation constant for muscarone from this extract is 2.4×10^{-6} M. The number of binding sites calculated is 70 n moles/g of fly head protein. Seventeen cholinergic compounds, including eserine (an inhibitor of AChE), were effective as competitors at 10^{-5} - 10^{-4} M for the binding sites, while of 11 noncholinergic compounds tested, only 4 exerted an effect at 10^{-4} M. Of various phospholipases and proteases tested, only trypsin and chymotrypsin were effective in destroying binding capacity, suggesting that the binding material is protein. Disc gel electrophoresis of the

homogenate was performed and sections of the gel were subjected to binding experiments. Only the basal slice showed activity, indicating the material was of very high molecular weight.

Eldefrawi and O'Brien note in this work (35) that there are similarities in the binding data for fly head and Torpedo electroplax extracts in regard to affinity (more that 3 times greater in the electroplax), reversibility, and the action of cholinergic agents. A point of difference is that whereas the fly extract is not affected by phospholisase C, this enzyme destroys 48% of the binding activity in Torpedo tissue extracts.

Extending this type of binding study, Eldefrawi et al. (36) investigated the specificity of binding of several cholinergic agents to particulate fractions of electric organ from E. electricus. In this tissue, they found apparent dissociation constants of 10^{-9}-10^{-8} M for the ligands tested, based on Scatchard plots. The four ligands tested were Muscarone, Nicotine, dimethyl-d-TC, and Deca. All were found to be completely reversible. The binding of muscarone and nicotine was reduced by the action of proteolytic enzymes and phospholipase C, while that of decamethonium was only reduced by proteolytic enzymes. Further, the binding of muscarone was reduced by the presence of any of the other three ligands.

In order to check specificity of binding, several proteins were subjected to these ligands at 10^{-8} M. A Krebs-Ringer buffer of salt concentration 0.2 M was used to minimize nonspecific interactions in these experiments. Only muscarone was specific for the eel

tissue; Deca, which has been widely used as a probe of the receptor, was the least specific, binding to 10 out of 28 proteins tested. Nicotine was reasonably specific; it also bound to butyrylcholinesterase. Dimethyl-d-TC bound to butyrylcholinesterase and phospholipase C. The results were interpreted as suggesting that muscarone and nicotine bind to a single site and compete, with similar dissociation constants:

Muscarone: $K_S = 5.5 \times 10^{-8}$ M, n = 0.021 n mole/g tissue

Nicotine: $K_S = 6.3 \times 10^{-8}$ M, n = 0.033 n mole/g tissue

The substance to which they bind was considered to be a phospholipoprotein due to the fact that binding capacity was destroyed by phosphlipase C and proteolytic enzymes.

A more promising attempt at isolation of the receptor than that of Takagi has been reported by Changeux' group (37-40). They showed that the innervated and non-innervated surfaces of the electroplaque were distinctly different in organization and function: the innervated face contains $2.5 - 5 \times 10^4$ synaptic terminals and is electrically and chemically excitable, while the non-innervated face is not excitable and is instead specialized for the transport of alkali cations. AChE is localized to the innervated face (37).

Beginning in 1970, several groups almost simultaneously initiated investigations on the receptor using neurotoxins derived from the venoms of elapid snakes (38, 39, 41-43). Several species of snakes have been used as sources for the toxins, all of which are

designated "α-toxins" and which act post-synaptically in a manner analogous to d-tubocurarine. The work of the Caltech group is the subject of this dissertation; accounts of studies published by other groups are reviewed in the concluding sections of this introduction.

Changeux' group reported working with a deoxycholate extract of a crude homogenate of electric organ, to which Mg^{++} and spermine had been added to remove most of the detergent and some protein. This extract was found by equilibrium dialysis to bind decamethonium at 60 n mole/g tissue with a $K_S = 8 \times 10^{-7}$ M. The bound Deca could be completely displaced by effectors of the cell in vivo, such as carbamyl choline, PTA and Hexa. Displacement was only 50-70% displaced by d-TC and flaxedil, a result which is in agreement with subsequent work using α-bungarotoxin (α-Bgt). In this work, Changeux et al. (38) found that the snake venom neurotoxin blocks 72% of the sites available to decamethonium (toxin conc = 20 μg/ml = 2×10^{-5} M. Deca conc = 5×10^{-7} M). Nonspecific Deca binding was reportedly avoided in these experiments by performing the dialyses in Ringer's buffer (ionic strength 0.18), but in considering these data it would seem wise to bear in mind O'Brien's results using Deca in the same buffer (36).

In a later paper, Changeux et al. (39) report further work on isolation of the receptor using α-Bgt. The toxin acts in vivo very much like curare in that it blocks transmission but does not cause depolarization; in contrast to curare, however, this action cannot be reversed. If d-TC is present before treatment with the toxin,

the cholinergic receptors are protected from irreversible blockade.

Changeux was able to repeat these results in vitro using radioactive Deca as the ligand in equilibrium dialysis. Under conditions where Deca binding is blocked by α-Bgt, no effect is observed on the activity of AChE.

Changeux states that, on adding an equal amount of membrane fragments (based on protein) from the noninnervated face to the liquid bathing the innervated face of a living cell, 30% more α-Bgt is necessary to produce an equal blockade of Deca binding. This was intended to show that Deca binding capacity which is sensitive to α-Bgt blockade is exclusively localized to the innervated face. This may well be the case, but the error in measurement is so large (Changeux estimates 30%, probably due to contamination of his non-innervated face fragments with innervated face material) that the interpretation remains open to question.

In vitro, Changeux et al. found a residual 28% of Deca binding which could not be displaced by α-Bgt. This 28% can be displaced by PTA, Carb and Hexa. This suggests that the binding of α-Bgt may be more specific than that of the smaller ligands. The work of Eldefrawi and O'Brien (36), indeed, shows that Deca in particular is quite nonselective with respect to its affinity for proteins, even in the presence of buffer of ionic strength 0.2 M.

Changeux' group subsequently showed (Meunier et al. (40)) that the recognition site of the receptor and the active site of AChE were located on different molecules, by means of differential thermal

inactivation and by selectively adsorbing the receptor to immobilized α-toxin from N. nigricollis. In the latter experiment, the toxin was coupled to Sepharose 4B, and then the protein extract from electric organ in 1% DOC was run through the column. Under their conditions, 75-100% of the receptor was retained while 85-100% of the esterase washed through. This suggests a potentially successful method of obtaining purified receptor, but no mention was made of such an attempt. Changeux' work has been summarized in a recent review (72).

The fact that α-Bgt seems to be much more specific in labeling the receptor than small molecules makes it potentially useful as a "tag" in isolation schemes. Miledi et al. (41) made use of radioactively labeled toxin in their attempt at isolation of the receptor from the tissue of T. marmorata. In their studies on electric tissue homogenates, they found that the nonionic detergent Triton X-100 adequately solubilized the receptor and toxin/receptor complex, and permitted binding of toxin to solubilized receptor. Molecular weight determinations by Sephadex G-200 chromatography and density gradient centrifugation suggested a weight somewhere between 240 K and 600 K, with subunits of approximately 88 K (determined in the presence of 0.5% SDS). Although a claim of near purity is made, no criteria are given. "Preliminary studies" on the "nearly pure" material gave a ratio of 1 μg toxin bound per 10 μg protein; from this, they calculate the subunit weight to be 80 K.

The last attempt at isolation to be discussed is that by Reich et al. (42). Basically, this procedure is similar to that of Miledi et al., in that detergent extraction is used and that a neurotoxin provides the "tag". However, conventional methods (i.e., DEAE cellulose) are used to achieve a partial purification. Again, no criteria of purity were offered for the isolated material.

A cautionary note has recently been sounded by Chavin (44) regarding labeling specificity. He points out that although criteria for active site specificity have been satisfied for many enzymes, there is little evidence that labeling has occurred exclusively at the desired site. It is probably true that, in transport or receptor systems, the protein of interest accounts for less than 1% of the total protein present. Specific labeling of a single protein has yet to be demonstrated when it is a minor component of a complex mixture. Some causes for this difficulty include the presence of other proteins of similar specificity, or a higher concentration of another protein which, although it has a higher K_m, may mask the smaller amount of the protein in question. In some cases, the desired protein may have a high K_m, which in itself tends to defeat specific labeling. The best possible way around such difficulties would seem to be in the use of a low mole ratio of label to protein, coupled with short reaction times.

The present study began as an attempt to isolate the acetylcholine receptor from electrogenic tissue of the electric eel, Electrophorus electricus, by using radioactive affinity labels.

The first attempts involved low molecular weight compounds; however, specificity was not achieved and Chavin's recent cautionary note (44) proved valid. Later attempts took advantage of the apparent specificity of α-bungarotoxin. Affinity chromatography using anti-toxin immunosorbents or Sepharose columns containing covalently bound α-Bgt, as well as more conventional protein isolation procedures, indicated that the actual isolation of the receptor may well be a long term project. Therefore, attention was turned to more fully characterizing the toxin-binding entity. This account traces work from the initial affinity-labeling attempts to a partial chemical and enzymological characterization of the toxin-binding material in electric tissue from both Electrophorus electricus and Torpedo marmorata, a marine electric skate.

Chapter I

Low Molecular Weight Affinity Labels

In view of Karlin's electrophysiological studies on intact electroplaque cells using MPTA (26), it seemed likely that this compound could be used as a specific affinity label for the receptor.

It was not known with any certainty at the time these experiments were begun whether the receptor would be found in the soluble portion of a homogenate or tightly bound to the membranes. In the intact cell, of course, the receptor is, by definition, a membrane-bound entity; however, some enzymes which are associated with membranes in the native state can be solubilized to a greater or lesser extent (e.g., acetylcholine esterase (45)). For this reason, the experiments to be described were performed on centrifugal supernatants and pellets, as well as homogenates of the whole electric organ. The "supernatants" described here actually contain a small amount of membrane fragments. These fragments are removed in the first ammonium sulfate fraction (to be described below); therefore, it is these fractions in which one would most likely find specific labeling if it occurred.

Synthesis of MPTA. (Methyl-^{3}H)-4-(N′-maleimido)-phenyltrimethylammonium iodide (MPTA) was synthesized essentially as described by Karlin and Winnik (26). In a 250 ml 3-neck flask equipped with mechanical stirrer and reflux condenser were dissolved

9.8 g (0.1 mole) maleic anhydride in 125 ml of dry diethyl ether. When solution was complete, 13.1 ml (13.6 g, 0.1 mole) of freshly distilled 4-(N, N-dimethylamino)-aniline in 25 ml of ether were run in rapidly but without overloading the condenser. Stirring was continued for 1 hr at room temperature; then the thick maroon suspension was cooled in an ice bath and filtered by suction. The residue was washed twice with small portions of cold ether and air-dried. Yield was 22.6 g (97% of theory).

The tertiary amine precursor of MPTA, MDA, was prepared by cyclizing the maroon adduct in acetic anhydride. One g adduct, 0.3 g of anhydrous sodium acetate and 2.5 ml of acetic anhydride were heated for 10 min in a stoppered flask on a steam bath. The solvent was then removed under vacuum on a rotary evaporator at 65-70°. The resulting material was dissolved in a 60/40 mixture of cyclohexane and dichloromethane and run onto a 1.5 × 20 cm column of silica gel equilibrated with the same solvent. 500 ml of this solvent mixture were run through, followed by 1 liter of dichloromethane. When orange color began to emerge, collection was begun and continued until all of the solvent had passed through the column. Impurities generated during cyclization are retained tenaciously by the silica gel, while the MDA is slowly eluted by dichloromethane. Solvent was removed under vacuum and the last traces were "chased" with diethyl ether, to avoid interference in the next step by dichloromethane. The orange residue was air-dried.

500 mg of the powder obtained was put into a heavy-walled ampoule of about 3 ml volume, and about 2 ml of diethyl ether were added. Trapped air was removed by careful application of aspirator vacuum. A few pieces of freshly scraped copper wire were added to scavenge any free I_2 formed during the methylation and to act as agitators. The ampoule was then cooled in dry-ice/acetone (not liquid N_2) and sealed onto a small vacuum line. Also sealed onto this line was a breakseal ampoule containing ^{3}H-methyl iodide (nominally 237 mg, 60 mCi/mmole). The system was evacuated and sealed. Then the breakseal was shattered and the CH_3I was condensed into the MDA/ether suspension. The system was allowed to warm once to room temperature, then was re-cooled and the reaction ampoule sealed off. The suspension was tumbled end-for-end in a steam bath for 14 hr.

The ampoule was then cooled and opened. The contents were poured into a large amount of ether and filtered. The insoluble residue was washed extensively with ether, then recrystallized from spectro-quality acetonitrile and benzene. The yellow crystals were washed with ether and air-dried. The yield was 427 mg (71% of theory based on methyl iodide) with a specific activity of 36.8 Ci/mole. A melting point and nmr spectrum were not determined for the hot compound, but it was tested for its ability to react with thiols; the test was positive (nitroprusside color test). Cold MPTA prepared the same way had the correct melting point and nmr spectrum as well as the capability of reacting with thiols.

Experimental Rationale. Preliminary experiments showed that ^{3}H-MPTA alone did not exhibit chemical specificity when tested on extracts of electric tissue. For this reason, experiments were planned in which N-ethyl maleimide (NEM) was present in 50-100-fold molar excess over ^{3}H-MPTA. It was hoped that the NEM would react at about the same rate as MPTA with sulfhydryls generally, but that MPTA would react with the sulfhydryl near the anionic site of the receptor at 300 times the rate of NEM (26). Thus, one would expect to see a constant and presumably low background of ^{3}H activity over a protein profile except at the point containing the receptor; here one would expect to see a substantial increase in ^{3}H activity.

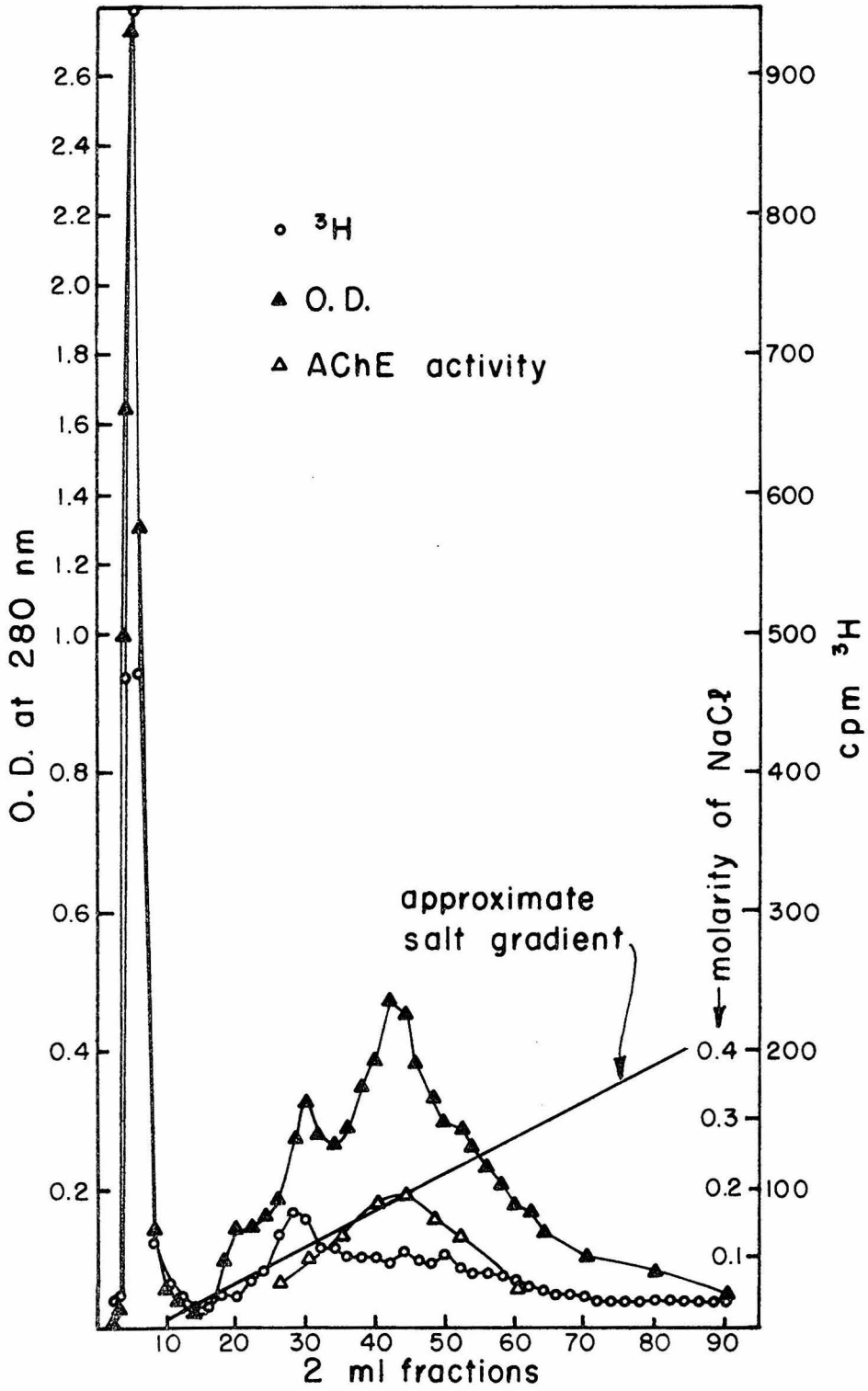
Preparation of Tissue Extracts and Use of MPTA and NEM. Extracts of electric organ from Electrophorus were prepared by grinding diced tissue under liquid N_2, then suspending the pulverized tissue in 0.1 M phosphate buffer, pH 7, and centrifuging at about 12,000 xg for 10 min.

The resulting supernatant was separated into two lots; one was pretreated with 10^{-3} M DTT at pH 8 under a stream of N_2 and then dialyzed exhaustively against pH 7 buffer under N_2 to remove excess reagent. This procedure, according to Karlin (26), reduces a disulfide bond near the anionic site to a free sulfhydryl group which can react covalently with maleimides; in the absence of prior reduction these reagents exert no permanent physiological effect.

Following dialysis, a mixture of NEM and ^{3}H-MPTA in a molar ratio of 50-100 was added to both samples. The MPTA concentration was fixed at about 1-5 $\times 10^{-6}$ M to minimize nonspecific interactions; 10^{-6} M is the concentration at which it elicits the half-maximal electrophysiological inactivation after DTT treatment (26). Reaction with the extract was allowed to proceed for about one min, whereupon it was quenched with an excess of 2-mercapto ethanol. The labeled extract was chromatographed on either DEAE-cellulose at pH 8, using a linear salt gradient from 0.01 to 0.5 M (see Figure 1), or fractionated by ammonium sulfate precipitation and subsequent chromatography of the fractions on Sephadex G-200. Only the portion precipitated by 10% (w/v) ammonium sulfate is shown (Figures 2a, b) since the labeled receptor, if present, would be expected to be in this fraction. The remaining ammonium sulfate fractions showed virtually no change in their radioactivity/protein ratio.

Results. Very little specificity of labeling was observed in the elution profiles from either DEAE cellulose or G-200; recovered radioactivity rather closely followed absorbance at 280 nm. The ion exchange column results showed, however, that AChE activity was unaffected by the treatment described, and that ^{3}H activity did not parallel AChE activity. This is presumptive evidence that the receptor and esterase functions are not located on the same molecule, and is in agreement with the results of other investigators (40, 41).

Figure 1: Chromatography of ^{3}H-MPTA-labeled extract on DEAE cellulose. Buffer: 0.01 M Tris Cl, pH 8. Gradient: 0-0.5 M NaCl, begun as indicated. Column dimensions 0.9 × 15 cm.


o ³H
▲ O. D.
△ AChE activity
O. D. at 280 nm
cpm ³H
molarity of NaCl
approximate
salt gradient
2 ml fractions
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
900
800
700
600
500
400
300
200
100
0.4
0.3
0.2
0.1
10
20
30
40
50
60
70
80
90

Figures 2a, b: Chromatography of ^{3}H-MPTA-labeled supernatant precipitable by 10% (w/v) ammonium sulfate on G-200. Column dimensions: 2.5 × 41 cm. Buffer: 0.1 M sodium phosphate, pH 7.

Figure 2a: pretreated with 10^{-3} M DTT before labeling.

Figure 2b: no DTT pretreatment before labeling.

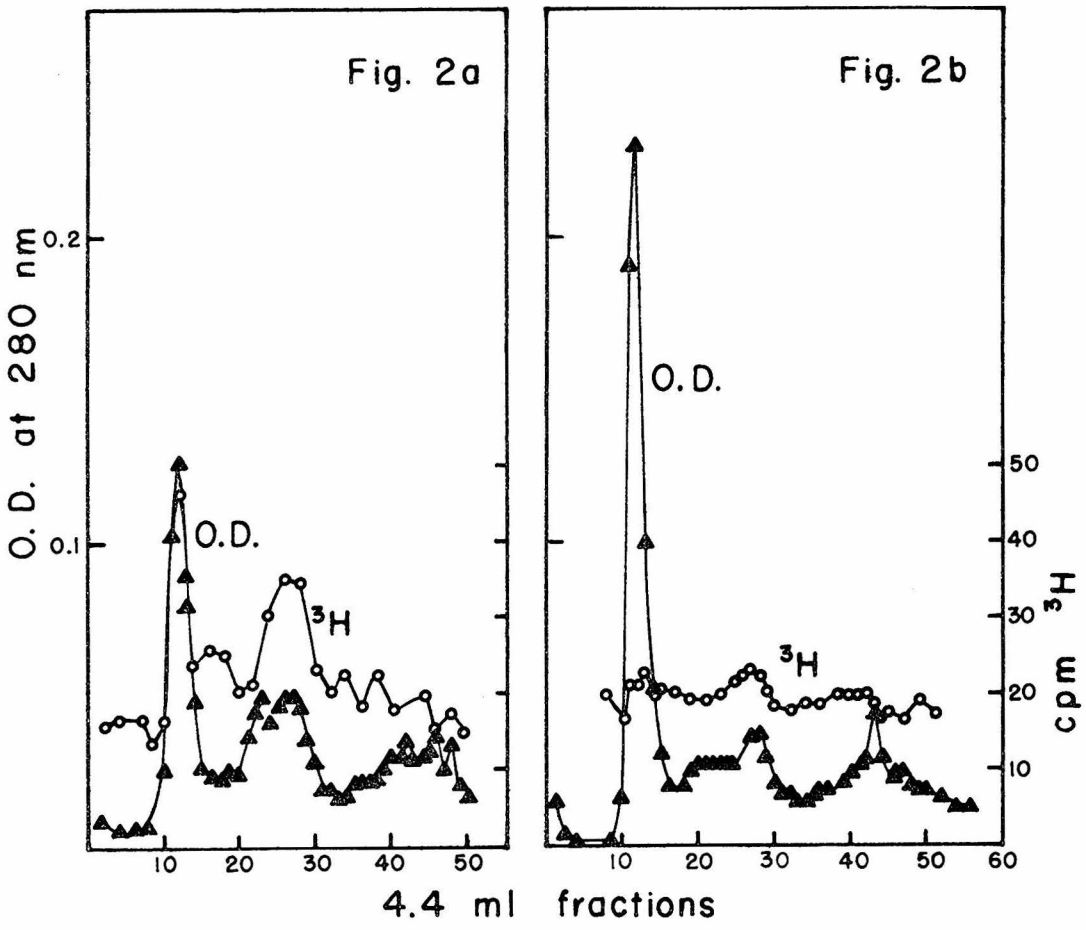


Fig. 2a
Fig. 2b
O. D. at 280 nm
0.2
0.1
O.D.
^{3}H
O.D.
^{3}H
10
20
30
40
50
60
cpm ^{3}H
10
20
30
40
50
4.4 ml fractions

Results from Labeling of Membrane Pellet. Similar experiments were performed on the pellet from the centrifugation step. Counting of weighed amounts of lyophilized membrane fragments likewise did not indicate any specificity of labeling.

Discussion. It was concluded that there are too many proteins present in both the supernatant and pellet which contain free sulfhydryl groups capable of reacting with maleimide derivatives, and that reduction with DTT merely increases this number. A method was needed which would discriminate more cleanly between nonspecific and specific labeling.

Refinement of Double-Label Experiments--Rationale. It was thought that a double-label experiment involving ^{3}H-MPTA and ^{14}C-NEM would provide this cleaner distinction. Instead of the radioactivity-to-protein ratio, the important quantity would be the $^{3}H/^{14}C$ ratio. Nonspecific labeling would be expected to give a low and fairly constant $^{3}H/^{14}C$ ratio, while specific labeling of the receptor would hopefully give a high $^{3}H/^{14}C$ ratio; if free of contaminants in a protein profile this ratio would be expected to be 300-fold greater than that for nonspecific labeling (26).

Experimental. Experiments to test this hypothesis were carried out essentially as above, except that ^{14}C-NEM was substituted for the cold reagent. After dialysis to remove unreacted reagents, the labeled extracts were fractionated by ammonium sulfate and these fractions chromatographed on G-200.

