To the Graduate Council:

I am submitting herewith a thesis written by Ruanglakana Vajanabukka entitled "The Nicotinic Acetylcholine Regulator of Torpedo californica: Purification and Reconstitution." I recommended that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biochemistry.

Leaf Huang

Leaf Huang, Major Professor

We have read this thesis
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Robert Bryant

Accepted for the Council:

L Evans Roth

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Graduate Studies and Research

THE NICOTINIC ACETYLCHOLINE REGULATOR OF *TORPEDO CALIFORNICA*: PURIFICATION AND RECONSTITUTION

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Ruanglakana Vajanabukka

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ABSTRACT

Acetylcholine receptor was extracted from the electric tissue of Torpedo californica by sodium cholate. The purification was performed by using density gradient centrifugation instead of affinity chromatography. The purified receptor was then incorporated into lipid vesicles by gel filtration on Sephadex G-50. The reconstituted vesicles were unilamellar, as revealed by negative staining electron microscopy. The vesicles were heterogeneous in size with an average diameter of 0.39 ± 0.18 μm. Approximately 50% of the receptor was oriented towards the outside in these vesicles. Sodium dodecyl sulphate gel electrophoresis showed four bands of apparent molecular weight of 17,000, 40,000, 60,000, and 90,000 daltons from purified receptor either before or after reconstitution.

The function of incorporated acetylcholine receptors was tested by measurement of $^{22}Na^{+}$ influx into the reconstituted vesicles. The influx was increased in the presence of 0.02mM carbamylcholine, a cholinergic agonist of the receptor. This excitability was blocked by preincubation with 2mM d-tubocurarine, an antagonist of the receptor.

These results, therefore, demonstrated that not only the specific neurotransmitter binding sites but also the molecular elements necessary for ion translocation were reconstituted into the artificial lipid vesicles.

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CHAPTER I

INTRODUCTION

Acetylcholine is one of the best known neurotransmitters and its action on various categories of cells has received considerable attention for almost a century. Until recently, however, many scientists doubted that its "receptive substance" would ever be isolated in the test tube. Indeed, lipids, polysaccharides, proteins and even nucleic acids were equally considered as potential receptors, but definite proof that any one of these compounds could account for the physiological action of acetylcholine was consistently missing. The difficulty rested on the lack of a firm theory and a reasonable method of characterization and isolation.

In 1955, Nachmansohn (1) proposed that the membrane receptor for acetylcholine is a protein and that a conformational transition is responsible for the command of the permeability change. Since then the acetylcholine receptor has been isolated, purified and studied in several laboratories (2-6).

Acetylcholine Receptor Protein and Ionophore

Binding of acetylcholine to an excitable membrane triggers the all-or-none opening of discrete channels or pores through which Na^+ and K^+ ions flow along their electrochemical gradient. The elementary functional unit that accounts for this regulation, the acetylcholine regulator,

therefore comprises at least two categories of distinct sites: 1. the acetylcholine receptor site which binds acetylcholine (and related compounds) and snake venom α-toxins, and 2. the site of ion translocation. The macromolecular units carrying each of them are referred to, respectively, as acetylcholine receptor protein and acetylcholine ionophore.

Historically, the term acetylcholine receptor was used to refer to acetylcholine regulator system which composed of both acetylcholine receptor protein and acetylcholine ionophore. In the present study, to avoid confusion, the terms acetylcholine receptor and acetylcholine ionophore were used to refer to the receptor moiety and the ionophore moiety of the acetylcholine regulator, respectively.

The acetylcholine receptor protein is a rather well characterized macromolecule. Yet, the identity of the ion gate, or ionophore, remains to be established without ambiguity. In particular, is the acetylcholine ionophore part of the purified receptor protein or, on the opposite, an entirely distinct macromolecule, highly coupled with the receptor protein in the subsynaptic membrane <u>in situ</u> ?

One evident approach to answer this question is to try to dissociate the excitable membrane fragments into their elementary components followed by their reassociation into membrane vesicles which exhibit both the characteristic selective permeability to cations and the sensitivity to acetylcholine. This type of approach might lead to the

identification of the components responsible for the selective permeability change caused by the cholinergic agents.

Electric Organ and Its Excitability

The biochemical studies on the cholinergic receptor have been done primarily with the electric organs from electric eel, Electrophorus electricus and electric ray, Torpedo californica (and also T. marmorata) which are very rich in cholinergic postsynaptic membranes. Electric organs are made of cells called electroplaxes. These electroplaxes derive from muscle cells. They retain the electrically excitable outer membrane of muscle but have lost the contractile apparatus (7).