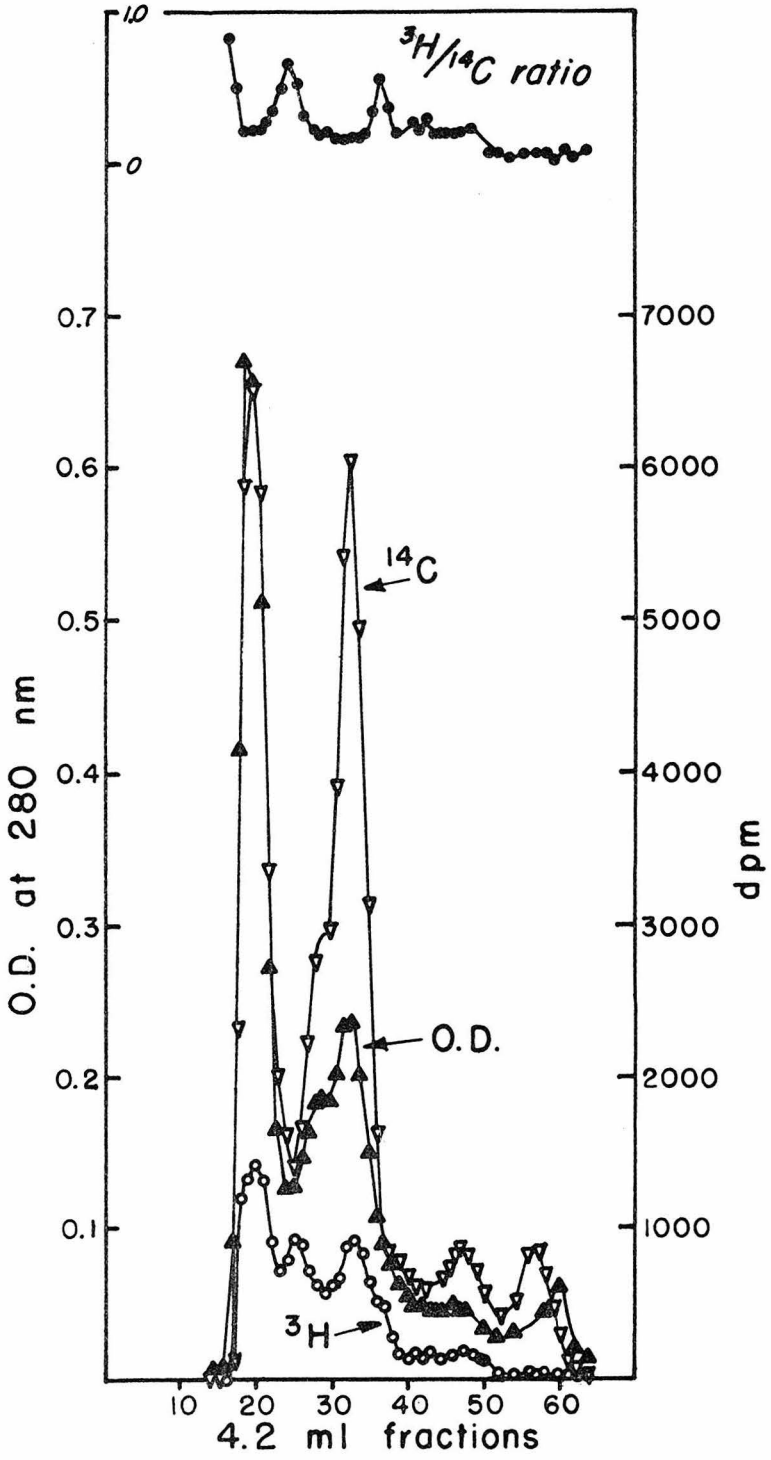
Results and Discussion. Analysis of the radioactivity profile showed no case where the $^{3}H/^{14}C$ ratio changed by more than a factor of 2, which would mean that the receptor, if in such a peak, would comprise less than 0.7% of the labeled protein present. Clearly, specificity was still lacking.

Final Refinement Attempt. In a final effort to increase specificity, the reaction time was reduced to 15 sec, and labeling was performed in the presence of 10^{-4} M DTT to help reduce non-specific reactions with protein sulfhydryl groups.

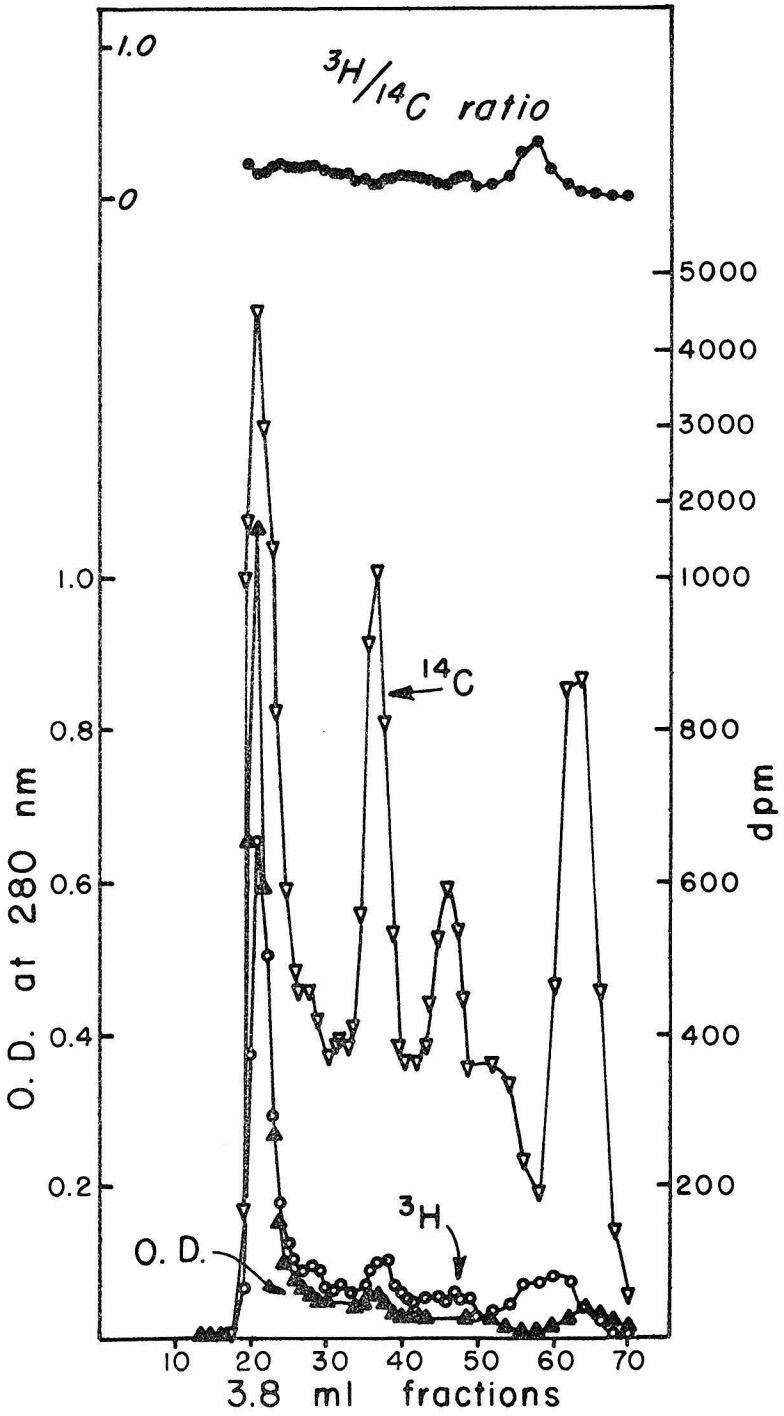
The labeled ammonium sulfate fractions were chromatographed as before on G-200. Furthermore, the labeled pellet from the centrifugation step was extracted first with chloroform-methanol 2:1 and then with 2.5% sodium dodecyl sulfate (SDS). The organic extract was concentrated and chromatographed on LH-20, while the SDS extract was separated on a Sepharose 4B column in 0.5% SDS.

Results and Discussion. The organic and detergent extracts of the pellet were chromatographed to determine whether the labeled receptor might be solubilized from the membrane only under more vigorous conditions, since little specific labeling had been observed in the supernatant fractions. The results of the three kinds of separations are shown in Figures 3-5, and indicate that, again, no great specificity was achieved. The conclusion drawn was that sufficient specificity is not attainable with free MPTA acting on a crude preparation of electric tissue.

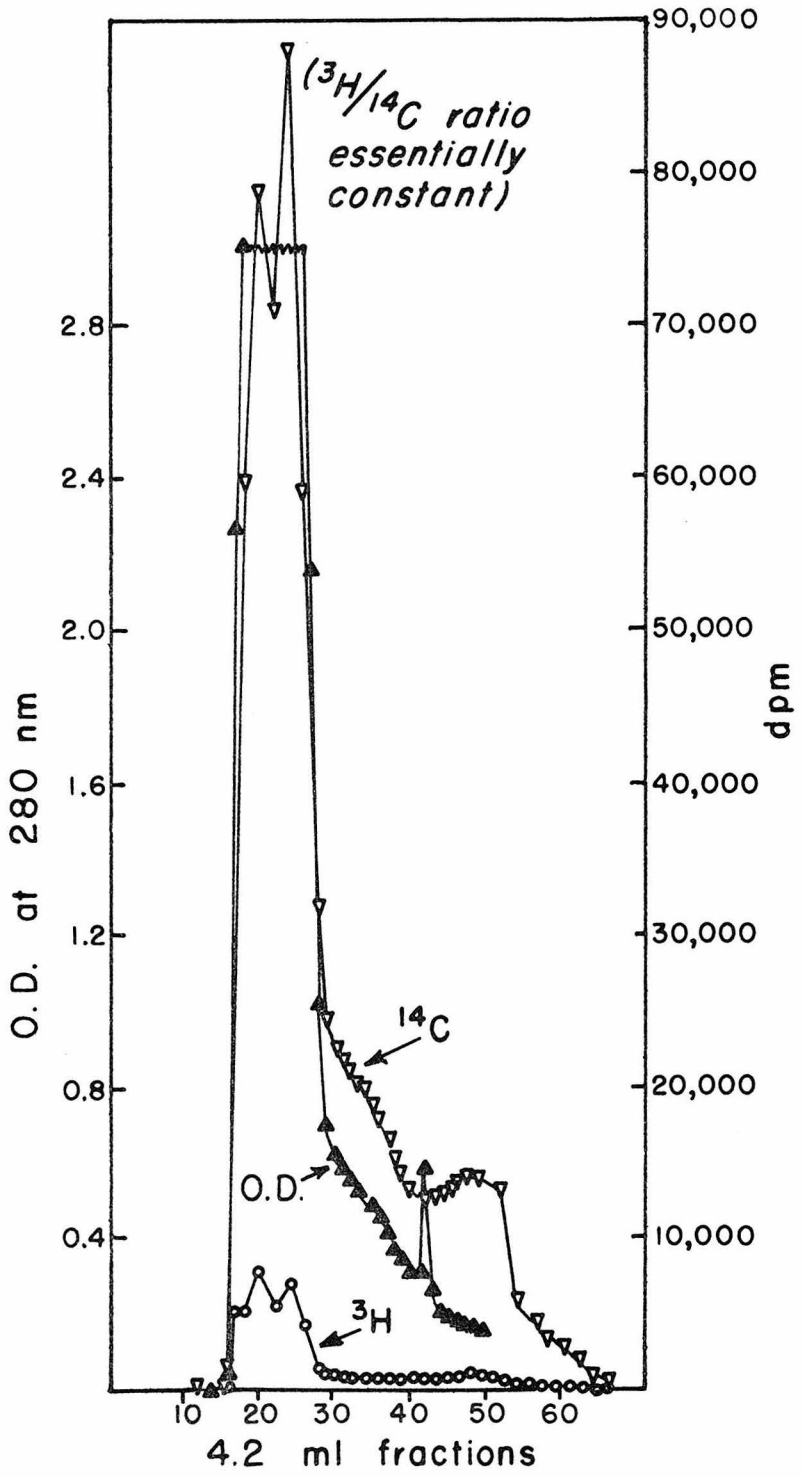
Figures 3a-g: Chromatography of ^{3}H-MPTA and ^{14}C-NEM labeled total supernatant and ammonium sulfate fractions thereof. Numbers beneath individual figures followed by "% AS" refer to material precipitating in that range of w/v% solid ammonium sulfate added.
Column: 2.5 × 41 cm G-200 in 0.1 M sodium phosphate, pH 7.



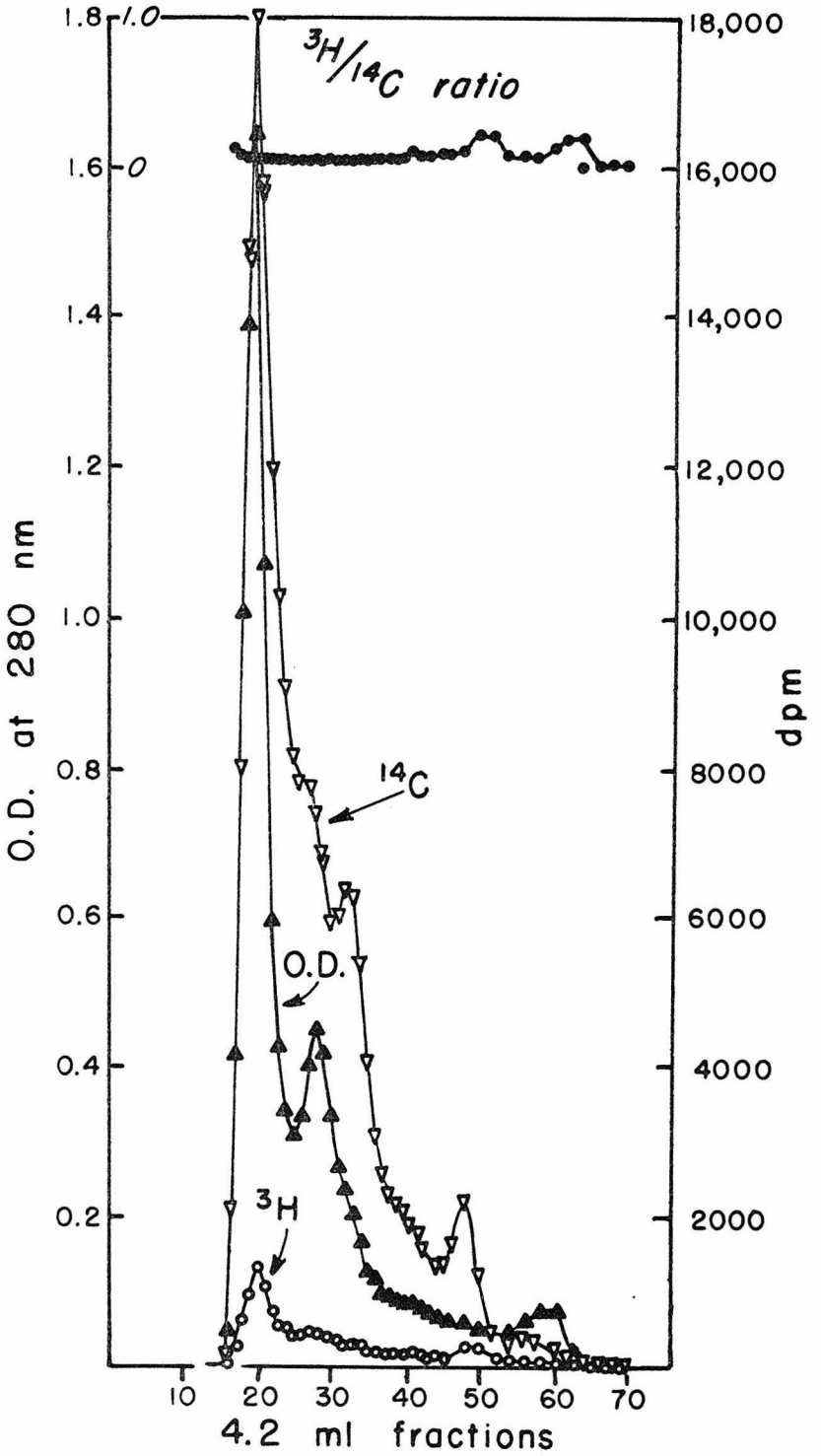
Total Supernatant


1.0
0
$^3H/^{14}C$ ratio
5000
4000
3000
2000
1000
800
600
400
200
dpm
1.0
0.8
0.6
0.4
0.2
O. D. at 280 nm
^{14}C
^3H
O. D.
10
20
30
40
50
60
70
3.8 ml fractions

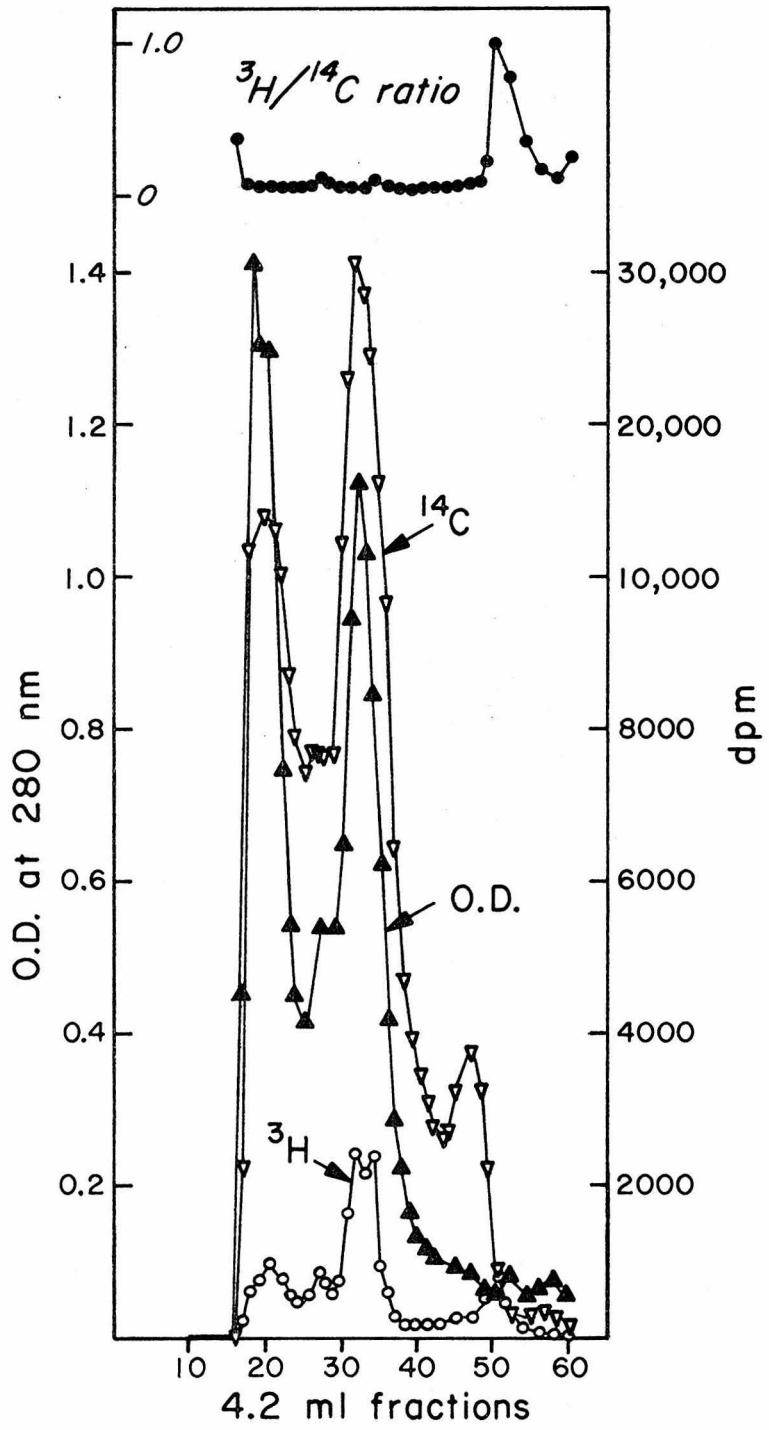
0-10 % AS


(3H/14C ratio essentially constant)
90,000
80,000
70,000
60,000
50,000
40,000
30,000
20,000
10,000
dpm
2.8
2.4
2.0
1.6
1.2
0.8
0.4
O. D. at 280 nm
14C
O.D.
3H
10
20
30
40
50
60
4.2 ml fractions

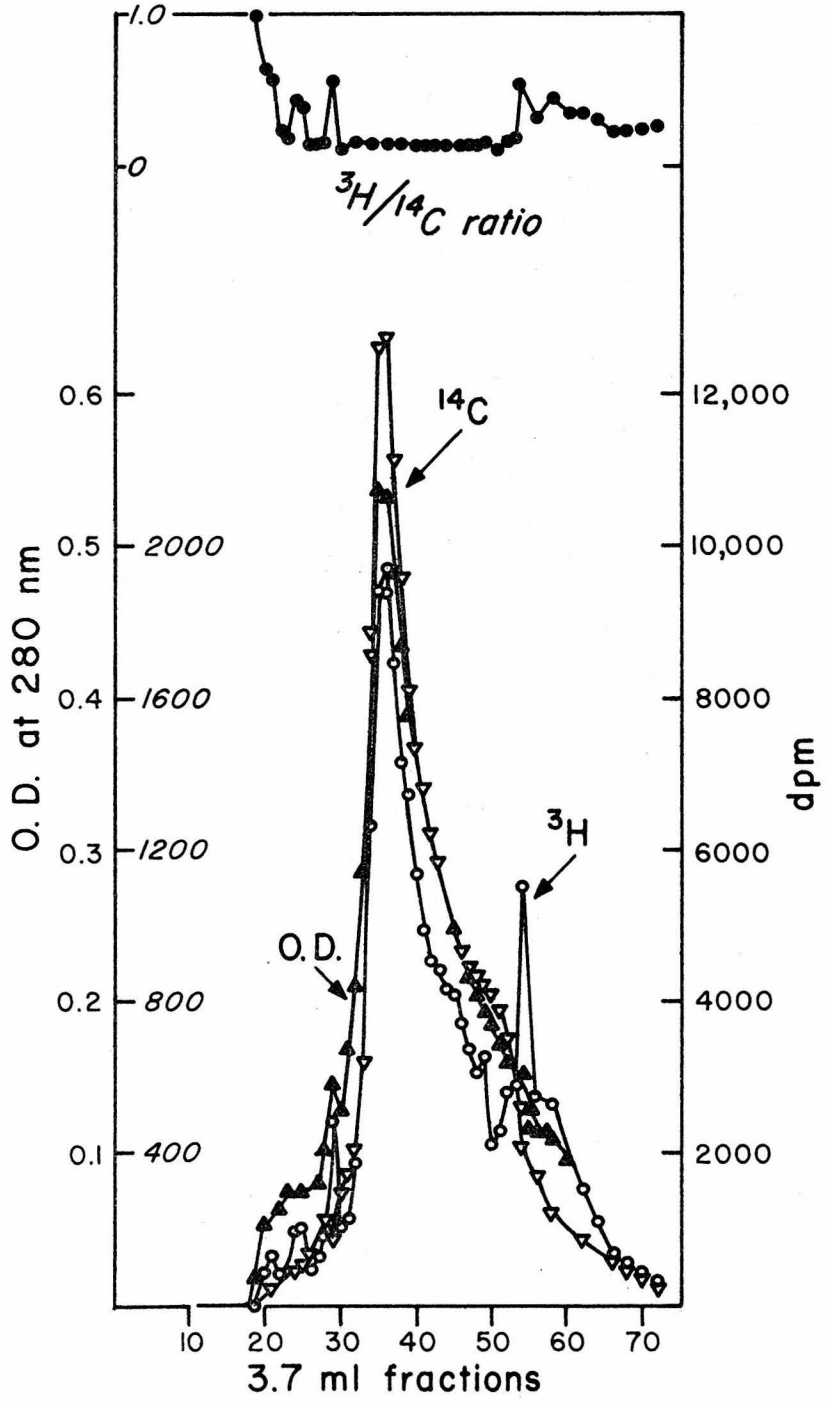
10-20% AS


1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
1.0
0
18,000
16,000
14,000
12,000
10,000
8000
6000
4000
2000
dpm
O.D. at 280 nm
$^3H/^{14}C$ ratio
^{14}C
O.D.
^3H
10
20
30
40
50
60
70
4.2 ml fractions

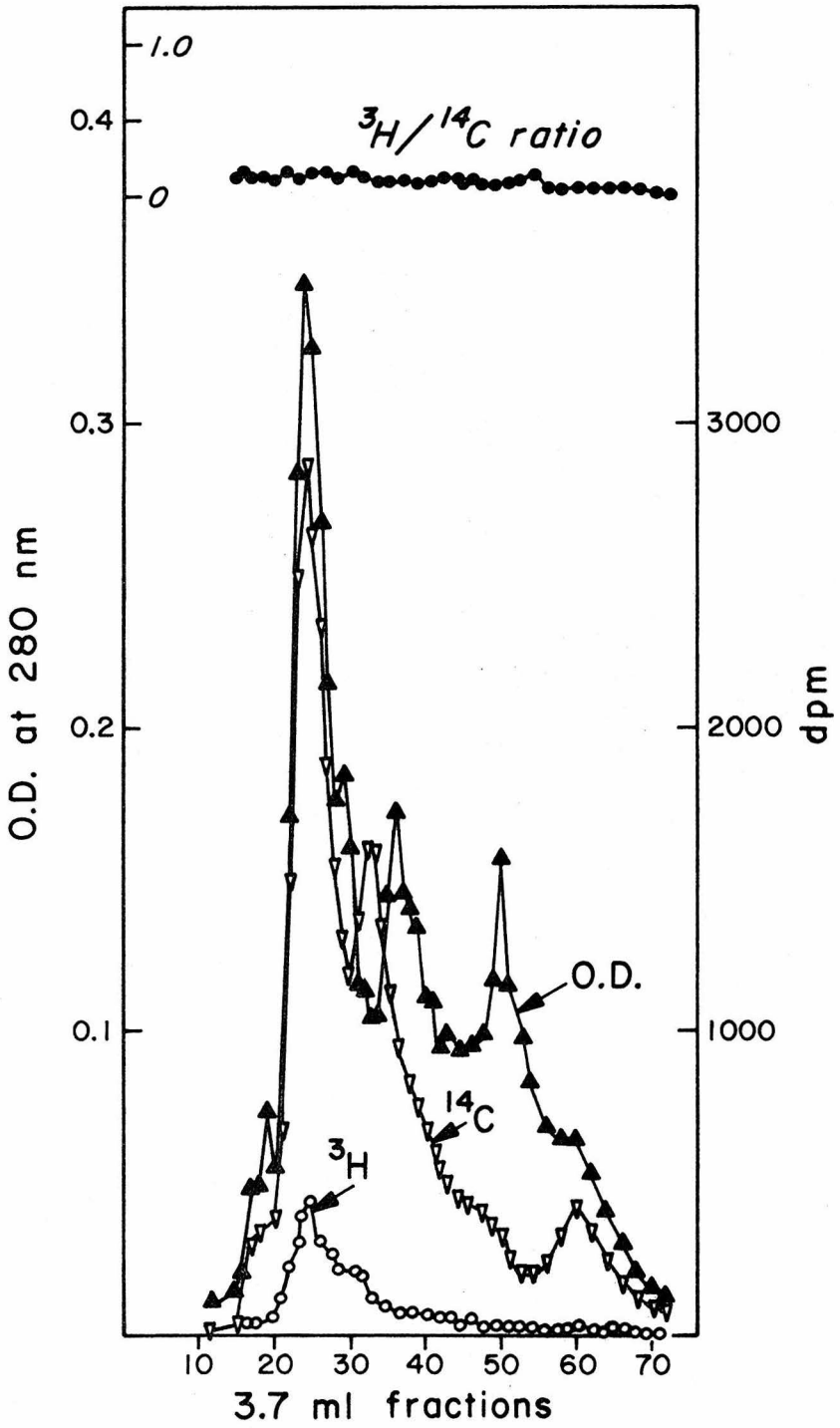
20-30% AS


1.0
0
$^3H/^{14}C$ ratio
1.4
1.2
1.0
0.8
0.6
0.4
0.2
O.D. at 280 nm
30,000
20,000
10,000
8000
6000
4000
2000
dpm
^{14}C
O.D.
^3H
10 20 30 40 50 60
4.2 ml fractions

30-40% AS


1.0
0
$^3H/^{14}C$ ratio
0.6
0.5
0.4
0.3
0.2
0.1
2000
1600
1200
800
400
O. D. at 280 nm
^{14}C
^3H
O.D.
12,000
10,000
8000
6000
4000
2000
dpm
10
20
30
40
50
60
70
3.7 ml fractions

40-50% AS


1.0
0.4
$^3H/^{14}C$ ratio
0
0.3
0.2
0.1
O.D. at 280 nm
3000
2000
1000
dpm
O.D.
^{14}C
^3H
10 20 30 40 50 60 70
3.7 ml fractions

50-60% AS

Figure 4: Chromatography on LH-20 of material extracted from the ^{3}H-MPTA and ^{14}C-NEM labeled pellet (same experiment as described in Fig. 3) by chloroform-methanol 2:1. Elution solvent: toluene-methanol 1:1.

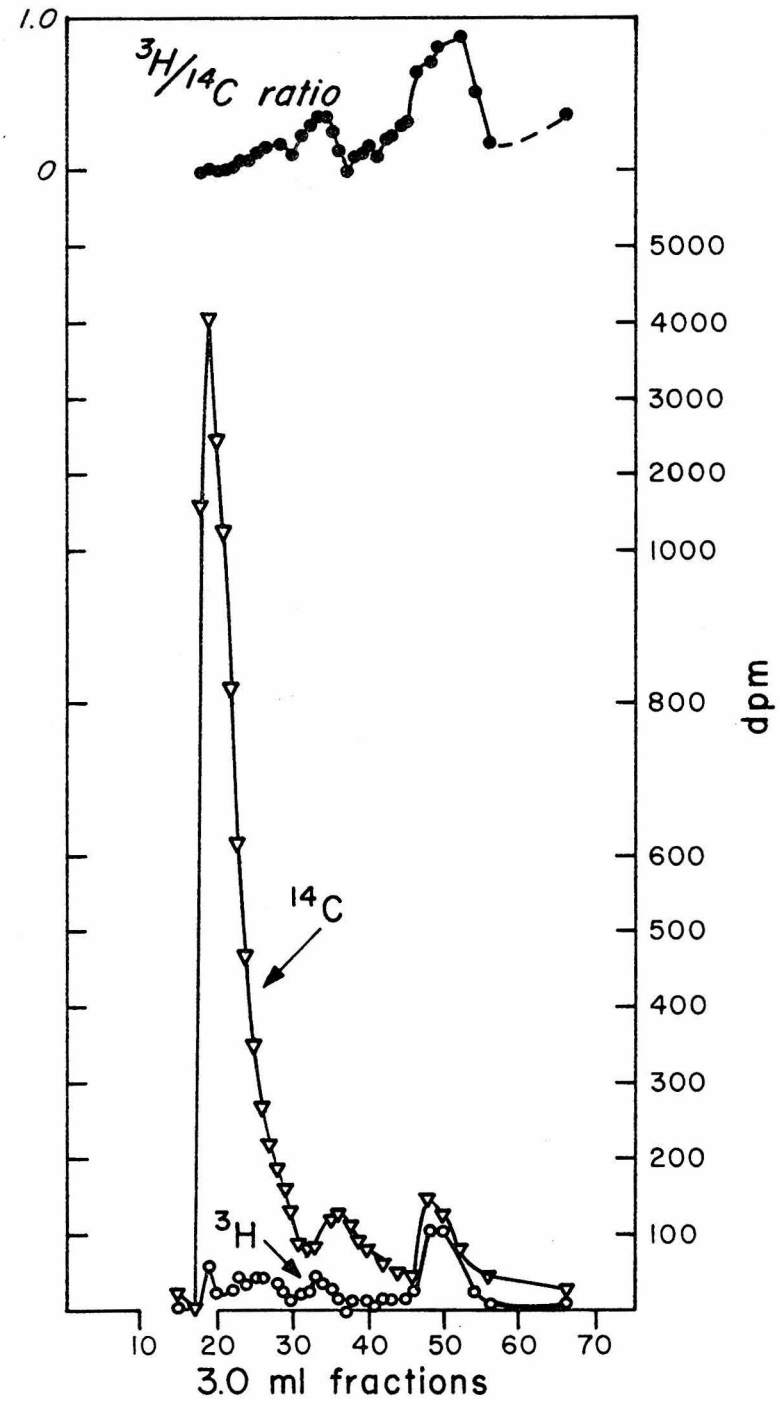
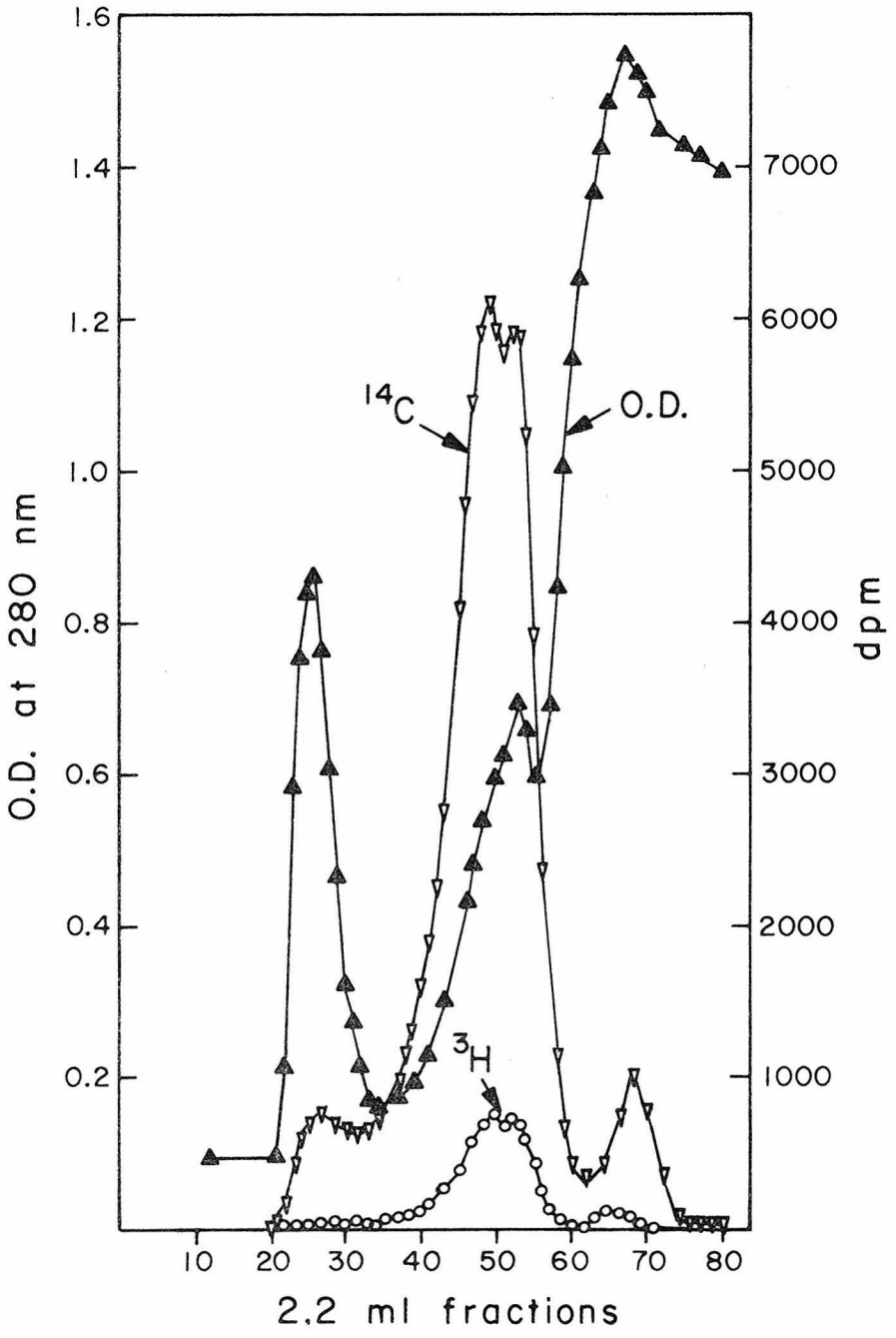

1.0
0
$^3H/^{14}C$ ratio
5000
4000
3000
2000
1000
800
600
500
400
300
200
100
dpm
^{14}C
^3H
10
20
30
40
50
60
70
3.0 ml fractions

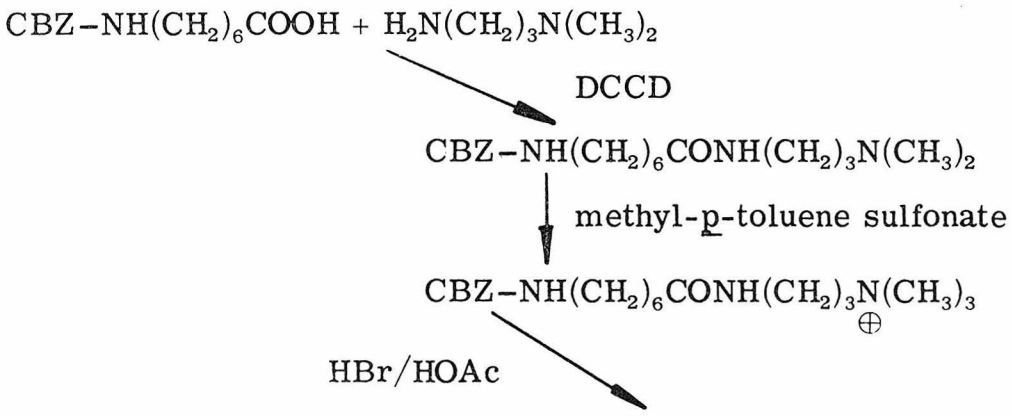
Figure 5: Chromatography of 2.5% SDS extract of lipid-extracted pellet (see Fig. 4 legend).
Column: Sepharose 4B, 2.5 × 41 cm, in 0.5% SDS + 0.05 M Tris Cl, pH 8. $^{3}H/^{14}C$ ratio was essentially constant (about 0.1) and was not plotted.

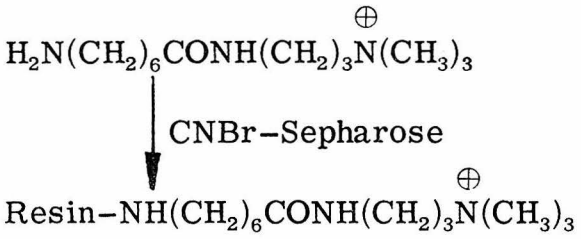

1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
O.D. at 280 nm
7000
6000
5000
4000
3000
2000
1000
dpm
^{14}C
O.D.
^{3}H
10
20
30
40
50
60
70
80
2.2 ml fractions

Affinity Chromatography. An alternative approach to affinity labeling was through the use of such labels covalently attached to an insoluble support. It seemed likely that the receptor would bind reversibly (and with high affinity) to an acetylcholine-like molecule so immobilized, and would thus be retarded, while other proteins would either pass through the column unhindered or would bind with a much lower affinity than the receptor. In either case, it seemed likely that contaminating proteins could be washed out, leaving the receptor "stuck" to the column. The latter could then be released by adding a reversible inhibitor or even acetylcholine itself to the column eluant.

Several such adsorbents were tried; they were generally synthesized by the method of Cuatrecasas (46), using cyanogen bromide-activated Sepharose and coupling the quaternary ammonium derivatives through long arms. Two such adsorbents will be briefly described.

The first was synthesized according to the reaction sequence



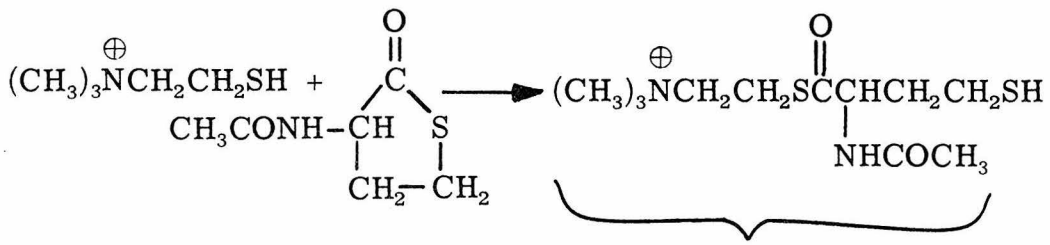


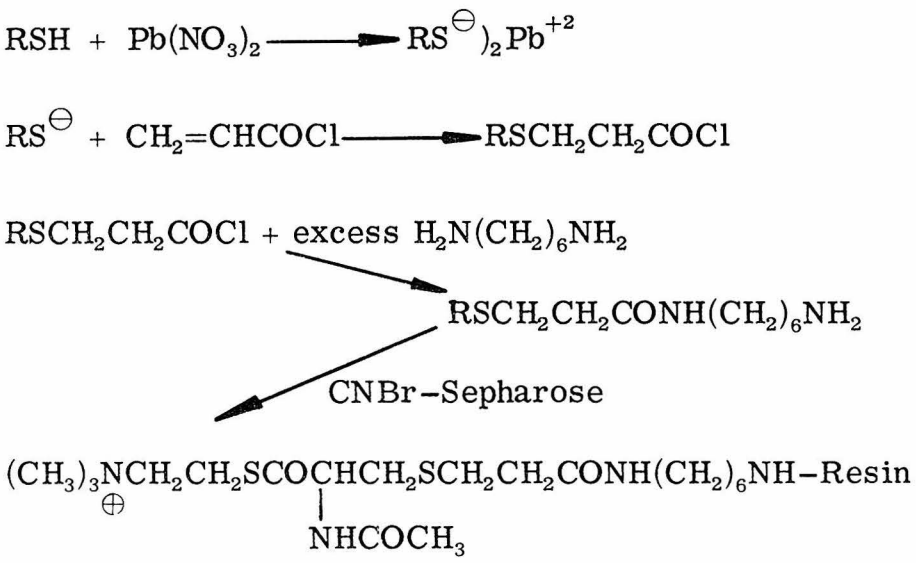
CBZ: carbobenzoxy group used to protect primary amines

DCCD: dicyclohexyl carbodiimide

Several experiments showed that this resin removed AChE from a homogenate of electric tissue in active form, but bound it so tenaciously that it could not be removed by 1 M ACh or 0.1 M hexamethonium. Since the receptor has an even higher affinity for acetylcholine than does the esterase, this resin was not practical for isolating the receptor.

Another adsorbent was prepared whose quaternary ammonium function could be hydrolyzed at alkaline pH. The rationale here was that the resin with adsorbed receptor could be extensively washed to free it of contaminating proteins, and then the ionic group itself could be hydrolyzed off the resin to release the bound protein. The reaction sequence was as follows:





Unfortunately, this column also retarded a large amount of protein; only 10% of the AChE activity initially present could be washed off with 0.1 M hexamethonium. It was concluded that hydrolysis of the column would be futile due to the large amounts of contaminating proteins still bound.

Evidence for Nonidentity of AChR and AChE. It was possible to show that proteins which could be covalently labeled with MPTA (under conditions of prior DTT reduction) were distinct from AChE, using the column just described. Electrophorus electric tissue homogenized and labeled as previously described was loaded onto the column and eluted first with 0.1 M phosphate pH 7, then with a linear gradient to 0.1 M hexamethonium in the same buffer. Analysis of the effluent revealed that none of the radioactivity was retained but that the esterase was recovered at the start of the hexamethonium gradient, free of radioactivity. This result again suggests that the esterase and receptor active sites are located on

different molecules.

Conclusion. The conclusion drawn from these experiments with low molecular weight acetylcholine analogues is that small ionic ligands lack specificity in the chemical sense even though they may possess it as judged by measurement of physiological responses. This approach was therefore abandoned in favor of the apparently more specific snake neurotoxin label.

Chapter 2

Purification and Characterization of α-Bungarotoxin

Several neurotoxins from the venoms of elapid snakes have been used in studies related to characterization and isolation of the cholinergic receptor (38, 39, 41-43). The apparent specificity and irreversibility of α-bungarotoxin (α-Bgt) (38, 41, 43) recommend it as a possible label for use in this effort.

In order to be certain that α-Bgt is in fact a useful label, it was first necessary to purify and characterize it. C. Y. Lee reported the amino acid composition of the α- and β-toxins from the venom of B. multicinctus (47); however, the purification of α-Bgt had not yet been described in full (39, 47) at the start of our investigation. This chapter (which has been published (48)) describes the purification and characterization, by both chemical and physiological criteria, of α-Bgt, as well as preparation of toxin-specific antibodies.

Experimental

Materials. Crude venom of B. multicintus (Bgv) was obtained from two sources: Sigma Chemical Co., lots 40B-2170 and 49B-0830 and Miami Serpentarium Laboratories, lot BM 12345F. The carboxymethyl cellulose used was Whatman CM-52. Sepharose 2B and Sephadex gels used were from Sigma, and the potato starch

used was "Swan" brand, from Stein-Hall and Co., New York. Reagent grade chemicals were used throughout.

Male rabbits were obtained from Hill Top Labs, Chatsworth, California, Freunds Adjuvant (complete) from Difco and 0.9% nonpyrogenic saline was obtained from McGraw Laboratories.

^{125}Iodide, carrier-free, was obtained from New England Nuclear and all samples were counted in a dioxane-based scintillator on a Packard TRI-CARB model 3375 liquid scintillation counter.

Ampholine, pH range 7-10, was an LKB product.

Methods

Electrophoresis on Potato Starch. Resolution of the components of Bgv by starch electrophoresis was performed in a 3.5 by 42 cm hemicylindrical glass trough as decribed by Chang and Lee (49). Constant-voltage electrophoresis (300 V) was conducted at 4° for 40 hr. Upon completion of the run, the starch was divided into and removed in sections of 0.5 cm, each of which was washed with 3 ml of water. Protein concentration in the supernatant was determined by the method of Lowry et al. (50) and the extinctions of each fraction at 260 and 280 nm were determined. These data are summarized in Figure 6. Various fractions were tested for their effectiveness in blocking the response to ACh in frog rectus abdominis muscle, and their AChE activity was determined by the method of Ellman et al. (51). The desired starch fractions were each washed twice more with water, pooled, concentrated by pressure dialysis

using an Amicon UM-2 membrane, and further purified by gradient-elution chromatography as described below.

Chromatography on Carboxymethyl Cellulose. Various components from potato-starch electrophoresis were pooled, desalted, lyophilized and dissolved in a minimal volume of 0.05 M NH_4OAc, pH 5.0 and applied to a 2.5 by 20 cm column of Whatman CM-52, equilibrated with the same buffer. Elution was carried out using a linear 1 ℓ gradient of NH_4OAc from 0.05 M, pH 5.0 to 1.0 M, pH 6.8. Fractions of 5 ml each were collected. The protein concentrations of fractions were determined by the Lowry et al. (50) method and their optical densities at 260 and 280 nm were recorded as shown in Figure 7 for a sample of α-Bgt. Various fractions were tested for (a) acetylcholine receptor blocking activity using the frog rectus abdominis preparation as described in a later section and (b) acetylcholine esterase activity (Ellman et al. (51)).

As an alternative to preliminary starch electrophoresis, followed by CM-52 chromatography, a sample of 100 mg of crude Bgv was dissolved in a minimum volume of 0.05 M NH_4OAc, pH 5.0, and was subjected to gradient-elution chromatography as described above. The elution profile of this chromatography is shown in Figure 8.

Fractions either from electrophoretic or CM-52 preparations of Bgv were rechromatographed using CM-52 equilibrated with 0.1 M NH_4OAc, pH 5.5, by application in the same buffer and elution with a 1.2 ℓ gradient of NH_4OAc, pH 5.5 from 0.1 to 0.4 M.

Lastly, after iodination of α-Bgt by the method of Pressman (52), ^{125}I-α-Bgt was separated from unreacted native toxin on Whatman CM-52. Desalted, lyophilized iodinated toxin was dissolved in 0.1 M NH_4OAc, pH 5.8, and applied to a CM-52 column 2.5 by 30 cm equilibrated with the same buffer. A 1.2 ℓ NH_4OAc gradient, 0.1 M-0.4 M, pH 5.8, was run and 6.6 ml fractions collected. Optical density at 280 nm of the fractions was recorded and radioactivity of 10 microliter aliquots of the individual fractions determined. The resulting elution profile is shown in Figure 9.

The specific radioactivity obtainable by the Pressman procedure (52) was insufficient for some of the binding assays to be described in a subsequent chapter. Iodination of microgram quantities of protein with carrier-free radioiodide is possible through the use of the oxidizing agent chloramine-T (which was obtained from MC&B). The procedure followed was essentially that of Hunter and Greenwood (53), except that instead of adding bovine serum albumin to the iodinated protein to prevent adsorption to glass, we used a 100-fold excess (over reacted toxin, about 1 mg) of native toxin, and performed all operations in plastic apparatus. The mixture of iodinated and native toxin was desalted on a small G-25 column and used without further purification. The mixture had a specific activity of 10^9 cpm/mg.

Characterization of Purified α-Bgt. 5.9 mg of the purified protein were taken for an end group analysis following the method of Stark and Smyth (54) for amino termini. The only modifications

were (a) the use of 5 M guanidine HCl as the denaturing agent and (b) determination of the presence of hydantoins by monitoring the effluent from Dowex 1 columns at 230 nm instead of taking the suggested volume cuts.

Amino acid analyses of the alkaline hydrolysis products of the recovered hydantoins were done according to the method of Spackman et al. (55).

Purified α-Bgt buffered at pH 8.0 was tested for the presence of free sulfhydryl groups by the method of Ellman (56) using DTNB.

Electrofocusing of ^{125}I-α-Bgt. 2 μg of purified, salt-free, ^{125}I-Bgt (sp. act. = 42×10^6 cpm/mg) was applied to a 110-ml electrofocusing column (LKB 8101) and electrofocused at 6° according to the method of Vesterberg and Svensson (57). The pH range of ampholine used was pH 7-10, at a final ampholine concentration of 1%, w/v. Voltage was maintained at 400 volts. At the end of 20 hr, the current was steady, and 0.6 ml fractions were collected. Every other fraction was analyzed for radioactivity and the pH of every fifth fraction was measured with a Radiometer pH meter 26 at 6°C.

Preparation and Purification of Antisera to α-Bgt. Anti-α-Bungarotoxin serum was prepared according to the following method. 0.1 mg of purified toxin in Freund's adjuvant (complete) was injected weekly into male rabbits. Immunization of the animals was begun by injecting the toxin dissolved in nonpyrogenic 0.9% saline subcutaneously along the backs of the animals; subsequently,

intramuscular injections were made weekly into the thighs of the rabbits for a period of three months. Rabbits exhibited appreciable titer, as demonstrated by the precipitin test, one week after initiation of the intramuscular injections and were thereafter bled weekly six days following the intramuscular injection.

Immunoglobin G was precipitated from pooled rabbit sera by the addition of saturated ammonium sulfate to 40% saturation. The resulting precipitate was dissolved in 0.15 M NaCl, dialyzed against 0.15 M NaCl and after clarification of the dialyzate by centrifugation was reprecipitated by addition of saturated ammonium sulfate to 33% saturation. The immunoglobin thus obtained was either (a) dialyzed against 0.15 M NaCl and used without further purification or (b) dialyzed against 0.10 M Tris · HCl, pH 8.0 containing 0.15 M NaCl. The latter (from procedure (b)) was then chromatographed on G-200 in 0.10 M Tris · HCl, pH 8.0 buffer containing 0.15 M NaCl. Fractions were pooled as indicated (Figure 10) and only the second pool (fraction 35-53) was used in further studies.

Anti-serum specific for α-Bgt was isolated by precipitation of the specific immunoglobulin by iodinated (^{125}I)α-Bgt. 5 ml of anti-Bgt serum (4.40 mg/ml) which had been purified by ammonium sulfate fractionation and chromatography on Sephadex G-200 was precipitated by the addition of 178 micrograms of ^{125}I-α-Bgt, specific activity 25.8×10^6 cpm/mg. The toxin-antibody solution was incubated at 37° for 8 hr, then at 4° overnight. The precipitate was then centrifuged, washed twice with cold 0.15 M NaCl and the

washed precipitate dissolved in 2 M deionized urea containing 0.55 M formic acid. The dissolved antigen-antibody complex was chromatographed on Sephadex G-100 (2.5 by 45 cm) equilibrated in the above buffer resulting in an elution profile as shown in Figure 11. The amount of toxin bound to immunoglobin was determined by ^{125}I counts while protein concentration was determined, after pooling the appropriate fractions and dialyzing against 0.15 M NaCl, by the method of Lowry et al. (50) using BSA as a standard.

Preparation of α-Bgt Immunosorbent. Coupling of α-Bgt antiserum to Sepharose 2B was performed according to the method of Givol et al. (58). Sepharose containing 25 or 77 mg of antiserum protein per ml of packed gel was used to determine ^{125}I-α-Bgt binding capacity and conditions for removal from the immunosorbent. 0.5 ml of immunosorbent was packed in a Pasteur pipette plugged with glass wool, equilibrated with buffer in 1% Triton X-100. An aliquot of ^{125}I Bungarotoxin was added, and thirty-two column volumes of buffer were then washed through the column at room temperature, finishing with a buffer containing 0.1% Triton X-100. The toxin was subsequently eluted from the immunosorbent column using conditions of low pH, high salt or both. Regeneration of the gel after such treatment was measured by its ability to again adsorb toxin. The conditions and results of these experiments are shown in Table I.

Physiological Characterization of Purified Toxin. Purified α-Bungarotoxin and ^{125}I-α-Bungarotoxin were assayed for biological activity on the frog rectus abdominis. The muscle was dissected out and immersed immediately in frog Ringer's solution, (0.11 M NaCl, 1.9 mM KCl, 1.2 mM $CaCl_2$, 2.4 mM $NaHCO_3$). One end of the muscle was pinned to a beeswax-filled cup and the other end hooked by means of a Nichrome wire to an FT03 force transducer. Solutions of acetylcholine freshly prepared in Ringer's were pipetted onto the dorsal side of the muscle, and the muscle contractions were recorded with a Grass polygraph. In each assay, the responses of the muscle to 10^{-6} g/ml, 10^{-5} g/ml, and 10^{-4} g/ml acetylcholine were first recorded. After thorough rinsing in Ringer's, the muscle was immersed in the toxin solution and allowed to soak for 30 minutes. After this time, the toxin solution was removed and the response of the muscle to the acetylcholine solutions was determined. In those cases where the degree of reversibility of Bungarotoxin-blocking was tested, the muscle was rinsed with Ringer's dripping at a constant rate from a separatory funnel into the cup.

The effect of α-Bungarotoxin antibody on toxin binding to the frog rectus abdominus muscle was tested in two ways. In one series of experiments, the antibody was added to the muscle-toxin preparation after the muscle had soaked 30 minutes in the toxin solution. In a second series of experiments, antibody was added to the toxin solution and the mixture allowed to stand 10 minutes before immersing the muscle in the mixture for 30 minutes.

Results and Discussion

Purification of α-Bungarotoxin. Purification of crude Bgv by starch electrophoresis has been shown by Chang and Lee (49) to separate the crude venom into components designated α, β and γ, as well as an AChE component. The separation of crude Bgv by starch electrophoresis into its components, in our hands, gave an additional two components designated I and II (Figure 6). Components I and II did not give positive Lowry tests and were presumably not detected by Chang and Lee (49). Assays for AChR blocking activity showed that only the component labeled α was effective against frog rectus abdominis muscle as described in a later section. Chromatography of the combined supernatants from the center fractions of this peak on Sephadex G-50 resulted in a single major peak and a small component emerging in the void volume which exhibited a high level of AChE activity. The major peak from Sephadex G-50 chromatography was slightly asymmetric, and chromatography of this peak on CM-52 (Figure 7), using gradient elution, revealed the presence of approximately five very minor contaminants in addition to the major fraction.

Fractionation of crude Bgv on CM-52 (Figure 8) by gradient elution gave eight well-defined components and four or five minor fractions as determined at 260 and 280 nm. Lowry analysis for protein in the effluent agreed with the direct uv observations except in the case of components I and II which were not detected;

components I and II also gave higher extinction at 260 nm than at 280 nm. Peak V in this fractionation of crude Bgv corresponds to α-Bgt as previously isolated by starch electrophoresis followed by gradient elution on CM-52. On testing for AChR blocking activity, maximum effect was found in component V (Figure 8) with partial activity in component IV. AChE assays revealed two activity maxima, one before and one after peak V.

Gradient elution chromatography of iodinated α-Bgt on CM-52 (Figure 9) cleanly separated ^{125}I-α-Bgt from native toxin and toxin decomposition products produced during the course of the iodination reaction. The elution profile, as determined by the optical density of the eluant fractions at 280 nm, revealed that the yield of ^{125}I-α-Bgt was, in this instance (Figure 9), 35 per cent of the toxin reacted. Indeed, chromatography of several different lots of iodinated toxin has demonstrated the variability of the yield of iodinated toxin between 10 and 35 per cent. Clearly, the resulting enhancement of specific activity and elimination of decomposition products achieved by this final purification step will be of great utility in future neurochemical investigations.

Chemical Characterization. An amino acid analysis of a 24 hr 6 N HCl hydrolysis of 30 nM of α-Bgt purified by starch electrophoresis followed by gradient elution on CM-52 is shown in Table II. The results agree well with those of Lee (47) with the exception of valine and possibly tryptophan. Within experimental error, no free sulfhydryls could be detected with the Ellman reagent

(DTNB) and as a consequence the cysteine content of the toxin is listed in Table II as half cystine. The minimum molecular weight calculated from these data is 7904 and agrees well with that known for other elapid neurotoxins (47) as well as with an approximate molecular weight of 8000 obtained by electrophoresis of purified α-Bgt in 12.5% polyacrylamide gels (10% cross linked, containing 0.1% SDS and 8 M urea) (59).

Additionally, the basic nature of purified α-Bgt was demonstrated by the amino acid content and agrees well with a pI of 9.19 obtained from the electrofocusing experiments on ^{125}I-α-Bgt.

Determination of the N-terminus of the purified α-Bgt by the cyanate method of Stark and Smyth (54) resulted in release of equimolar quantities of isoleucine and allo-isoleucine. The amino terminus of α-Bgt is therefore isoleucine in agreement with Lee (47). We estimate on the quantitative basis of this result that there can be no more than 1% impurity present in α-Bgt prepared by starch electrophoresis followed by chromatography on CM-52.

Toxin Specific Antibody. Following ammonium sulfate fractionation of pooled anti-α-Bgt sera, remaining macroglobulin (IgM) was separated from the lower molecular weight immunoglobin G (IgG) on Sephadex G-200. Appropriate fractions were pooled, and pool II, Figure 10, composed only of IgG was used for the remaining studies.

Isolation of antibody specific for ^{125}I-α-Bgt from the above purified anti-α-Bgt serum was attempted for the purposes of

(a) demonstrating the antigenicity of ^{125}I-α-Bgt towards anti-α-Bgt antibody and (b) establishing what the approximate fraction of the total IgG pool was composed of toxin-specific IgG.

Figure 11 illustrates the partial separation of the toxin-specific antibody-antigen complex into free and high-molecular-weight-bound ^{125}I-α-Bgt, and IgG. Previous studies by Chang and Yang (60) on antibodies made against purified cobrotoxin showed that, of the immunoglobins, only IgG was produced against that protein toxin. The fact that in this study IgM had also been removed prior to precipitation of antibody by antigen leads to the conclusion that the radioactive peak of ^{125}I-α-Bgt chromatographing with the principal protein peak as monitored by optical density at 280 nm must represent a solubilized toxin-IgG complex which was not dissociated in 2 M urea-0.55 M formic acid.

The principal protein peak in Figure 11 as monitored by optical density at 280 nm represents 1.2 mgs of IgG as determined by Lowry assay (50). This quantity of toxin-specific antibody represents 5% of the total IgG pool.

Physiological Activity. Purified α-Bungarotoxin in concentrations of 10^{-5} g/ml and 10^{-6} g/ml was effective in completely abolishing the muscle response to all concentrations of acetylcholine. Rinsing with 1 liter of Ringer's for 2 hr did not restore the muscle response. 10^{-5} g/ml and 10^{-6} g/ml ^{125}I-α-Bgt also completely and irreversibly abolished all muscle response. Approximately 5×10^{-4} g/ml of anti-α-Bgt antibody added to muscle which had incubated 30

minutes in either 10^{-6} g/ml or 10^{-5} g/ml ^{125}I-α-Bgt was unable to reverse the acetylcholine response inhibition by the toxin.

After preincubation for 10 minutes with antibody at concentrations up to 3×10^{-4} g/ml, 10^{-5} g/ml α-Bgt was still completely effective in blocking the acetylcholine response. These results indicate that at least some of the antigenic sites on α-Bgt probably do not overlap with the acetylcholine receptor-specific sites of the toxin. Therefore with the long-range goal in mind of using antibody affinity columns to isolate a toxin-receptor complex we next investigated the preparation of Sephadex 2B conjugated antibody columns and their toxin binding properties.

Toxin-specific Immunosorbent. Immunosorbent was successfully produced from twice-ammonium-sulfate-fractionated IgG after chromatography on Sephadex G-200 (Figure 10); coupling to Sepharose 2B was accomplished in yields of 95-99%.

Conditions for eluting bound toxin from the immunosorbent (Table I) indicate the necessity of low pH or high concentrations of selected salts. Previous studies on immunosorbents (58, 61) have used 8 M urea, 20% formic acid, guanidine hydrochloride or 0.8 M ammonium hydroxide to elute bound antigen. Our experience with anti-α-Bgt immunosorbent indicates that conditions of high salt are not as useful as low pH conditions, as they either do not elute toxin or irreversibly deactivate the gel-bound antibody. Bound toxin is eluted quantitatively by 0.55 M formic acid (pH 1.8) and nearly quantitatively by 1-2 M acetic acid (pH 2.5). Both conditions leave

the immunosorbent fully regeneratable. However, our desire to reduce the harshness of the eluting conditions in order to minimize release of Sepharose-bound antibody, as observed by ourselves and others (58), lead us to prefer 2 M acetic acid elution.

Conclusion

Starch electrophoresis of crude venom from B. multicinctus followed by carboxymethyl cellulose gradient elution chromatography of an appropriate fraction yields pure α-Bungarotoxin which is physically homogeneous and chemically well characterized, in agreement with the results of Lee (47). α-Bungarotoxin can be iodinated under mild conditions and the native and iodinated proteins can be separated in pure form by carboxymethyl cellulose chromatography. Both the native and iodinated toxins display full physiological effectiveness against the acetylcholine receptor in frog rectus abdominis muscle, and both react with toxin-specific antibodies produced against native toxin. Thus, iodinated α-Bgt, which was shown to be essentially identical with the native toxin in physiological and antigenic characteristics, should be a useful label for probing the nature of the acetylcholine receptor.

Figure 6: Starch electrophoresis of crude Bgv at constant voltage at 4° for 40 hr. Following completion of electrophoresis, starch was removed in 0.5 cm sections, eluted with water and absorbance at 260 and 280 nm determined. The designations of peaks α, β, γ are those of Chang and Lee (49) while peaks I and II are components which gave negative Lowry tests.

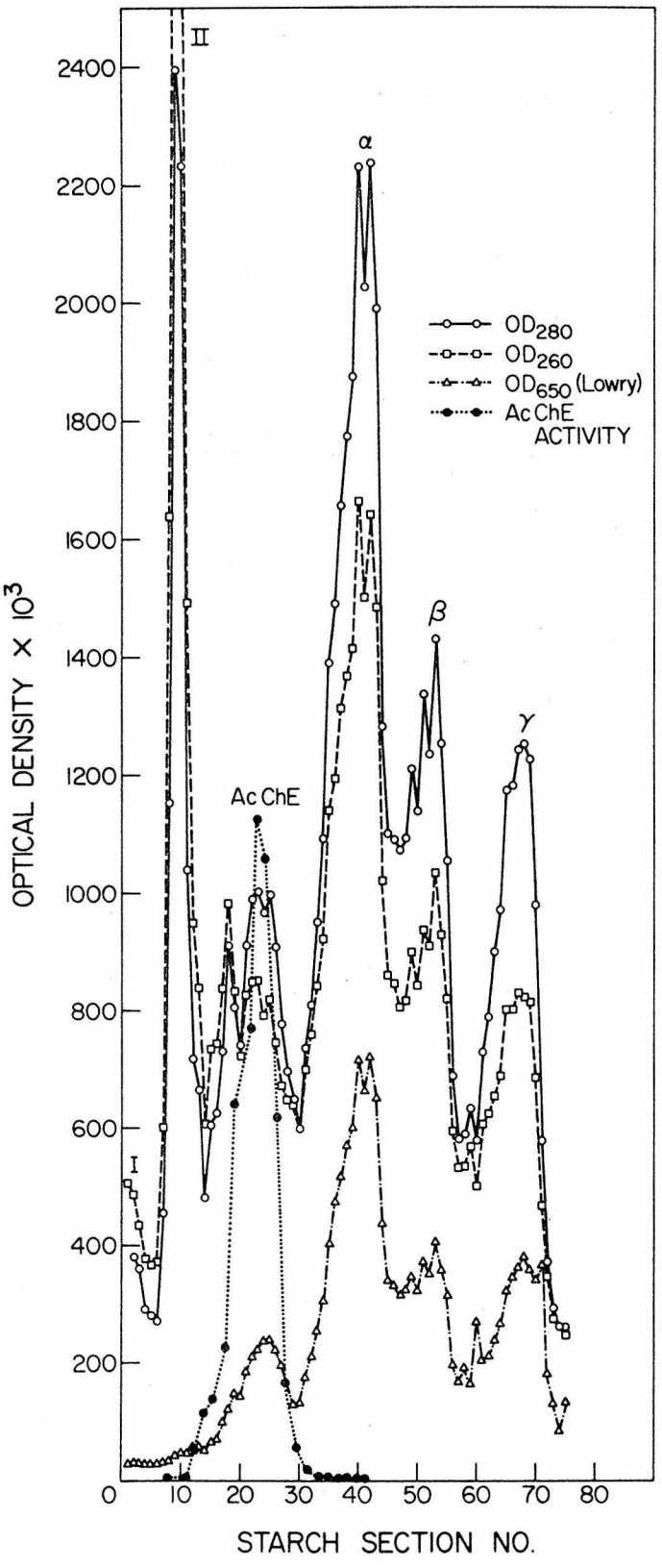

OPTICAL DENSITY X 10^3
2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
10
20
30
40
50
60
70
80
STARCH SECTION NO.
I
II
α
β
γ
Ac ChE
OD_{280}
OD_{260}
OD_{650} (Lowry)
Ac ChE ACTIVITY

Figure 7: Gradient elution chromatography on carboxymethyl cellulose using a column 2.5 by 20 cm and an ammonium acetate gradient from 0.05 M, pH 5.0, to 1.0 M, pH 6.8. The minor peaks are due to contaminants from Bgv remaining after starch electrophoresis and subsequent chromatography on Sephadex G-50.

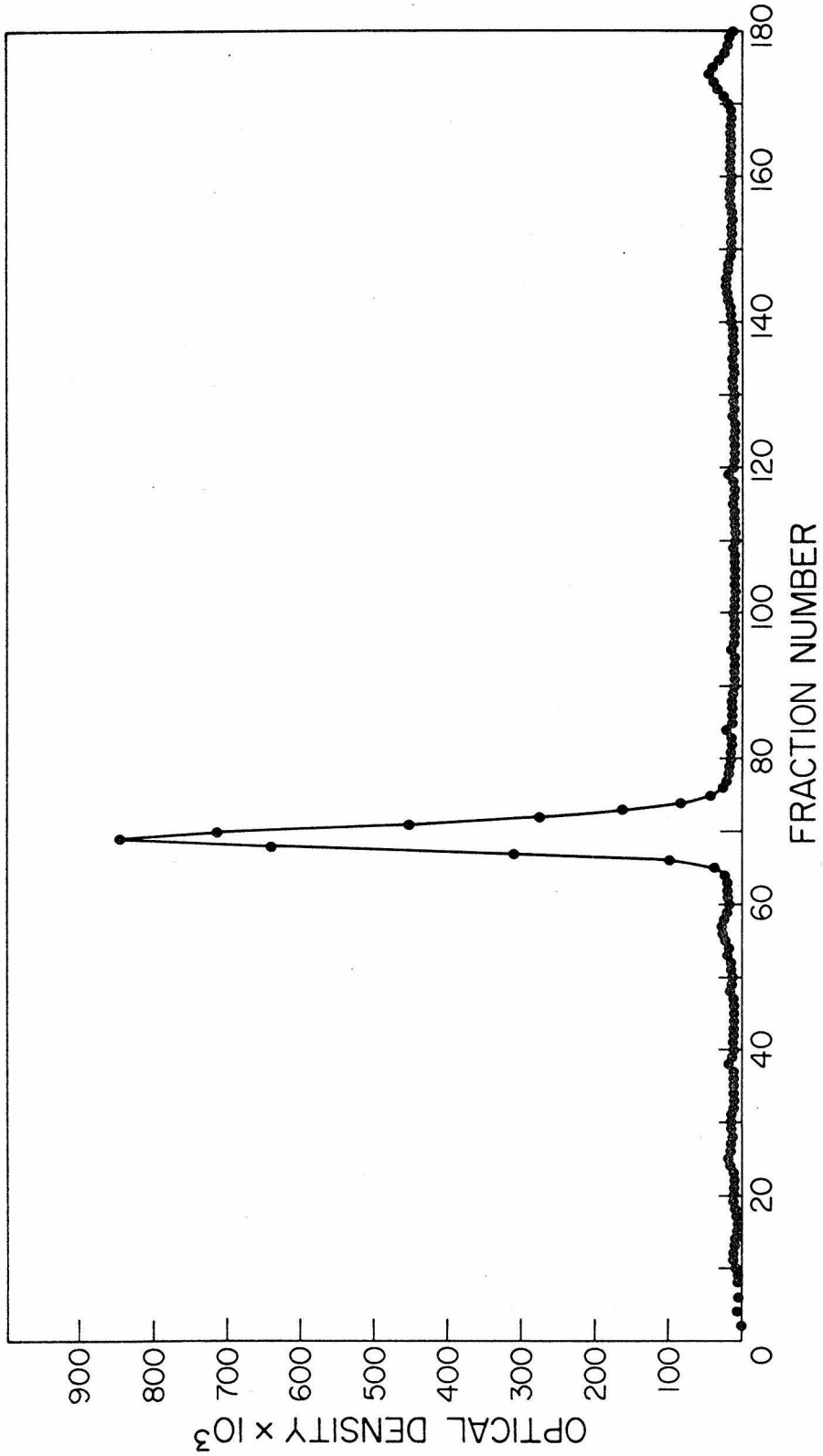

OPTICAL DENSITY x 10³
900
800
700
600
500
400
300
200
100
0
20
40
60
80
100
120
140
160
180
FRACTION NUMBER

Figure 8: Gradient elution chromatography of crude Bgv on carboxymethyl cellulose using a column 2.5 by 20 cm and an ammonium acetate gradient as described in Fig. 7. Peak V was found to be α-Bgt.

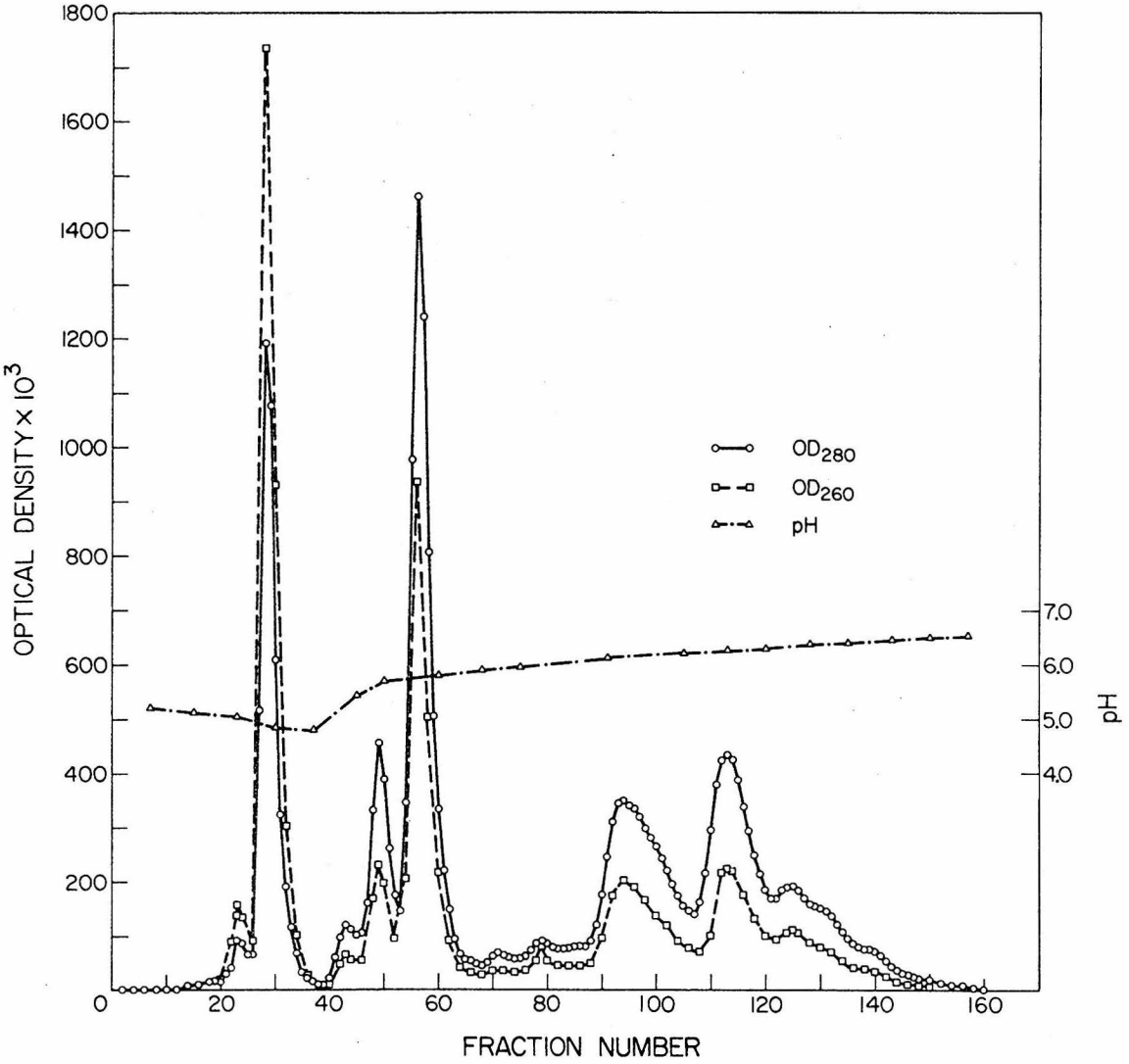

OPTICAL DENSITY X 10^3
1800
1600
1400
1200
1000
800
600
400
200
0
20
40
60
80
100
120
140
160
FRACTION NUMBER
OD_{280}
OD_{260}
pH
7.0
6.0
5.0
4.0
pH

Figure 9: Gradient elution chromatography on carboxymethyl cellulose of the reaction products from the iodination of α-Bgt. Elution was carried out on a 2.5 by 30 cm column using a 1.2 ℓ pH 5.8 gradient from 0.1 M to 0.4 M ammonium acetate. Fractions of 6.6 ml were taken, absorbance of the fractions at 280 nm determined and 10 λ aliquots of the appropriate fractions taken for the determination of radioactivity.

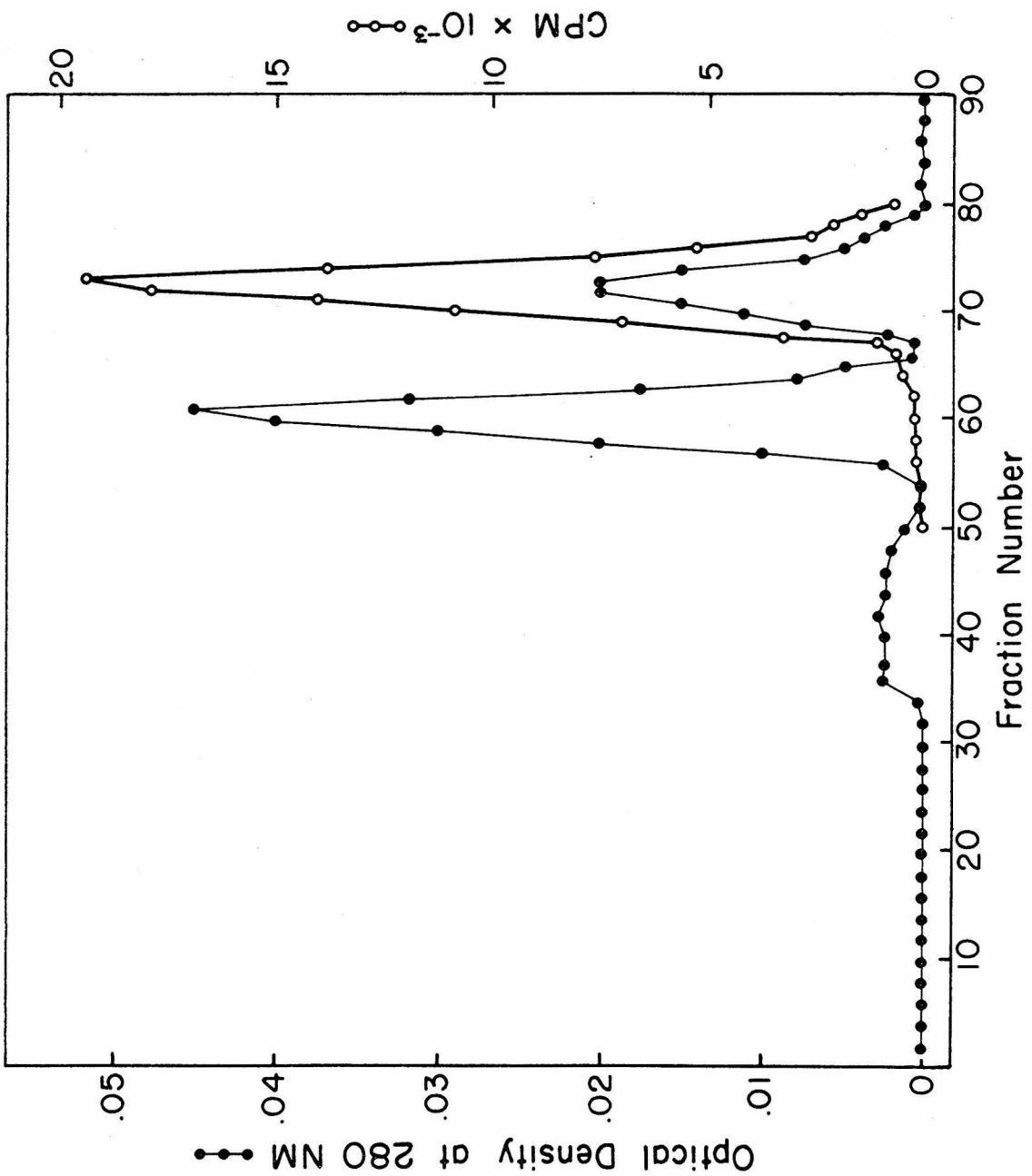

Optical Density at 280 NM
CPM x 10⁻³
Fraction Number
0
.01
.02
.03
.04
.05
5
10
15
20
10
20
30
40
50
60
70
80
90

Figure 10: Chromatography of twice-ammonium-sulfate-fractionated anti-α-Bgt serum on a Sephadex G-200 column, 2.5 by 70 cm, using 0.10 M Tris · HCl, pH 8.0, containing 0.15 M NaCl. 4.5 ml fractions were taken. Protein was pooled as indicated. The second pool was essentially free of macroglobulin (IgM).

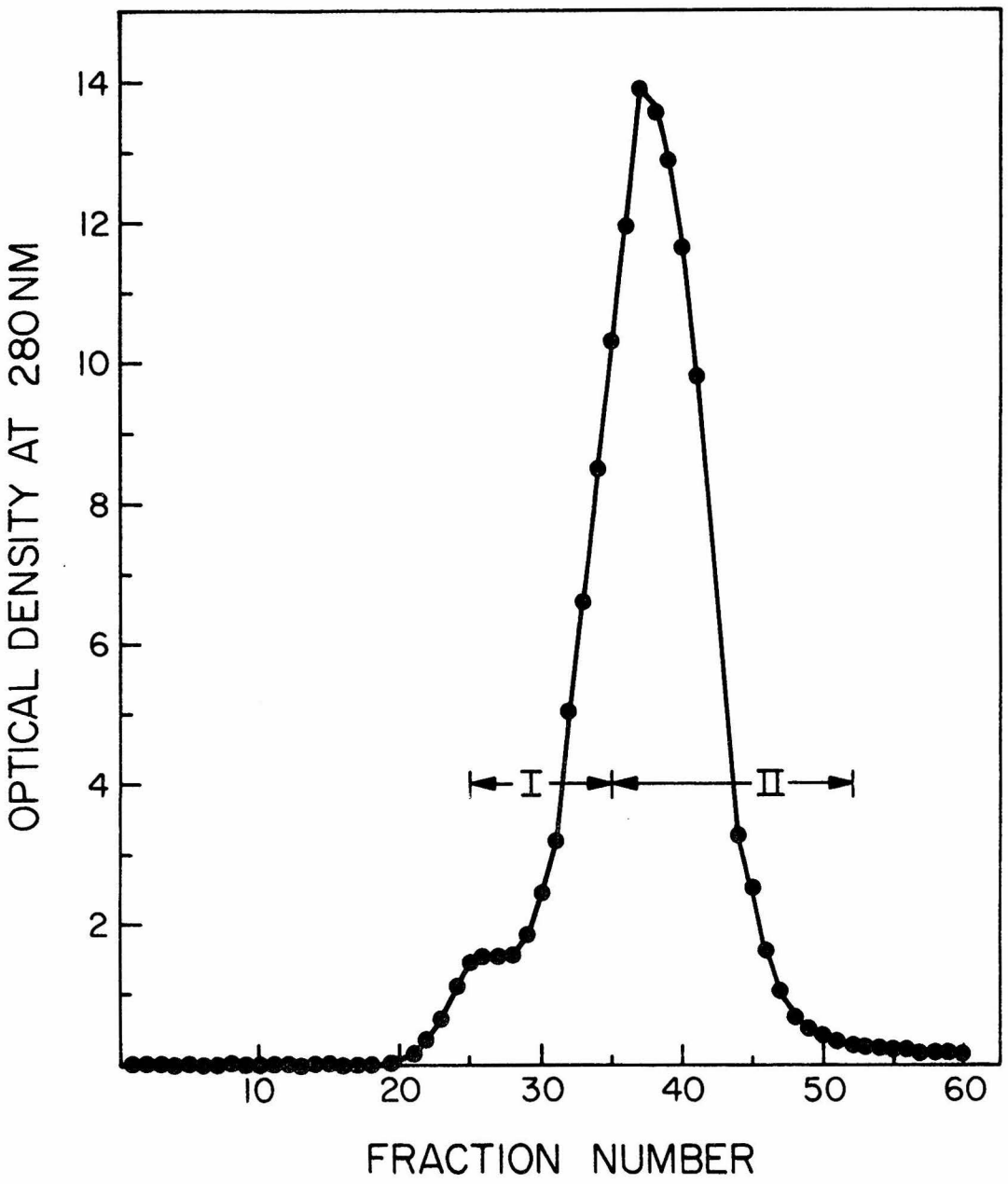

OPTICAL DENSITY AT 280 NM
14
12
10
8
6
4
2
I
II
10
20
30
40
50
60
FRACTION NUMBER

Figure 11: Partial separation of ^{125}I-α-Bgt from anti-α-Bgt serum which had been ammonium sulfate fractionated and chromatographed on Sephadex G-200. Chromatography at 4° was carried out on Sephadex G-200 using a 2.5 by 45 cm column eluting with 2 M urea - 0.55 M formic acid. 2.0 ml fractions were taken.

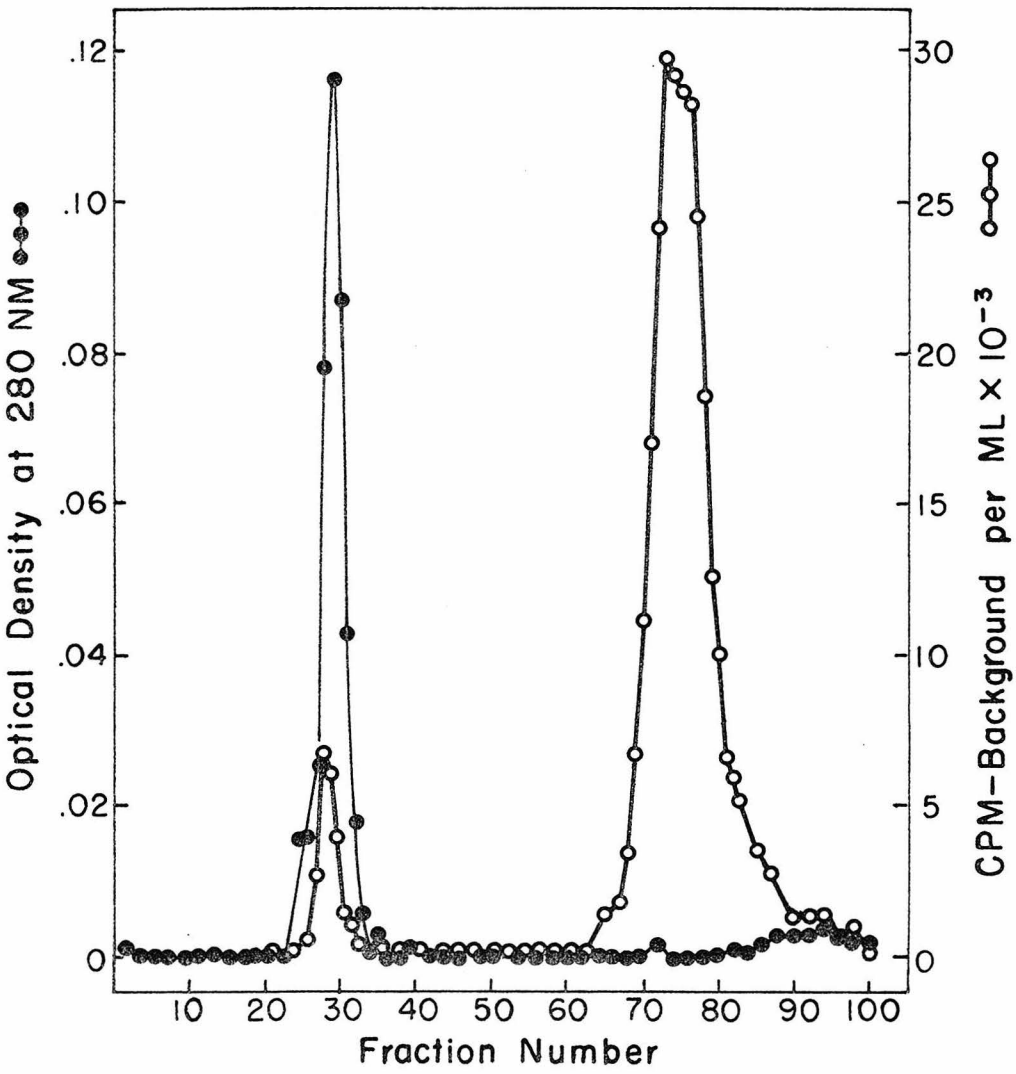

Optical Density at 280 NM
.12
.10
.08
.06
.04
.02
0
CPM–Background per ML X 10⁻³
30
25
20
15
10
5
0
10 20 30 40 50 60 70 80 90 100
Fraction Number

Table I. Binding Capacity, Conditions of Toxin Elution, and Regeneration Characteristics of Anti-α-Bgt Immunosorbent

Immunosorbent	Toxin μg	% of toxin bound	Eluant	% of Bound counts eluted under conditions 1-9	% Regeneration
77 mg antibody per ml Sepharose 2B	177	80	1	100	–
	277	80	1	100	50
	540	66	1	100	65
	750	80	1	100	–
25 mg antibody per ml Sepharose 2B	17	82	1	100	90
	17	95*	2	66	100
	17	97*	3	78	100
	17	95* **	4	81	100
	17	85	5	6	–
	17	97*	6	58	–
	17	86	7	50	–
	17	77	8	100	20
	17	98*	9	3	–
	17	95* **	4	75-81	100

(Continued to next page)

Table I. (Cont'd)

* Rate of toxin load onto immunosorbent column decreased by a factor of 3 to 17 microgram toxin/ml/minute.

** Resin prewashed with 8 M deionized urea, reequilibrated in 1% Triton-Ringer's.

Eluants:
1. 0.55 M formic acid, 0.15 M NaCl, 0.1% Triton.
2. 0.50 M acetic acid, Ringers solution, [1] 0.1% Triton.
3. 1.0 M acetic acid, Ringers solution, [1] 0.1% Triton.
4. 2.0 M acetic acid, Ringers solution, [1] 0.1% Triton.
5. 0.10 M $(NH_4)_2CO_3$, pH 10.0, 0.1% Triton.
6. 0.90 M NH_4OH, 0.15 M NaCl, 0.1% Triton.
7. 3 M guanidine · HCl, pH 6.0, 0.1% Triton.
8. 6 M guanidine · HCl, pH 6.0, 1% Triton.
9. 8 M urea (deionized), 0.1% Triton.

[1] Reference 62.

Table II. Amino Acid Composition of α-Bgt

Amino Acid	nmoles found		Mole ratio based on Phe or lysine* (nearest integer)	
	A	B*	A	B*
Asp	133.5		4	
Thr	208.4		7	
Ser	181.7		6	
Glu	166.2		5	
Pro	275.5		8	
Gly	133.6		4	
Ala	159.8		5	
Cys	337.1		10	
Val	130.9		4	
Met	31.1		1	
Ilu	48.0		2	
Leu	65.3		2	
Tyr	65.0		2	
Phe	34.1		1	
His	59.7	38.4	2	2
Lys	195.0	133.8	6	6
Arg	99.1		3	
Trp	–	43.8	–	2
Calculated molecular weight**			7904	

* Calculated by hydrolyzing in the presence of mercaptoacetic acid and comparing to lysine.

** Exclusive of amide NH_3.

Chapter 3

Specificity and Irreversibility of α-Bungarotoxin as a Receptor Label

Several investigators have used α-Bungarotoxin (α-Bgt) and related neurotoxins for labeling the cholinergic receptor (38-43), assuming chemical specificity on the basis of physiological specificity. It has been shown that α-Bungarotoxin (α-Bgt) irreversibly blocks acetylcholine-mediated neuromuscular depolarization (49, 63, 64), that it blocks excitation of the monocellular electroplax of Electrophorus electricus (39) and that it abolishes miniature potentials in electric tissue of Torpedo marmorata (41). In vitro, α-Bgt competes with tubocurarine and carbamylcholine binding to crude membrane preparations from Torpedo (41), it blocks the effect of cholinergic ligands on microsacs from Electrophorus electroplax (39) and it is competitive with binding of cholinergic compounds to detergent-solubilized preparations of Electrophorus electroplax membranes (39). Association of α-Bgt with Torpedo (41) and Electrophorus (39) preparations has recently been shown, by gel-filtration and density gradient centrifugation, to be due to complexation with high molecular weight material(s) present in detergent extracts of membranes. Little evidence is, however, available regarding the homogeneity of such complexes.

Despite physiological specificity, the problem of chemical specificity of α-Bgt association with cholinergic synapses is unresolved: α-Bgt (65) and related neurotoxins are, like all cholinergic compounds, highly basic and could bind to acidic synaptic components. If α-Bgt is to be regarded as a useful receptor label with any degree of confidence, it must fulfill two criteria:

1) it must be specific for the receptor, even in the presence of large amounts of other protein and membrane constituents, and
2) it must be irreversibly or nearly irreversibly bound under the experimental conditions used for assays.

The work described in this chapter was designed to establish whether or not α-Bgt met these two criteria, and to attempt to determine whether the high molecular weight complex with α-Bgt was unique.

Experimental

Materials. Chromatographically pure ^{125}I-α-Bgt was prepared (48) from Bungarus multicinctus venom (Sigma). Cobrotoxin was isolated (66) from Naja naja atra venom (Sigma). Electrophorus electricus was from World Wide Aquarium, Venice, California. Ampholine (40%, pH range 3-10) was from LKB, BRIJ detergent from Pierce Chemical Company and NCS solubilizer from Amersham-Searle.

Methods

Membrane Fractions. Freshly excised electric organ was homogenized with one volume of Ringer's (62) in a Waring blender, passed through cheese cloth and centrifuged at 12, 000 xg for three hr. The pellet was resuspended in an equal volume of distilled water (Waring blender) and centrifuged as before. Following a second resuspension in distilled water it was centrifuged at 45, 000 xg for twelve hr. The pellet was resuspended in Ringer's (ca. 0.3 ml/gm original tissue) and was further diluted as necessary.

Filter Disk Assay. 0.5 ml membrane suspension, representing 0.1 gm electroplax, was incubated with 0.01 ml of ^{125}I-α-Bgt (3.3 mg/ml) at room temperature for up to 30 minutes. Three ml Ringer's, containing 1% BSA, were added and the suspension passed through a filter disk (Schleicher and Schuell No. 593-A) pretreated with Ringer's-BSA. The use of Ringer's-BSA is essential to minimize nonspecific adsorption of toxin to membrane fragments and filter disks. The filters were incubated with 1 ml NCS at 50°C for two hr prior to counting.

Preparation of Labeled Extracts. Membrane suspensions were incubated with ^{125}I-α-Bgt (1 μg/ml suspension) for one hr at 25°C, then made 1% (v/v) in Triton X-100 (Sigma), stirred four hr at 4°C and centrifuged at 30, 000 xg for 60 min. The supernatant was chromatographed on Sephadex G-75 or Sepharose 6-B. Alternatively, extraction and centrifugation were completed prior to labeling.

Isoelectric Focusing. This was performed (67) using an LKB column (110 ml), pH gradient 3-10, 5% ampholine, 1% BRIJ, until constant current (2 mA) at 500 V was obtained. 1.1 ml fractions were analyzed for radioactivity (50 μl), pH and absorbance at 280 nm.

Results

Binding of ^{125}I-α-Bgt to membrane fragments was time-dependent and reached maximum values of 0.36 ± 0.02 μg/gm original electroplax in about ten min. Figure 12 shows a double-reciprocal plot of the binding data. The concentration of ^{125}I-α-Bgt necessary for half-saturation of toxin binding sites (7×10^{-8} M) is in good agreement with electrophysiological studies on unlabeled (39, 49, 63) or labeled α-Bgt (48) and related neurotoxins (68). Regarding the specificity of ^{125}I-α-Bgt binding to membranes it is evident from Figure 13 that lysozyme and cytochrome c were ineffective in preventing association of this labeled toxin (8×10^{-7} M) even at concentrations 10^2-10^3 in excess. These proteins were chosen because of their basicity and small size, two features which would tend to make them competitive with α-Bgt for nonspecific binding sites. Unlabeled α-Bgt and cobrotoxin, at low concentrations, gave half-saturation values of 10^{-8}-5×10^{-7} M in competing with the labeled toxin. In addition, there seem to be a smaller number of low affinity sites or nonspecific sites for toxin binding.

Extraction of ^{125}I-α-Bgt labeled membranes with 1% Triton-X-100 and subsequent gel-filtration on Sepharose 6-B (Figure 14) demonstrated a high molecular weight complex between the toxin and extracted membrane material(s). The apparent Stokes radius of the complex corresponds to that of β-galactosidase. Acetylcholinesterase present in the samples was aggregated due to low ionic strength (69) and emerged ahead of the labeled complex. The extractable complex corresponds to about 0.3 μg ^{125}I-α-Bgt/gm original electroplax.

Labeling experiments performed on either membranes prior to detergent extraction or on Triton extracts of membrane suspensions yielded about the same value of 0.3 μg of α-Bgt complexes in soluble high molecular weight form per gram of original tissue. In terms of extractable high molecular weight complex, therefore, it is immaterial whether labeling is performed before or after Triton extraction.

Assignment of the toxin-binding component exclusively to the membranes was made on the basis of labeling experiments done on whole organ homogenates. While some high-molecular-weight-bound toxin was detected in the supernatants of relatively low speed spins (e.g., 12,000 xg for 15 min), none was found after high speed centrifugation (45,000 xg for 12 hr). This indicated that a small amount of membrane fragments produced during homogenization were small enough to remain in suspension during the low speed centrifugations which were done in the course of the first part of this investigation.

The ^{125}I-α-Bgt complex from gel filtration on Sephadex G-75 was electrofocused in a pH 3-10 gradient, in the presence of detergent (67). Apart from a radioactive peak of pI = 9.5, representing free labeled toxin, a major radioactive component of pI = 5.15 (± 0.15) was obtained (Figure 15).

Discussion

These results indicate that α-Bgt specificity for a membrane-bound site in electric tissue is substantial but not complete. Care must therefore be taken to include a proper control in binding studies to determine the "background level" of nonspecific α-Bgt binding.

The amount of labeled toxin bound to membrane fragments (0.36 ± 0.2 μg/gm electroplax) corresponds with the amount of high molecular weight complex (0.3 μg/gm electroplax) extractable by 1% Triton-X-100. The stability throughout gel filtration of the complex is in agreement with the physiologically observed irreversible cholinergic blockade of native (39, 48, 49, 63, 64, 68) and ^{125}I-labeled toxin (48). From both membrane binding and gel filtration experiments the concentration of toxin binding sites is about 35 pM/gm electroplax, in close agreement with values determined from muscarone binding to electroplax membrane fragments (36).

The value for the Stokes radius of the complex (M.W. $\sim 6 \times 10^5$) from Sepharose 6-B chromatography is not in agreement with values obtained from density gradient centrifugation (S value 9.4) (J. Schmidt and M. A. Raftery, unpublished), where the

complex appears to be slightly smaller than catalase (M.W. $\sim 2.6 \times 10^5$). This anomaly has also been observed recently elsewhere and postulated as perhaps due to the non-globular nature of the membrane component which complexes the toxin (70).

It is considered likely that the asymmetry toward the leading edge of the peak of complex on Sepharose 6-B chromatography is due to aggregation since lower concentrations of detergent magnify this effect. Good evidence for homogeneity of the complex was obtained by isoelectric focusing (Figure 15). The symmetry and width at half height of the peak of complex, compared with similar separations of pure proteins, are highly suggestive of such homogeneity. The majority of materials in the extract are highly acidic, but show no association with the labeled toxin. Preliminary experiments on electrofocusing of unlabeled extracts of membranes, followed by assay with ^{125}I-α-Bgt (Sephadex G-75 columns) indicate that the macromolecular species which interacts with the toxin has a pI ~ 4.7 (D. G. Clark and M. A. Raftery, unpublished). The available evidence therefore suggests that the toxin binds to a unique component of electroplax membranes.

Studies designed to partially characterize the toxin-binding component of electroplax membranes, taking advantage of the specificity and irreversibility of the α-Bgt label, are described in the following chapter.

Figure 12: Reciprocal plot of ^{125}I-α-Bgt binding to crude membrane fractions. The ordinate shows the reciprocal of membrane-bound radioactivity, the abscissa shows the reciprocal of ^{125}I-α-Bgt concentration (specific activity = 1.7 ci/mmole). Binding was assayed by the filter disk method in the experimental section.

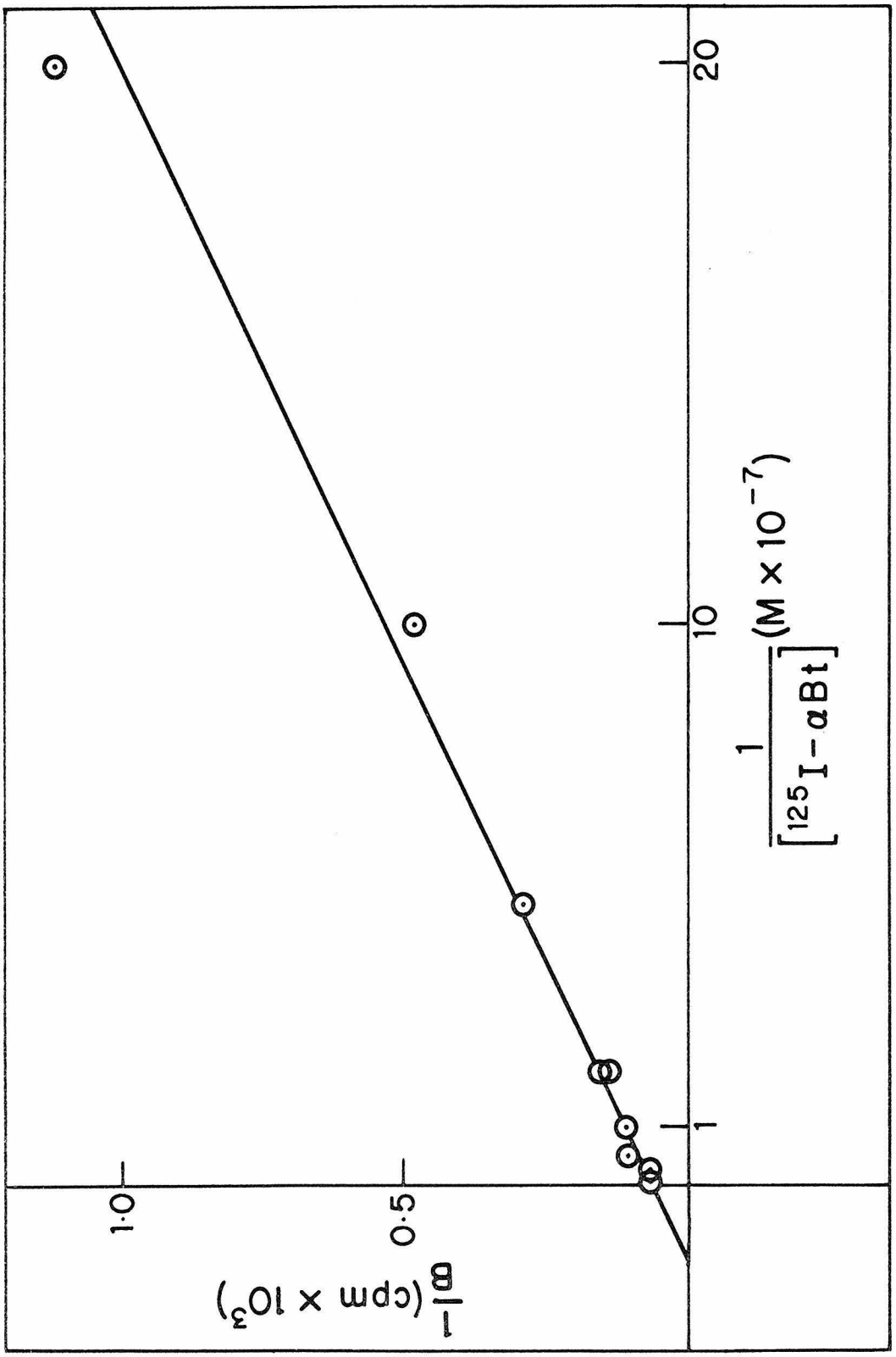

1·0
0·5
$\frac{B}{B^1}$ (cpm x 10^3)
1
10
20
$\frac{1}{[^{125}I - \alpha Bt]}$ (M x 10^{-7})

Figure 13: Competition of increasing concentrations of various basic proteins with ^{125}I-α-Bgt (8×10^{-7} M) for membrane binding sites. All values were measured on amounts of membranes corresponding to 0.1 gm electroplax tissue. Binding assayed by filter-disk method.

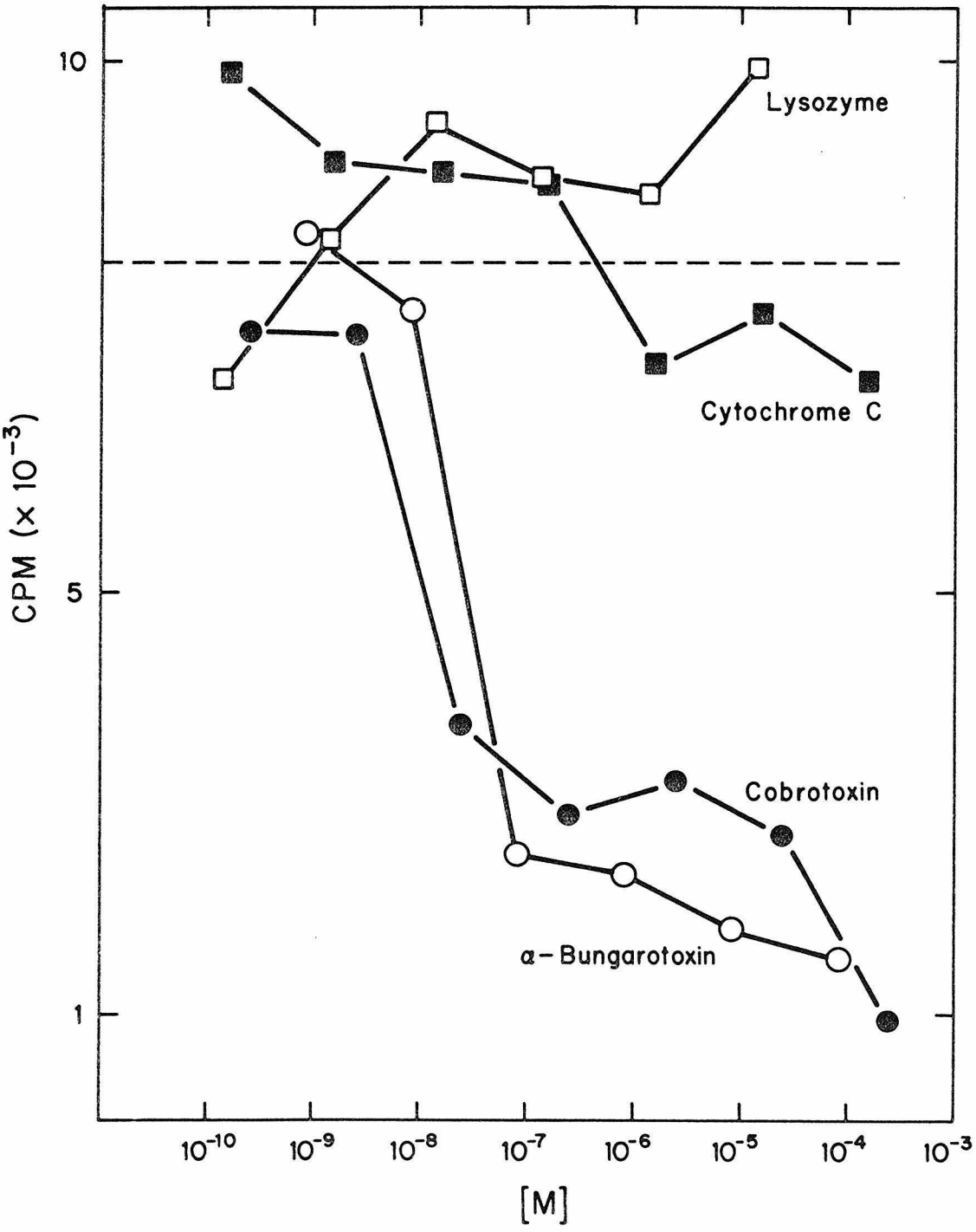

10
5
1
CPM (x 10⁻³)
Lysozyme
Cytochrome C
Cobrotoxin
α-Bungarotoxin
10⁻¹⁰
10⁻⁹
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
10⁻⁴
10⁻³
[M]

Figure 14: Sepharose 6-B chromatography (column 1.5 × 80 cm) of 2 ml Triton X-100 extract (desalted on Sephadex G-75). Eluent was 1% Triton in 10 mM Na_2HPO_4 pH 7.5. Calibration was accomplished with the marker proteins shown at top. Fraction size was 1.6 ml. 1 ml aliquots were counted (Packard 3375) in 15 ml Bray's solution. 0.5 ml was used for protein determination. AChE, acetylcholine esterase; β-Gal, β-galactosidase; GPD, glyceraldehyde-3-phosphate dehydrogenase; α-CT, α-chymotrypsinogen; α-BT, α-bungarotoxin.

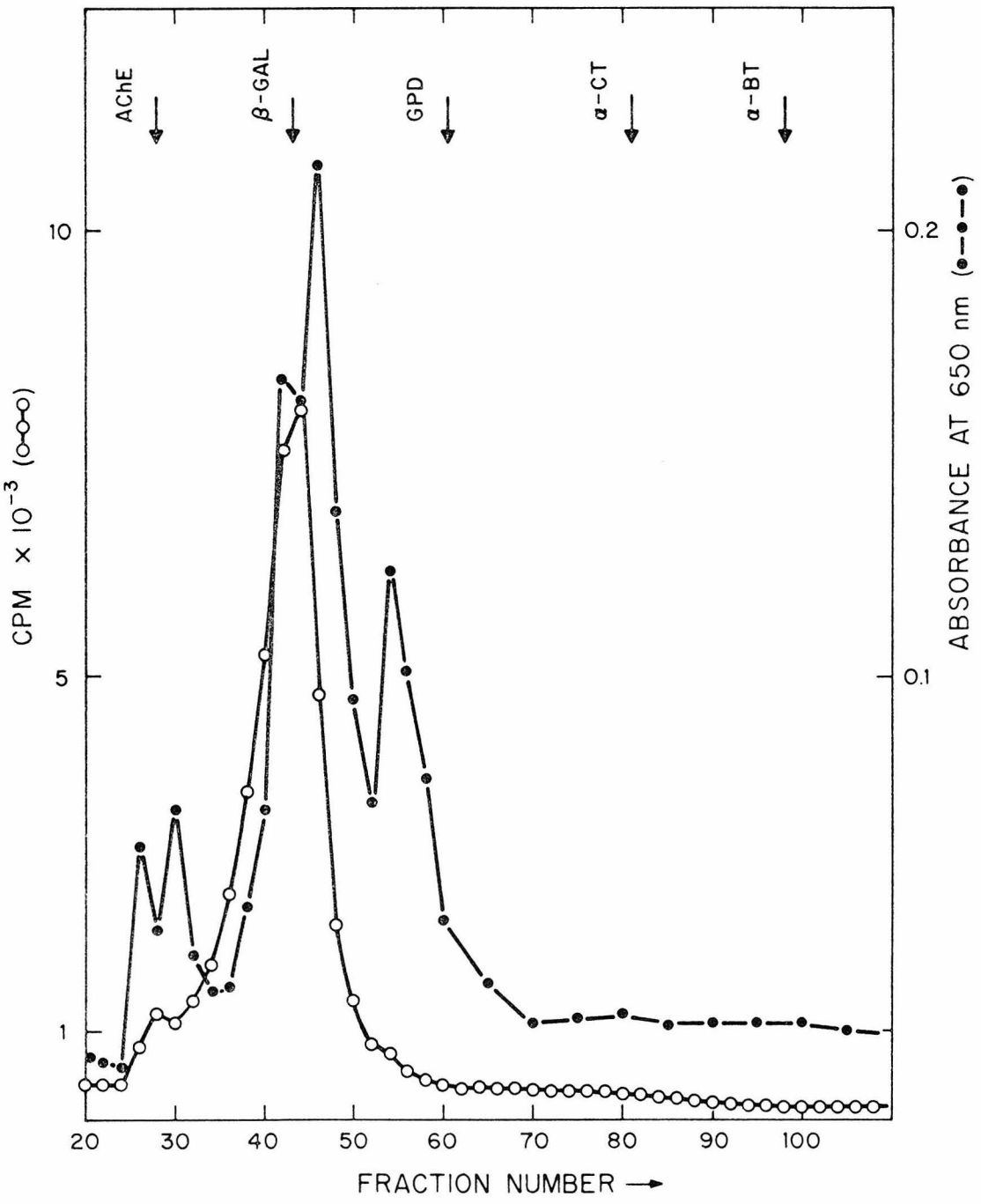
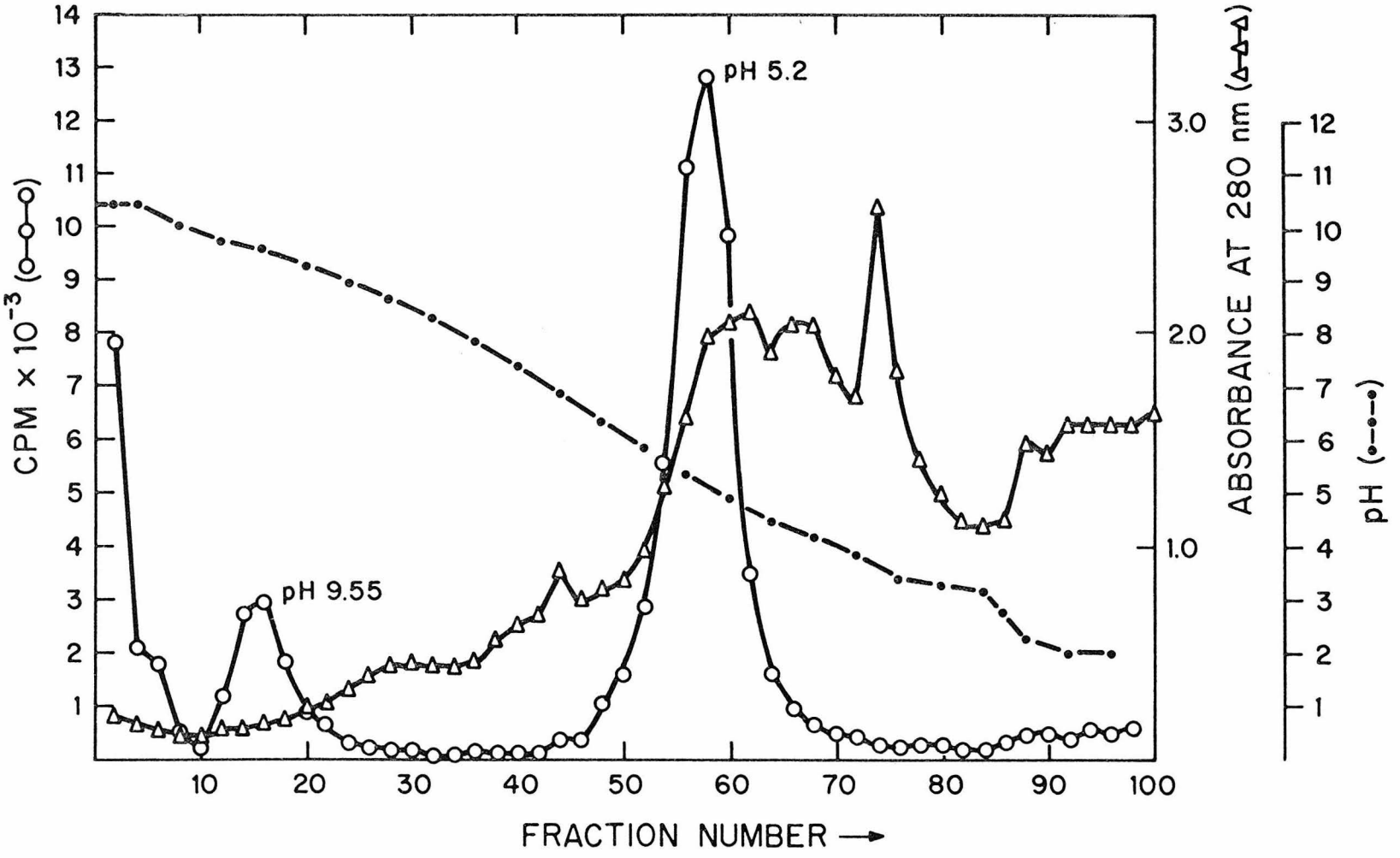

AChE
β-GAL
GPD
α-CT
α-BT
CPM x 10⁻³ (o—o—o)
ABSORBANCE AT 650 nm (•—•—•)
10
5
1
0.2
0.1
20
30
40
50
60
70
80
90
100
FRACTION NUMBER →

Figure 15: Isoelectric focusing of ^{125}I-α-Bgt complex from Sephadex G-75 chromatography. Fractions of ~1 ml were collected, 50 λ taken for scintillation counting, pH determined on the remainder and absorbance at 280 nm read after dilution with 1 ml water.


pH 5.2
pH 9.55
CPM x 10⁻³ (o—o—o)
ABSORBANCE AT 280 nm (Δ—Δ—Δ)
pH (•—•—•)
FRACTION NUMBER →

Chapter 4

Partial Characterization of the α-Bungarotoxin-Binding Component

The postulated receptor for acetylcholine has been described as a protein (7, 11-13), an acidic mucopolysaccharide analogous to hyaluronic acid (15), a sialoprotein (19), a proteolipid (33, 34), a phospholipoprotein (36) and as a prostaglandin-RNA complex (71). It is important to know the exact nature of the receptor in designing isolation schemes, and since there appears to be such a wide diversity of opinion on the topic, it was felt that an effort should be made to characterize the receptor.

The results reported in the preceding chapter suggest that α-Bungarotoxin, labeled at high specific activity, is a useful label for an apparently unique macromolecular component of electroplax membrane. Because of the possible connection between this component and the postulated cholinergic receptor, experiments were planned to determine the chemical nature of this material. These involved treatment of membrane suspensions and detergent extracts of membrane fragments with the enzymes DNase, RNase, phospholipase C, trypsin, neuraminidase and hyaluronidase, to test for the possible involvement of nucleic acids, phospholipids, protein, sialic acid, and hyaluronic acid; and the reagents, DTNB, PCMB and DTT to test for sensitive thiol or disulfide moieties which might be involved in the toxin recognition site (22, 23, 26-28).

Materials and Experimental Procedure

The enzymes were obtained from Sigma except for trypsin, which was from Mann Research Labs. All were assayed before use and found to be active even in the presence of 1% Triton-X-100, the non-ionic detergent used to extract membrane fragments. DTT was a product of Nutritional Biochemical Co., DTNB was from Aldrich, and PCMB was obtained from Calbiochem.

α-Bungarotoxin was prepared as described in Chapter 2, and iodinated either by the method of Pressman (52) or the method of Hunter and Greenwood (53), as described in that chapter.

Sephadex G-75 was a product of Pharmacia; filter disks were 2.5 cm circles from either Schleicher and Schuell or the Millipore Corp. Other materials used were of reagent quality.

Membrane suspensions were obtained from homogenates of electric tissue of E. electricus or Torpedo marmorata (a skate) as follows:

Freshly excised electric organ (36 g) was rinsed free of superficial blood and slime, then ground in a meat grinder. The resultant thick suspension was homogenized in an equal volume of water using a VirTis 23 homogenizer at full speed for 3 min. Water was then added to the homogenate to bring it to 120 ml and the suspension was centrifuged for 30 min at 15,000 rpm in a Sorvall SS-34 rotor.

The supernatant was discarded and the pellet resuspended in about 60 ml of water using the VirTis at top speed (3-4 min). Water was again added to bring the suspension to 120 ml, and it was then centrifuged for 1 hr at 15,000 rpm in the SS-34 rotor.

The light, fluffy pellet was resuspended in 30 ml of water with the VirTis (top speed, 2 min), and then made to 36 ml with water. This suspension was filtered through 8 layers of cheesecloth to remove large fragments of tissue, and water was squeezed through the cheesecloth until the filtrate volume was 36 ml. This resulted in a fairly finely divided suspension of osmotically shocked membrane fragments in which 1 ml corresponded to about 1 g of original tissue.

To obtain fragments of still smaller size, the suspension from the eel was forced through a French press (Amicon) at about 3000 psi. The suspensions were stored at 4° with a few drops of toluene to inhibit microbial growth.

To prepare the detergent extract of eel membranes, the above procedure was followed through the cheesecloth filtration step, except that generally only 2 layers were used. At this point, sufficient Triton X-100 in water was added to bring the final detergent concentration to 1%, and the mixture was stirred at 4° overnight. Then sufficient 4 M NaCl was added to bring the salt concentration to 165 mM. This causes expanded and therefore less dense tissue fragments to shrink, and is an aid in the subsequent centrifugation. This spin was performed twice at 15,000 rpm for 30 min in the SS-34 rotor. The resulting clear supernatant was stored at 4°.

Toxin binding assays were carried out as follows. Aliquots of membrane suspensions corresponding to 0.6 g (eel) or 0.3 g (skate) of original tissue were suspended in 3 ml of an appropriate buffer. Dilutions were arranged so that the final volume of membrane suspension was 3.3 ml. Of this, half served as a control and half was treated with one of the enzymes or reagents previously mentioned.

The concentration of each enzyme used was about 1 mg/ml except for DNase (0.5 mg/ml), trypsin (0.3 mg/ml) and neuraminidase (2.5 mg/ml). The solid enzyme was added at zero time, whereupon incubation was begun. The DNase and RNase experiments were incubated at 24° for one hr; the remaining four at 35° for 1 hr.

In order to avoid digestion of toxin, trypsin was poisoned before the labeling step by addition of a large excess of di-isopropyl fluorophosphate (DFP). This addition was made to both the trypsin-treated sample and the control. Incubation was for 15 min at 24° before addition of ^{125}I-α-Bgt.

DTT, DTNB and PCMB were prepared as 10^{-2} M stock solutions. Incubation was begun by adding an amount of stock solution equal to 10% of the desired final volume, and was carried out at 24° for one hr. Buffer in all three cases was 0.04 M Tris-Cl, 0.01 M $CaCl_2$, pH 8.0.

After completion of incubation, α-Bgt was added in 6-10-fold excess calculated on the basis of 0.3 μg/g of original tissue for the eel (43) or 2 μg/g original tissue for the skate (J. Duguid, unpublished). A larger excess gives prohibitively large values for

the blank, while smaller amounts do not saturate the binding sites. Incubation was for 15 min (Triton extract) or 30 min (membranes). In the phospholipase C experiments, an amount of cold toxin equal to twice the amount of hot toxin used was added to the labeled membranes just prior to dilution (see below) in order to minimize nonspecific adsorption.

The blank mentioned above was necessary to correct for non-specific adsorption of ^{125}I-α-Bgt to membrane fragments and to the filter disks used. The correction factor was obtained by pre-incubating membrane fragments for 1/2 hr at 24° with an amount of cold α-Bgt equal to twice the amount of hot toxin to be added; then adding the hot toxin and incubating a further 1/2 hr at 24°. The blank was subsequently processed in exactly the same manner as the samples.

At the end of the incubation with hot toxin, 1.5 ml of 1% bovine serum albumin in a modified Ringer's saline (62) (BSA/R) were added to each tube, and the resulting suspension filtered on either Schleicher and Schuell No. 593-A paper (2.5 cm), in the case of the eel, or on an 0.45 μ Millipore filter (HAWP02500) (skate). The filter disks were invariably pre-soaked in BSA/R before use. This procedure is essential to minimize nonspecific adsorption of toxin.

After filtration of the sample under gentle aspirator vacuum, the disks were washed once (skate) or twice (eel) with 1.0 ml of BSA/R, then air-dried in a 75° oven on a pin board. The dry disks

were counted in vials containing 5.0 ml of a toluene-based scintillation fluid using a Packard Model 3375.

Results

For clarity of presentation, the buffer systems used are shown in Table III. The numbers correspond to numbers listed in the "buffer" column of the tables which follow.

Table III. Buffers Used

Buffer number	Composition	pH
1	0.04 M Tris-maleate, 0.03 M $MgCl_2$	7.5
2	0.05 M potassium phosphate	7.9
3	0.04 M Tris-Cl, 0.04 M $CaCl_2$	7.35
4	0.04 M Tris-Cl, 0.01 M $CaCl_2$	8.0
5	0.05 M sodium acetate	5.0
6	0.1 M sodium phosphate, 0.15 M NaCl	5.5

The results of specific ^{125}I-α-Bgt binding to membrane fragments from E. electricus and T. marmorata are given in Tables IV and V.

Similar experiments were performed on a 1% Triton X-100 extract of eel membrane suspension. Sample and control experienced identical incubation conditions. Following incubation, ^{125}I-α-Bgt was added in about 2-fold excess (calculated on the basis of 0.3 μg/g original tissue) and incubated a further 15 min at 24°. Then an

Table IV. Electrophorus Membrane Fragments: Each Value Represents the Average cpm of Triplicate Samples, Each Corresponding to 0.1 g Original Tissue

Treatment	Buffer	Enzyme -blank (S)	Control -blank (C)	S/C	Error †
DNase	1	6344	6956	0.91	±0.1
RNase *	2	508	312	1.63	0.6
Phospholipase C	3	7421	12370	0.60	0.1
Trypsin	4	1276	5525	0.23	0.14
Neuraminidase	5	13693	11757	1.16	0.05
Hyaluronidase	6	12967	13762	0.94	0.1
DTT 10^{-3} M	4	4858	3965	1.23	0.5
DTNB 10^{-3} M	4	4356	4677	0.93	0.2
PCMB 10^{-3} M	4	4836	5499	0.88	0.2
Blank		2360			

* Low recovery for RNase due to unexplained partial solubilization of membrane suspension in buffer 2 before RNase addition. Experiment was repeated several times and always showed little change in binding capacity upon RNase treatment.

† Calculated by adding the ± cpm from sample, control and blank, and comparing the sum to the net control (C) cpm.

Table V. T. marmorata Membrane Fragments: Each Value Represents the Average Counts/min of Triplicate Samples, Each Corresponding to 0.05 g Original Tissue

Treatment	Buffer	Enzyme -blank (S)	No Enzyme -blank (C)	S/C	Error
DNase	1	432859	339792	1.27	0.44
RNase	4	288512	301514	0.96	0.03
Phospholipase C	3	256838	309147	0.86	0.03
Trypsin	4	231847	303090	0.76	0.04
Neuraminidase	5	397801	266900	1.49	0.05
Hyaluronidase	6	437828	442771	0.99	0.04
DTT 10^{-3} M	4	160154	218743	0.73	0.04
DTNB 10^{-3} M	4	222502	226240	0.98	0.04
PCMB 10^{-3} M	4	222502	226240	0.98	0.04
Blank		23267			

aliquot representing 1 g of original tissue was loaded onto a 0.8 × 25 cm G-75 column equilibrated with 1% Triton X-100 in 1 mM phosphate, pH 7.8. The amounts of radioactivity recovered in the exclusion volume for both treated sample and control were corrected for losses and then compared. The effect of the various treatments is shown in Table VI.

Table VI. Electrophorus Triton X-100 Extract

Treatment	Buffer	Enzyme (S)	Control (C)	S/C	Error
DNase	1	64900	67500	0.98	0.1
RNase	0.04 M Tris, pH 8.0	71700	71690	1.00	0.1
Phospholipase C	3	21901	18249	1.20	0.1
Trypsin	4	10742	34434	0.34	0.1
Hyaluronidase	6	29619	31140	0.95	0.1
PCMB 10^{-3} M	0.04 M Tris, pH 8.0	35053	49080	0.72	0.1

Although the action of trypsin is clear, the above experiments do not allow one to distinguish between two possible modes of action of phospholipase C: the enzyme could either be destroying the toxin binding site or it could be merely degrading the membrane into units small enough to pass through the filter while still leaving the toxin-binding entities functional. To clarify this point, the same experiment as described for Tables IV and V was performed on membrane fragments in buffer 3, except that an aliquot of the filtrate (0.5 ml) was chromatographed exactly the same way as the detergent extracts on an 0.8 × 25 cm G-75 column. The results are shown in Table VII, and indicate that recovery of toxin lost from membrane-bound sites as soluble high molecular weight complex is less than 5% of the

Table VII. Refinement of Phospholipase C Experiment Values in cpm

	Phospholipase C - blank (S)	Control -blank (C)	Blank	S/C	Error
Filter disk assay for membrane fragments	84484	116425	13512	0.73	±0.03
G-75 column-recovery of cpm in void volume	597	1283	964		
"Expected" G-75 void volume recovery (1/2 of loss from membrane	15006	(0)	(964)		

value expected if there were no damage to such toxin binding sites.

Discussion

The results of the experiments, as described in Tables IV-VII, are summarized in Table VIII. The symbols "+", "-" and "0" indicate, respectively, a definite increase, decrease, or no change in toxin binding capability, while the symbol "δ" indicates a small or statistically insignificant effect.

α-Bungarotoxin was chosen as the radioactive label in these experiments because of (a) its apparent high degree of specificity (39, 41, 43) and (b) its irreversibility when bound to the toxin-specific site. These two characteristics allow a comparison of ligand bound to well-washed membrane fragments to that bound in a

Table VIII. Summary of Treatment Results

(Blank spaces indicate that no test was performed.)

Treatment	*Torpedo* membranes	*Electrophorus* membranes	*Electrophorus* Triton extract
DNase	δ+	0	0
RNase	0	δ+	0
Phospholipase C	—	—	δ+
Trypsin	—	—	—
Neuraminidase	+	δ+	
Hyaluronidase	0	0	0
DTT 10^{-3} M	—	δ+	
DTNB 10^{-3} M	0	0	
PCMB 10^{-3} M	0	δ-	—

soluble extract without the possibility of losses by dissociation.

The results indicate that only trypsin and phospholipase C have clear-cut effects on toxin binding capacity. Trypsin causes a dramatic reduction in both *Electrophorus* and *Torpedo* membranes, phospholipase C causes a lesser but still significant reduction.

An anomalous situation exists in the *Electrophorus* Triton extract. Here, repeated experiments showed no reduction in specific toxin-binding capacity upon phospholipase C treatment; if anything, treatment *increased* binding capacity. Yet when the filtrate from a

membrane experiment was assayed for bound toxin, virtually none was found (<5% of the observed loss in toxin capacity from treated membranes could be accounted for as high molecular weight labeled material in the filtrate). One possible explanation is that the detergent supplies the essential phospholipid interactions to the toxin-binding protein; since no detergent is present in the membrane experiments, the essential phospholipid interaction is lost there.

The effect of neuraminidase is to increase binding capacity in Torpedo and perhaps in Electrophorus as well, and may be due to an "unmasking" of binding sites covered by sialic acids, either in the intact organ or as an artifact of the homogenization process.

Finally, PCMB at 10^{-3} M appears to effect a significant reduction in binding capacity in Electrophorus Triton extract (a result observed in two experiments in which incubation times were 30 and 60 min). This reduction is not so apparent in the membranes. It is possible that the effect is masked by the large statistical variation of the values for the membrane assay.

From these results it is possible to conclude, for Electrophorus, that the toxin-binding entity is a membrane-bound protein or phospholipoprotein which does not require nucleic, sialic or hyaluronic acids for its activity. Furthermore, this material is unaffected by DTNB in situ, but is susceptible to slight inactivation by 10^{-3} M PCMB when solubilized, suggesting the presence of at least one free thiol group which, however, is resistant to oxidation.

It can be concluded that the toxin binding site in Torpedo is likewise located on a phospholipoprotein or a protein with an essential phospholipid requirement, which has no requirements for nucleic, sialic or hyaluronic acids. It has, in addition, a disulfide bridge susceptible to reduction by 10^{-3} M DTT, which may be involved in stabilizing the conformation of the binding site.

Karlin has reported data from electrophysiological experiments (22, 26) which show that 10^{-3} M DTT inhibits the electroplaque response to cholinergic activators, such as acetyl choline or carbamyl choline. However, the effect of the reduction is quite subtle; hexamethonium, which is normally a competitive inhibitor of the acetylcholine response, becomes an activator in DTT-treated cells (26). It is therefore not surprising that DTT had no effect on the toxin binding capacity in Electrophorus membranes. It may be that low molecular weight cholinergic agents could serve as more sensitive probes of the more subtle chemistry of the physiological receptor, once this entity has been isolated.

Chapter 5

Conclusions and Discussion

The early part of this work showed that low molecular weight ionic ligands are useless as chemically specific labels in a total electric tissue homogenate. It also indicated that affinity chromatography, using derivatized agarose as the adsorbent, was not a suitable method for selectively removing the receptor from such a homogenate. Cuatrecasas, who has recently published an extensive treatment of the method (46), has only reported success in the use of such adsorbents when the ligand attached is essentially nonionic in character (73-75). It may be that the problem with ionic ligands is general in nature: Sepharose modified by attachment of such groups is capable of functioning as an ion exchanger, able to interact with any molecule bearing a group of opposite charge. Sepharose which is presently commercially available has in fact recently been shown to carry ionic groups and to function as an "ion-retarder" with basic polypeptides (76). These problems with lack of chemical specificity led to the investigation of α-Bungarotoxin as a possible labeling agent.

This neurotoxin proved to be quite specific, easy to radioactively label and easy to purify, both in native and iodinated form; futhermore, both forms were found to be equally active physiologically. Membrane assays by a filter disk method showed that α-Bgt binds to Electrophorus membranes to the extent of 0.36 μg/g

original tissue, to Triton X-100 extracts of these membranes at 0.3 μg/g, and to Torpedo membranes at about 2 μg/g (J. Duguid and R. G. Wolcott, unpublished). The complex from Electrophorus has been shown to be of high molecular weight by G-75 and Sepharose 6B chromatography. It emerges in the void volume of G-75 columns and has an apparent molecular weight of 600,000 on Sepharose 6B. However, it appears to have a molecular weight of slightly less than 260,000 by density gradient centrifugation. The disagreement is probably due to a non-globular shape of the complex.

Preliminary evidence for homogeneity and therefore uniqueness of the toxin complex was obtained from electrofocusing experiments, where a symmetrical peak of radioactivity at pI ~5.1, and separated from the bulk of the protein, was observed in addition to the free toxin peak at pI 9.5. The unmodified toxin-binding material would be expected to exhibit a lower pI than its toxin complex, and there is evidence that this is in fact the case: binding experiments on fractions from an electrofocusing experiment revealed toxin binding capacity around a pI of approximately 4.7.

Studies using several enzymes established that the material of interest from both Electrophorus and Torpedo contains trypsin-- and phospholipase C--susceptible structures essential to its ability to specifically bind α-Bgt. No other susceptibilities were detected. However, repeated experiments involving phospholipase C and a Triton X-100 extract of Electrophorus electric tissue showed that, under the conditions used, the enzyme (demonstrated to be fully

active) has no effect on specific toxin binding capacity, although the latter can still be destroyed by trypsin. This puzzling result can be explained by postulating that the protein involved requires associated membrane phospholipids in order to maintain its proper conformation, and that Triton can supply the necessary interactions upon extraction. Thus, while it is tempting to assign the toxin-binding entity to the phospholipoprotein category on the basis of the membrane experiments, the detergent extract results tend to limit the assignment (at least in Electrophorus) to a protein with a membrane-dependent conformational requirement which can also be fulfilled by Triton X-100.

Chemical studies on this protein revealed dissimilarities between Electrophorus and Torpedo. In the skate, there was a marked sensitivity to the action of DTT, suggesting the involvement of an easily reducible disulfide bond. Eel tissue, on the other hand, showed no sensitivity of toxin binding to DTT, but was affected by PCMB. This implicated a sulfhydryl group; the fact that DTNB was without effect suggests that the group is resistant to oxidation.

The DTT results for Electrophorus are not in agreement with the electrophysiological results of Karlin (22, 23, 26, 28). A significant experimental difference that should be borne in mind is that he used low molecular weight cholinergic agents such as ACh and Carb for his studies, while in the present work a polypeptide of molecular weight 8000 was employed. The results have shown that α-Bgt is chemically far more specific than small molecules in interacting with

the protein in question, perhaps due to its greater number of contact points. Karlin pointed out (26, 28) that although DTT inhibits depolarization due to ACh and Carb, the compound Hexa, normally an inhibitor of the ACh response, becomes an activator, causing depolarization. This means that cholinergic agents must still be able to bind to the DTT-reduced receptor. All that has been altered is specificity; the protein is still capable of its physiological function. DTT probably produces a very subtle conformational change in the receptor, not large enough to cause a major change in the binding of the relatively large α-Bgt molecule. Thus, while small molecules may lack the chemical specificity which is available in the larger α-Bgt molecule, subtle changes in receptor conformation may only be detectable through the use of small cholinergic compounds.

Finally, antibodies specific against α-Bgt were produced in rabbits and purified by conventional techniques. Immunosorbents prepared by coupling antibodies to Sepharose 2B were shown to be capable of adsorbing iodinated α-Bgt nearly quantitatively from very dilute solutions, and to release the bound toxin almost quantitatively with an appropriate change in elution conditions.

The toxin-specific immunosobent has been used in this laboratory to attempt purification of the toxin complex from detergent extracts of electroplax membranes (D. G. Clark and R. G. Wolcott, unpublished). The immunosorbent was capable of removing a significant fraction of the radioactivity from preparations of high molecular weight complex (free of unbound toxin), while most of the

protein passed through unhindered. Most of the bound radioactivity could be released (after thorough washing of the immunosorbent) by using 2 M acetic acid. The major portion of the released label chromatographed in the void volume of G-75 columns, indicating it was still high molecular weight material. SDS-polyacrylamide gel electrophoresis of this material indicated the presence of subunits of about 40,000 molecular weight in addition to toxin.

Additional evidence for the formation of a ternary "protein-toxin-antibody" complex has been obtained in this laboratory from sucrose density-gradient centrifugation of a mixture of F_{ab} fragments of α-Bgt-antibodies and the high molecular weight toxin complex (R-Tx) (J. Schmidt, unpublished). It has also been obtained from Sepharose 6B chromatography of a mixture of R-Tx and divalent antibodies, where the radioactivity emerged in the void volume, while R-Tx alone is partially included in the gel and emerges later (J. Schmidt and D. G. Clark, unpublished). Other evidence includes our own work using antibody-pretreated α-Bgt on frog muscle (48), and that of Changeux' group, which used the α-Toxin from N. nigricollis (77). They reported in vivo labeling of single electroplaque cells with toxin, addition of anti-toxin IgG from rabbit, and lastly addition of fluorescent-labeled sheep anti-rabbit serum. Photographs of these preparations revealed fluorescing material localized on the innervated face, which has been shown by other methods to contain the acetylcholine receptor (37, 39). Control experiments using serum from non-immune rabbits revealed much

less fluorescence in this region.

However, the evidence presented above does not prove the existence of an antibody-toxin-protein complex. The gel filtration experiments were performed with divalent antibodies; thus the change in elution position of high molecular weight radioactivity could also be explained by antibody-assisted dissociation of the R-Tx complex followed by aggregation of antibody-toxin units (Ab-Tx) into multimolecular species such as -Ab-Tx-Ab-Tx-Ab-. This possibility is supported by results from gel filtration experiments involving R-Tx and F_{ab} fragments (where no shift to higher molecular weight can be shown) and by results from gel electrophoresis of complex eluted from immunosorbent columns, where bands corresponding to antibody subunits were often observed in addition to or in place of the 40,000 MW band referred to earlier.

To summarize, the toxin-specific antibody provides a very attractive and potentially successful means for isolation of the R-Tx complex in a relatively pure state. Technical problems still exist and must be overcome before the crucial question of whether or not a ternary complex exists can be unambiguously answered.

The data presented here are insufficient to allow a claim of identity for the physiological cholinergic receptor and high molecular weight α-Bgt complex demonstrated <u>in vitro</u>. Although evidence has been presented for the specificity of labeling and uniqueness of the complex, clarification of this point can only be achieved by isolation of the toxin-binding material in homogeneous form and by testing it

for specificity, affinity, and reversibility with the various cholinergic ligands. α-Bgt may well be the required tool to complete this isolation; only then can the low molecular weight compounds be used to (possibly) characterize the protein as the acetylcholine receptor.

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Abstracts of Propositions

Proposition I

A method is proposed for introducing a heavy atom into a protein molecule at a single, known location, prior to final purification and crystallization for X-ray diffraction studies. A basic polypeptide of 8000 MW, α-bungarotoxin, is used as the example.

Proposition II

A purification method for choline acetyltransferase using affinity chromatography is proposed. The immobilized ligand is coenzyme A attached to an organomercurial Sephadex.

Proposition III

Evidence from several membrane systems suggests that carboxyl activation may play an important role in biological energy transduction and utilization processes. It is proposed to investigate this possibility through the use of sensitive ^{18}O tracer techniques coupled with radioactive labeling of participating carboxyl groups. Isolation of such labeled groups following reactions carried out in ^{18}O-enriched water and analysis of their ^{18}O content would allow estimation of the degree of carboxyl activation in these processes.

Proposition IV

A study of substrate and inhibitor binding behavior on lysozyme is proposed using a gel filtration technique analogous to equilibrium dialysis. The results of such a study could be correlated with those obtained from X-ray crystallography and nuclear magnetic resonance techniques.

Proposition V

Mitochondria and chloroplasts exhibit many structural and functional similarities. The suggestion is made that these two organelles may have evolved from a common ancestor which parasitized host organisms but become a true symbiont in the course of time. A test is proposed for assessing the degree of homology between the DNAs of the two organelles.

Proposition I

α-Bungarotoxin, a component of the venom of the Formosan Banded Krait (Bungarus multicinctus) has been shown to be a post-synaptic blocking agent in vivo (1-3) and to bind in a specific, irreversible manner to a unique macromolecular component of electroplax membranes (4). This component may be the physiological receptor for acetylcholine, and it would be of interest from both physiological and chemical viewpoints to know the shape of the toxin molecule which interacts with it so strongly and specifically.

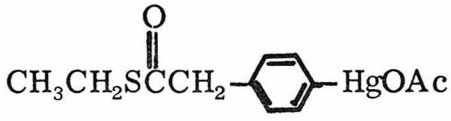
A study of the crystal structure of α-Bgt has already been proposed (5) using the native and mono-iodinated proteins; however, a second heavy-atom derivative is necessary. This proposal suggests the preparation of three possible derivatives for such an investigation by means of covalent attachment of the heavy atoms to single residues on the toxin.

It is reasonably certain that derivatives can be made where only a single residue on the protein has been modified. Barnard et al. (6) have prepared ^{3}H-acetylated α-Bgt by reaction of the toxin with ^{3}H-acetic anhydride at pH 7.5, and report finding 0.4-0.8 acetate molecules per toxin. This modified toxin retained full physiological activity. The fact that reaction proceeds at a pH near neutrality suggests that either the α-terminal amino group (pK ~7.2-7.5) or a histidine (similar pK) is the reacting nucleophile; the ε-amino

groups of the lysine residues, with pK 10.2-10.8, would be more than 99% ionized at pH 7.5 and would thus be relatively unreactive. The reaction sequence to be outlined will take advantage of the nucleophilicity of the reactive group near neutral pH.

Thiol esters of aliphatic compounds are especially reactive toward nitrogen nucleophiles (7). Two such esters, one of which can carry either of two heavy atoms, are suggested as reagents for modifying the toxin.

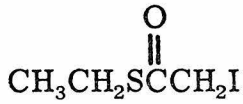
The first is the thioethyl ester of phenylacetic acid. Phenylacetyl chloride is commercially available and could be reacted with ethane thiol in ether or benzene with pyridine as a base. The product would probably be a liquid which could be purified by vacuum distillation. The purified ester could be reacted neat with mercuric acetate; this procedure gives rise, in the case of the methyl ester, to exclusive <u>para</u> mercuration (8). The product (I) could be purified by recrystallization.



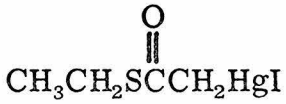
I

The second reagent could be prepared from commercially available iodoacetyl chloride and ethane thiol and purified by distillation. It could be used as purified (II), or as the mercury derivative (III), prepared by incubating the iodo compound with mercurous

sulfate at about 50° for 15-20 hr (9).



II



III

Any of these compounds could be reacted with α-Bgt at pH 7-7.5 under the conditions of Barnard et al. (6) to yield a mono-substituted derivative. Since reaction is unlikely to be complete or exclusive (reagents have limited water solubility, and a small number of ε-amino groups can be expected to react under these conditions), it would be necessary to purify the modified derivative. Chromatography on carboxymethyl cellulose has been shown to be capable of separating native and iodinated toxins (3), and would thus be the method of choice in separating native toxin from the modified species. It should also be possible to separate molecules modified on α- and ε-amino groups due to the different pK values of these functions, provided chromatography is carried out near pH 8.

The chromatographically pure derivative could be checked for physiological activity on an appropriate preparation (4) prior to crystallization. The advantage of such a pure compound, regardless of whether or not it displays physiological activity, is that the heavy atom is known to be located exclusively at one position throughout the crystal. This should simplify calculations from the X-ray diffraction pattern. It may also be a method of general application

for protein crystallography, provided that the protein in question possesses an accessible group chemically distinct from other groups on the same molecule. This assessment is easily made as a preliminary step by reacting the protein with the radioactive reagent of choice under suitable conditions and analyzing the product to determine the number of reagent molecules incorporated per protein molecule.

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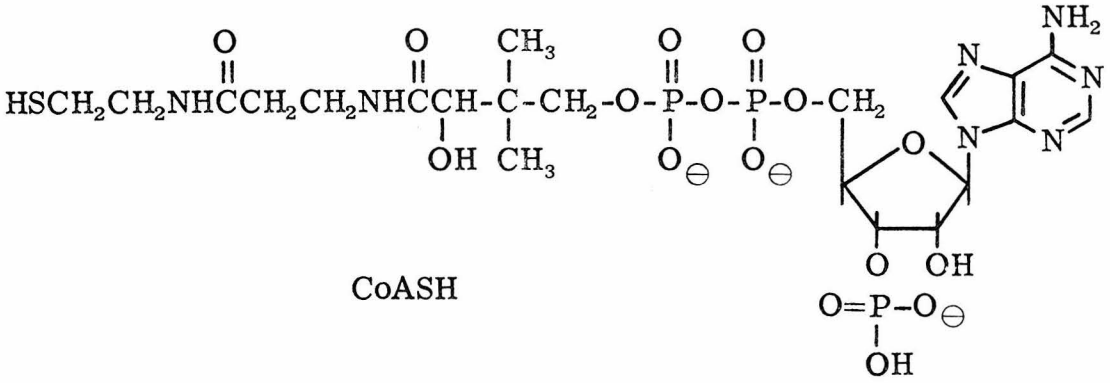
Proposition II

An account of a partial purification of choline acetyltransferase has recently been published (1). Although a considerable purification was achieved, complete homogeneity was not attained. This protein is thought to be localized in the presynaptic region of the nerve. Considerable effort is currently being expended in the study of the two postsynaptic proteins, acetylcholine esterase and the acetylcholine receptor; a more complete understanding of the acetyltransferase would help complete the picture of the process of impulse transmission across synapses. A homogeneous preparation would be very useful for mechanistic studies and indispensable for chemical characterization.

A purification method is proposed which should avoid some of the steps now used and which should help stabilize the protein (which has shown varying degrees of lability, depending on the source) during isolation. The method involves affinity chromatography, using coenzyme A (CoASH) covalently linked to an organomercurial Sepharose or Sephadex support. Numerous precedents for such procedures exist; see for example, references 2.

The purification sequence of Glover and Potter (1) contains



CoASH

the following steps: (a) homogenization, in a pH 5 buffer, of striate nuclei of bovine brain, (b) gradient elution chromatography on CM-Sephadex, (c) ammonium sulfate fractionation, and (d) gel filtration on Sephadex G-200. Although a purification factor of 570 was achieved, comparison of the protein and activity profiles from the final step revealed the presence of considerable extraneous protein. The purified material, in addition to choline acetyltransferase activity, also acetylated carnitine. However, a 30-fold purification of the former over the latter was attained.

Rechromatography of the most active fractions on G-200 yielded a molecular weight of about 65,000. The enzyme proved to be inactive below pH 5, and had a pH optimum of 7.5-10. Salts up to 150 m$\underline{M}$ seemed to have little effect on the purified preparation.

The enzyme was found to bind both acetyl CoA (K_m = 10 $\mu\underline{M}$) and free CoASH (K_I = 16 $\mu\underline{M}$ with acetyl CoA as substrate), and neither inhibition by products nor activation by choline was found. In contrast, the carnitine acetyltransferase present had a K_m = 400

μM. Although the two enzymes were completely specific for choline or carnitine, respectively, each product of the carnitine enzyme competes with at least one substrate (with K_i values approaching the substrate K_m values) while the choline enzyme suffers no product inhibition (1).

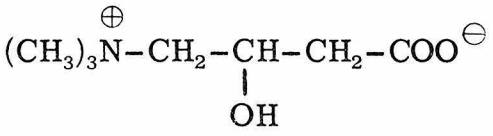
Since CoASH binds reversibly with a dissociation constant of about 10^{-5} M, it is proposed to prepare immobilized inhibitor on a Sepharose or Sephadex support. Eldjarn and Jellum (3) have described the preparation and use of an organomercurial polysaccharide (a derivatized Sephadex) which, in essence, contains a free -SH group at the end of a long chain whose opposite end is anchored to the gel matrix; the -SH group is then reacted with mercuric acetate or nitrate to obtain the organomercurial. This gel is quite stable and easily handled.

The authors report (3) that hemoglobin can be adsorbed to such a gel and quantitatively released either by adding 10^{-4} M cysteine to the eluant or by lowering the pH from 7.95 to 5.3. Cysteine-eluted hemoglobin was always recovered without any sign of denaturation by three criteria (absorption spectrum, sedimentation behavior and titrable -SH groups).

Such a resin could be prepared and reacted with free CoASH such that the final concentration of bound CoASH is of the order of 10^{-5} M in the gel (the extent of mercuration should be such that not many mercury atoms remain unreacted at this level of substitution). Fixing the ligand concentration near the value of the known dissociation

constant has two advantages: first, it minimizes interactions with proteins of the same specificity but lower affinity (e. g., the carnitine enzyme), and second, it minimizes the ion exchange properties of the column.

The bovine brain homogenate (supernatant) could then be passed through the column; only the choline acetyltransferase and other CoA-binding enzymes of similar affinity should be retained. Since the carnitine enzyme would probably be present to some extent, it would be advantageous to remove it before eluting the choline enzyme. Product inhibition of the former is known to occur. Therefore it is suggested that O-acetylcarnitine be added to the eluant; this should displace the contaminating enzyme from the matrix-bound CoASH. The compound could be readily synthesized by reaction of N-acetyl-imidazole with the alcoholate anion of carnitine, the latter being generated by the action of $NaNH_2$ on the commercially available



Carnitine

material (4).

Release of bound protein remaining after extensive washing could be effected by addition of cysteine to the eluant, lowering the pH or perhaps by raising the salt concentration (2, 3). Subsequent carboxymethyl cellulose chromatography should allow good resolution

of the eluted proteins; if necessary, a gel-filtration step could also be employed.

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Proposition III

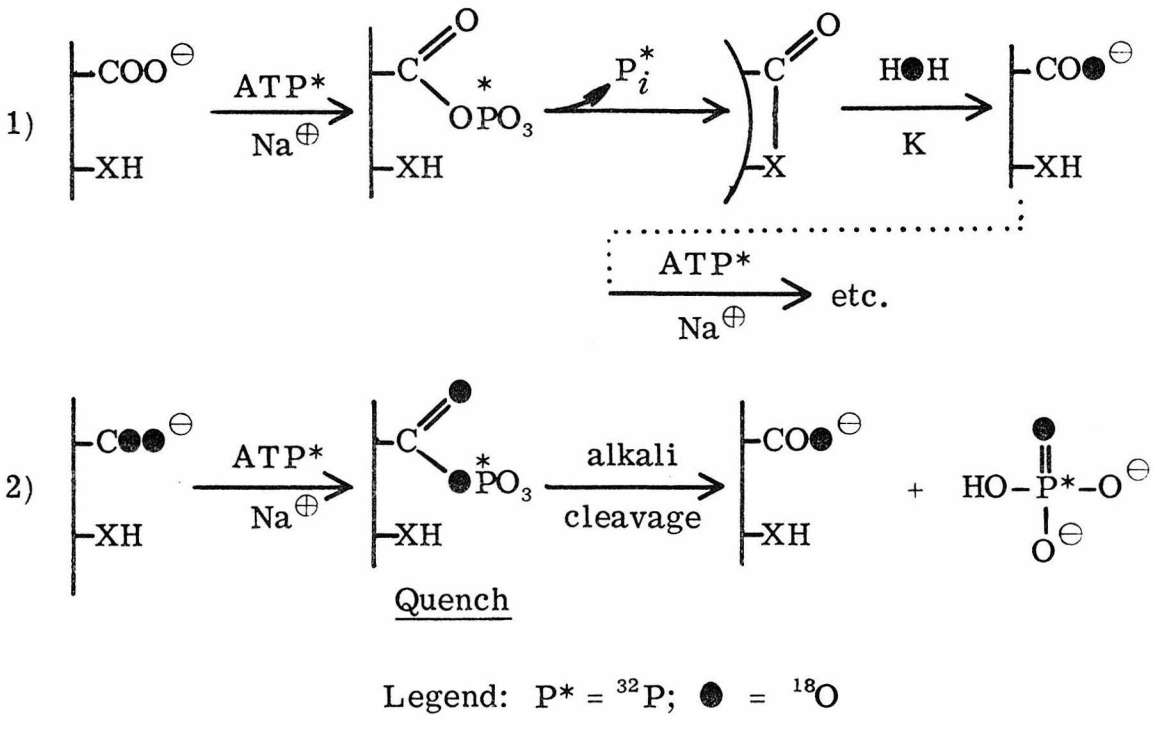
There is evidence from several biochemical systems suggesting that certain biological energy transduction and utilization processes (including transport phenomena) involve formation of an activated acyl intermediate. The interesting possibility exists, with the various systems, that upon energization carboxyl groups may form such intermediates with loss of an oxygen to water. Upon completion of the catalytic cycle these groups may become labeled with water oxygens.

The research proposal which follows involves the development of a methodology which would allow the isolation and characterization of specific carboxyl-containing compounds from membranes, and subsequent determination of their ^{18}O contents. The proposal is divided into two parts in the interest of clarity. The first section deals with recent evidence suggesting the presence of activated carboxyl intermediates, while the second proposes a method of attack on the problem of isolating these carboxyl groups.

Historical Background

^{18}O Labeling of Na^+, K^+-Dependent ATPases. Unpublished experiments of Boyer and Dahms with the Na^+, K^+-dependent ATPase from porcine kidney and Electrophorus (electric eel) electroplax tissue using $AT^{32}P^*$ and $H_1^{18}O$ show that, in short time periods, about one-half of the participating carboxyl groups become labeled with ^{18}O, possibly via an acyl-x intermediate. A possible mechanism for this

process is shown below:



Legend: P* = ^{32}P; ● = ^{18}O

*Abbreviations used: ADP, adenosine diphosphate; ATP, adenosine triphosphate; DCCD, dicyclohexyl carbodiimide; DNP, 2, 4-dinitrophenol; NAD, nicotinamide adenine dinucleotide; P_i, inorganic phosphate; β-TMG, methyl-β-thiogalactoside.

If such a mechanism were operative, one would expect the oxygens of participating carboxyls to become labeled with ^{18}O if the reaction were allowed to proceed for several cycles in ^{18}O-enriched water. Subsequent quenching and isolation of radioactive P_i released by alkali cleavage, followed by conversion of oxygen to CO_2 and analysis for excess ^{18}O in the mass spectrometer would reveal whether the carboxyls were exchanging oxygen with water. The fact that such exchange is observed is suggestive of an activated acyl intermediate.

Evidence for a Phosphorylated Intermediate in Mitochondrial Oxidative Phosphorylation. Studies by Cross et al. (1, 2) on mitochondrial oxidative phosphorylation indicate the presence of a phosphorylated intermediate. The terminal bridge oxygen in ATP derives from ADP, not inorganic phosphate (P_i), indicating that P_i enters the phosphorylation sequence prior to or at the same time as ADP. Results using oligomycin and aurovertin in the presence or absence of arsenate indicate that the two antibiotics block the phosphorylation pathway at different points; oligomycin blocks before and aurovertin after the incorporation of P_i. Experiments using $^{32}P_i$ show that aurovertin-blocked mitochondria contain higher levels of bound ^{32}P than do oligomycin-blocked organelles; this incorporated P_i is rapidly discharged by added cold P_i or DNP. Furthermore, Cross and Boyer (unpublished) have observed sensitivity to pH and hydroxylamine, as would be expected for an acyl phosphate.

Inhibition of Coupled Respiration and Covalent Labeling of Mitochondrial Membranes by a Carboxyl Reagent (DCCD). Beechey et al. (3) have shown that DCCD at 2 nmoles/mg protein inhibits coupled respiration in intact ox heart mitochondria. Arsenate will not relieve such inhibition, but uncouplers will. DCCD has no effect on substrate-linked phosphorylation associated with α-oxo-glutarate, but inhibits ATP-driven partial reactions of oxidative phosphorylation, ATPase, and ATP $\rightleftharpoons$ P_i exchanges in submitochondrial particles. The rate of onset of inhibition by DCCD is increased two-fold when the pH is changed from 8.68 to 7.3.

If ^{14}C-DCCD is used to label submitochondrial particles, it is found that they lose their ability to catalyze the ATP-driven reduction of $NAD^{\oplus}$ by succinate, and most of the covalently bound radioactivity is found in a proteolipid fraction consisting of four major components (4). Beechey (personal communication to P. D. Boyer) suggests a possible carboxyl derivative.

Sensitivity of Anaerobic Transport as well as ATPase to DCCD in S. faecalis. Studies on S. faecalis (5-10) show that anaerobic transport is sensitive to uncouplers of oxidative phosphorylation and to DCCD. The uncouplers neither inhibit glycolysis nor synthesis and turnover of ATP, but prevent utilization of metabolic energy for transport (6). DCCD is a potent inhibitor of the membrane-bound ATPase of S. faecalis. The enzyme can be solubilized from inhibited preparations in active form. However, when reconstituted into depleted membranes, it again becomes sensitive to DCCD inhibition (5). ATPase can be protected from DCCD inhibition by Mg^{++}, Na^{+}, and ATP (to varying degrees) (8).

Energy-dependent Transport in E. coli without ATP Intervention. Energy-dependent transport of proline and methyl-β-thiogalactoside (β-TMG) coupled to O_2 uptake, but without intervening ATP formation, has been demonstrated in E. coli (11-13). Membrane vesicles from the bacterium are capable of O_2-dependent transport of proline; this transport is independent of the presence of ATP or arsenate, or the absence of P_i, but is inhibited by uncouplers. These vesicles do not conduct oxidative phosphorylation. The

demonstrated insensitivity to arsenate makes unlikely the intervention of ATP. The same effects are observed in intact E. coli; furthermore, under anaerobic conditions proline transport is dependent upon ATP and can be abolished by adding arsenate.

E. coli can accumulate β-TMG against a concentration gradient under anaerobic conditions; this is abolished by uncouplers of oxidative phosphorylation even though the latter process is not occurring. Under these conditions, the carrier remains functional and facilitates equilibration across the membrane. The presence of uncouplers does not alter the steady-state level of ATP, suggesting that they act only to uncouple energy utilization in membranes from active transport of β-TMG.

Oxygen Exchange Suggestive of a Carboxyl Intermediate. Oxygen exchange studies suggest the involvement of a carboxyl intermediate in oxidative phosphorylation and the ATPase reaction. When arsenate is present to block recombination of P_i, oxygen exchange accompanying ATP cleavage by submitochondrial particles is nearly abolished. The data are suggestive of a mechanism involving dynamic reversal of ATP formation rather than labilization of phosphoryl oxygen (13).

Research Proposal

Further study of the nature of carboxyl activation is important for several reasons. Transport phenomena are fundamental to many biological processes; increased knowledge about the underlying

mechanisms of nutrient and ion translocation will help in our understanding of the basic processes of life at the molecular level. Elucidation of steps in the mechanisms of oxidative phosphorylation and transport will reveal possible fundamental relationships or similarities between these phenomena. One particularly intriguing aspect of such a study is in connection with impulse transmission in nerves; this phenomenon is intimately connected with the active transport of ions, and a study of possible acyl intermediates may do much to increase our understanding of this important process.

The number of carboxyl groups involved in membrane energy coupling systems is undoubtedly very small in comparison to total protein, and therefore it is important to first localize and identify these moieties. A promising approach to this problem is through the use of nucleophiles to label the suspected activated carboxyl group; compounds which are under consideration include N-methyl substituted hydroxylamines and hydrazines. A radioactive label could be introduced into the molecule via methyl amine or dimethyl amine, both of which are commercially available radiochemicals. Methods for the synthesis of N, N-dimethylhydrazine (14, 15), N, N-dimethylhydroxylamine (16) and N-methylhydroxylamine (17) have been published and appear to be fairly straightforward.

A possible reaction scheme would involve bacteria or isolated membrane systems supplied with energy sources, material to be transported, oxygen and other necessary factors; after a suitable short time period, the reaction could be quenched (by rapid freezing

and lyophilization) and the protein denatured, with subsequent addition of the nucleophile, or stopped by direct addition of the reagent. One or both of these methods should yield derivatives labeled at the site in question if an acyl intermediate participates.

It is extremely important to choose quenching and subsequent handling conditions such that there is no possibility of exchange of label from water or of discharge of an active acyl intermediate. For this reason, direct addition of the nucleophile may be the method of choice.

An alternate scheme which might prove to have greater specificity would involve reaction with DCCD. This reagent reacts with carboxyl functions to produce a reactive intermediate which is quite susceptible to nucleophilic attack. In the membrane system under study, a reaction would be started as outlined above, with substrate or other protective compound present in excess to inhibit the action of DCCD at the transport-related site (8). After the reaction had been started, DCCD would be added, followed by glycine or another small nucleophile. This would serve to derivatize any carboxyl groups accessible to the medium except those involved in the transport process. After such "cold" labeling, low molecular weight reagents could be removed by dialysis or centrifugation of the membrane system and replaced by DCCD and a radioactive small nucleophile. In this way, only those carboxyls directly involved in the transport process should bear radioactive labels.

After successful labeling, the membrane system would be subjected to tryptic digestion as described by Ingram (18), followed by a "fingerprint" peptide map (18, 19). In this way, label could be localized to one or more specific peptides either by radioautography or liquid scintillation counting, and the peptide(s) of interest eluted for further degradation (19). In this way the label could be localized to a specific amino acid.

Once this is done, model compounds can be prepared containing, in the carboxyl group, the amount of ^{18}O expected from the experiments proposed below, taking into account the amount of radioactive label found in the exploratory experiments already described. Methods for conversion to CO_2 of very small amounts of these compounds, corresponding to expected yields based on the exploratory work, will be investigated. It is possible that the method of pyrolysis in the presence of guanidine chloride, developed by Boyer et al. for the analysis of low atom percent excesses of ^{18}O in CO_2 (20), will be adequate, but it is probable that more sensitive methods will need to be developed.

As soon as a suitable methodology for detection of low levels of ^{18}O has been worked out, reactions involving $H_2{}^{18}O$ would be run as before and stopped by the appropriate radioactive nucleophile in the way that gave the best results in the exploratory experiments. The isolation procedure described would yield the labeled carboxyl group which would then be analyzed for ^{18}O. The presence of excess ^{18}O would implicate carboxyl activation via an acyl-X intermediate

(see the mechanism proposed in part A).

The nature of material to which the radioactive label binds could be characterized early in the isolation by various criteria, such as solubility in aqueous, organic, or detergent-containing media coupled with spectral, amino acid, sugar and nucleotide analyses. In this way it could be determined whether the energy-linking system were a protein, proteolipid, glycoprotein or nucleoprotein.

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Proposition IV

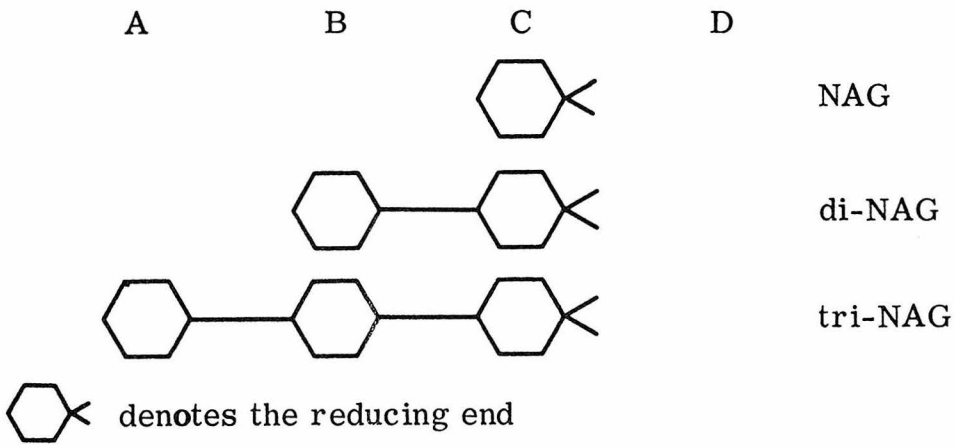
A gel-filtration method for the study of the interaction of inhibitors with enzymes which is analogous to equilibrium dialysis has been described by Hummel and Dreyer (1) and extensively investigated by Fairclough and Fruton (2). It consists of preparing a column of a gel whose pore size is small enough to completely exclude the macromolecule but large enough to include the inhibitor. The column is equilibrated with a buffer containing the inhibitor at the desired concentration, and then a known volume of the same buffer containing a known concentration of protein is loaded and eluted. The protein, being excluded from the gel beads, will run faster than the solvent and will emerge at the void volume of the column, having carried with it the fraction of inhibitor originally bound. The remainder of the loaded solution will emerge at the salt volume of the column, and will be correspondingly depleted in the amount of inhibitor. The degree of increase in level of inhibitor in the protein peak and of decrease at the salt volume should (ideally) be identical and equal to the amount bound. The ideal inhibitor concentration would be that at which half of the enzyme present is bound; this would produce the maximum change in the observed excess or deficiency of inhibitor, and would in fact be the K_S value, or dissociation constant, for the inhibitor on the enzyme. Mathematically the relationship is

$$K_S = \frac{[E][I]}{[E \cdot I]}$$

where E refers to free enzyme, I refers to free inhibitor, and $E \cdot I$ is the enzyme inhibitor complex. When [I] is at the value of K_S, half of the enzyme originally present would be complexed and half would remain free.

The method will be discussed with respect to lysozyme as an example of its utility in comparing results from X-ray structural determinations of inhibitor binding and the behavior of the enzyme-inhibitor system in solution. It is proposed that the competition between various inhibitors of lysozyme be studied and compared to the X-ray evidence of Blake et al. (3) and the nmr evidence of Raftery et al. (4, 5, 6).

The crystallographic results indicate the presence of three strong binding sites for saccharides in the cleft which appears to constitute the active site of the enzyme. These are designated A-B-C. In addition, there are at least two other, weaker, binding sites, designated D and E. The nmr evidence (4-6) shows that N-acetylglucosamine (NAG) monosaccharides bind exclusively in site C; that chitobiose (di-NAG) binds in B-C; and that chitotriose (tri-NAG) binds in A-B-C. This evidence is summarized diagrammatically below:



At present it is unclear from nmr methods where N-acetyl-muramic acid (NAM) binds to lysozyme. Fluorescence studies indicate that it may bind in either site B or D (7), and nmr evidence definitely excludes it from site C, since no chemical shift change is observed in its acetamido methyl resonance (6).

Penicillin has been shown to bind to lysozyme by crystallographic work (8, 9) and by the gel-filtration technique here described (R. G. Wolcott, unpublished). The latter work indicated that penicillin binds in the area of sites A and B due to the fact that, while no competition is observed with NAG, it is observed with di-NAG.

It is proposed that a study of the competition between penicillin and NAM be carried out by the gel-filtration technique. It has been shown (R. G. Wolcott, unpublished) that NAM competes (weakly) with α-methyl-NAG, which is known to bind in site C (4, 6). If NAM is found to displace penicillin, then it must bind in site B. On the other hand, if it does not affect penicillin binding, then it must bind in site D. Definite assignment to one or the other of these sites would

support Phillips' proposed binding orientation of the bacterial cell wall, in which NAG residues are located in sites A and C, while NAM residues (which alternate with NAG) occupy sites B and D (3).

The filtration method could also be used to obtain directly the binding constant for NAM (or any inhibitor, for that matter). The only requirement is that there be some way of detecting a change of concentration of inhibitor relative to volume eluted. In the case of the mono- and disaccharide inhibitors this could be accomplished by radioactive labeling. Such a procedure also has the advantage of requiring only small amounts of inhibitor and, hence, of enzyme.

A further use for the method would be to simultaneously follow the binding behavior of two competing inhibitors. For example, the interaction of NAM and α-methyl-NAG could be followed by double-channel counting of the differently labeled inhibitors (one could be labeled with ^{3}H and the other with ^{14}C). This would be especially desirable in view of the fact that two assumptions (that $I = I_0$ and $(1 - \frac{\delta}{\Delta}) = 1$, where δ is the observed chemical shift change and Δ is the total shift change of the inhibitor when bound to the enzyme) must be made to analyze the data from nmr measurements (4-6). No assumptions need to be made with the filtration method; EI and EI$'$ are known directly from the areas of the troughs or peaks observed, and hence E, I, and I$'$ can be calculated knowing E_0, I_0, and I_0'.

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Proposition V

Although the size and the internal and external morphology of chloroplasts and mitochondria vary to a large degree in different types of cells, the organelles always fulfill the same functions. Chloropasts absorb energy from light and utilize it to fix carbon dioxide in the form of carbohydrates; in other words, the absorbed light energy is converted to potential energy, stored as carbohydrates. Mitochondria are able to tap this source of potential energy through the process of oxidation and convert it to metabolically useful forms of energy, which ultimately emerge as so-called "high energy" compounds such as ATP.

There are certain striking similarities in the nature of the structure of mitochondria and chloroplasts. Each organelle is a small body enclosed by a double membrane (1). The outer surfaces are usually smooth, but the inner surfaces are generally highly invaginated.

In mitochondria these internal structures are called cristae; they have been seen in many forms, but are always attached at some point to the inner membrane of the organelle. The spaces between the folds of the cristae are filled with a soluble matrix. Many experiments have shown that the apparatus which controls oxidative phosphorylation (production of ATP) linked to electron transport (oxidation of substrates) is localized on the cristae, and is not part of the

soluble matrix. On the other hand, those enzymes responsible for the breakdown of carbohydrates to CO_2 (i. e., the Krebs cycle intermediates) are known to be localized in the soluble matrix (2).

In chloroplasts the regions of invagination take the form of grana, which on closer inspection consist of stacks of disc-like structures, known as lamellae. Some of the lamellae extend between the grana, thus connecting them. Indeed, in certain algae there are no grana, but lamellae extend throughout the entire plastid. In the electron microscope the disc-like structures are shown to be tiny sacks; hence the designation "thylakoids" from the Greek, meaning "sack-like". The space between the lamellae is occupied by a soluble matrix known as the stroma (3). As in mitochondria, the electron transport mechanism (here driven by light energy), again coupled to (photosynthetic) phosphorylation, seems to be localized on the insoluble thylakoid membrane. It has been shown that particles smaller than thylakoids can carry out the Hill reaction, which includes light absorption, release of oxygen and at least some electron transport. This is analogous to the finding that small pieces of mitochondria can carry out portions of electron transport coupled with oxygen uptake. Again, the enzymes of carbon chemistry seem to be contained in the soluble matrix (4).

Other similarities in the chemistry carried out by these organelles and in their biogenesis have been noted. In particular, both bodies develop from colorless proplastids, undergoing differentiation and maturation (5, 1, 6). Both bodies contain an electron

transport mechanism essential for ATP production. There is, of course, a difference in that one is driven by light energy while the other is driven by transfer of electrons to molecular oxygen. But the basic similarity of electron transfer *via* a cytochrome system from a reducing agent to an oxidizing agent coupled to ATP formation is perhaps quite significant (3, 5).

There is a good deal of evidence that both mitochondria and chloroplasts arise from pre-existing bodies; they are not formed *de novo* in the dividing cell (1, 7, 8, 28). Furthermore, both have been shown to contain DNA by a number of methods, including direct determination, cytological evidence and by following the transmission of defects to daughter bodies (1, 2, 3, 5, 9, 19). Mitochondrial DNA is almost certainly double-stranded and circular (9, 10, 11), and chloroplast DNA is probably double-stranded and may be circular (12, 13). All of this suggests that these bodies may have their own means of genetic control, independent of that of the nucleus.

In both cases the organelle DNA has been shown to be different from that of the nucleus. Chloroplast DNA is distinguishable from nuclear DNA by means of different buoyant density in CsCl gradients (13, 14, 29), different base ratios and "melting" temperatures (15, 16, 17) and, in *Euglena*, absence of 5-methylcytosine, which *is* present in *Euglena* nuclear DNA (18). Mitochondrial DNA has been shown to be different from nuclear DNA by similar tests (1, 20).

On the basis of the similarities in structure and basic function as well as the fact that both organelles contain DNA which is distinct

from nuclear DNA and seemingly functional in terms of protein synthesis (1, 5, 6, 7, 21, 22, 23), it is suggested that both organelles may have evolved from a common ancestor, which itself was a living organism which parasitized host cells and eventually evolved into a true symbiont as the present-day chloroplast or mitochondrion. Similar suggestions have been made separately for the chloroplast (5) and the mitochondrion (5, 24), but apparently no attempt has been made to relate the two.

It is proposed that a study be made of the hybridization capability of denatured chloroplast and mitochondrial DNA, with a view toward establishing whether or not there is similarity between the two. This technique is well known and has been used to establish complementarity between RNAs and DNA and RNA, and DNAs.

Mitochondria can be obtained free of nuclei and cell fragments by equilibrium centrifugation on a sucrose gradient, and can be freed of nuclear DNA by treatment of the intact organelle with DNase (10). Likewise, chloroplasts can be purified and their DNA extracted (25).

It is proposed that two batches of the same organism be grown. The organism must possess both mitochondria and chloroplasts. One batch would be labeled with either ^{14}C or ^{32}P, and the other not at all. After sufficient growth, one organelle would be isolated from one batch and the other from the second batch, and both would be freed of nuclear DNA by treatment with DNase. The organelle DNAs would then be isolated and purified by preparative ultracentrifugation. The pure DNAs would then be "nicked" with DNase to an extent

sufficient to open up a reasonable fraction of the double-stranded circles while still leaving a high proportion of long linear strands. A Poisson distribution based on the assumption that attack by DNase is random shows that an average of 2--4 "hits" per DNA molecule will give a reasonable amount of long linear material. Work done on polyoma DNA, which is also double-stranded and circular, has shown that such a procedure is feasible (26).

The unlabeled, nicked DNA could then be denatured by heat or dimethyl formamide and trapped in this state in a gel, such as agar. The hardened gel can be broken into small pieces and washed free of unbound strands of DNA and other contaminants. Retention of DNA in the gel is nearly quantitative (27).

This gel containing unlabeled DNA from one organelle could then be slurried in a buffer containing nicked, denatured, labeled DNA from the other organelle, and incubated under appropriate renaturation conditions. Unhybridized label could be subsequently washed away and conditions then changed so that any hybridized material would denature. This could then be eluted from the gel and counted.

If a significant number of counts above a suitable control (such as the above experiment run with unrelated nuclear DNA) is found, it woud indicate complementarity between the DNAs of mitochondria and chloroplasts and would suggest common ancestry for the two organelles.

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