At the electroplax synapse, the transmission of the nerve impulse is mediated by a neurotransmitter, in this case, acetylcholine, which after being liberated by the nerve terminal causes a transient and selective increase of permeability of the postsynaptic membrane to cations. Such fast opening of the ionic channels or "activation" takes place in the millisecond range. When the neurotransmitter is applied to the subsynaptic membrane in a prolonged and continuous manner rather than as a brief pulse, the channels become unresponsive and do not open again unless the neurotransmitter is removed and reapplied. This phenomenon is usually referred to as "pharmacological desensitization" which leads to a much slower overall transmembrane cation flux.

Acetylcholine and Related Compounds

Compounds such as acetylcholine, carbamylcholine or decamethonium (4,7) increase the permeability response when they bind to the acetylcholine receptor site and, therefore, act as agonists. The structural requirements for a compound to behave as an agonist are the presence of quarternary nitrogen with methyl groups around the nitrogen atom. The effects of the agonists are blocked by compounds, referred to as antagonists, which increase apparent dissociation constant without changing the maximal response. The antagonists, such as d-tubocurarine and flaxedil, also contain quarternary nitrogens but often with large hydrophobic areas. Antagonists bind with the acetylcholine receptor complex at the acetylcholine receptor site in a competitive manner to agonist binding. The other class of compounds is the noncompetitive blocking agents. They have little or no effect on apparent dissociation constant but decrease reversibly the amplitude of the permeability response. The noncompetitive agents, including local anesthetics such as procaine, lidocaine, quotane (8,9) and the columbian frog toxin, histrionicotoxin (10), bind to the local anesthetic site. They block ion translocation at the level of the acetylcholine ionophore and some evidence suggests that the local anesthetic site is carried by a membrane protein different from the acetylcholine receptor protein (11).

Snake Venom α-Toxin as Marker for Acetylcholine Receptor

Neurotoxins from the venom of a variety of snakes,

such as α-bungarotoxin from formosan snakes Bungarus multicinctus, or α-cobratoxin block neuromuscular transmission by binding to acetylcholine receptors on motor end plates, or on the innervated face of the electroplax of the electric organ. These toxins are small proteins having 74 or 61 amino acids and molecular weight of about 8,000 (12). The neurotoxins can be made highly radioactive by iodination (13), acetylation (14), or treatment with pyridoxal phosphate (15). Labeled toxins bind tightly to the acetylcholine receptor protein with a dissociation constant as low as 10^{-10}M and has a slow reversibility. Most important, it does not bind to other macromolecules in the postsynaptic membrane. Thus, the acetylcholine receptor can be specifically marked with a radioactive α-toxin.

Isolation of the Receptor and Reconstitution into Vesicles

Electric organ of Torpedo is rich in acetylcholine receptor which is an integral membrane protein. Detergents are necessary to release it into solution. Several nonionic detergents, such as Triton X-100, Tween 80, Emulphogen and negatively charged ones, such as deoxycholate or cholate, give efficient solubilization without loss of α-toxin and cholinergic ligand bindings (16). After solubilization, the receptor has been purified by affinity chromatography with a cholinergic ligand coupled covalently to a solid matrix. The ligands selected (7) are α-toxins and a variety of

quarternary ligand derivatives. This chromatography technique, however, was reported (17,18) to destroy the function of the receptor when it is recombined with lipid vesicles. To avoid this effect, the density gradient centrifugation was introduced and satisfying result was obtained.

To combine the purified receptor into lipid vesicles, the common method used to prepare single bilayer lipid vesicles is by sonication but the disadvantage of sonication is that this treatment may lead to degradation of receptor protein and irregularities in the order and packing of the lipid molecules. In 1976, Brunner et al. (19) described a technique of preparing single bilayer vesicles without sonication. They prepared single shelled lecithin vesicles by solubilizing unsonicated lecithin dispersion with sodium cholate. As the small lecithin-cholate micelles are passed through Sephadex G-50 column, lecithin begins to aggregate as soon as the cholate is removed by gel filtration and eventually single bilayer vesicles are formed. The gel filtration technique uses mild condition and so it is useful for the reconstitution of biological membranes.

In the present study, acetylcholine receptor protein (and probably also acetylcholine ionophore) was extracted, partially purified , and reconstituted into lipid vesicles by using the gel filtration method. The incorporated receptors were then examined for their orientation within the lipid bilayer, their polypeptide chain compositions,

and finally, their function for the regulation of cation translocation.

CHAPTER II

MATERIALS AND METHODS

A. ELECTRIC TISSUE

Torpedo californica electric tissue was purchased from Pacific Biomarine, Venice, California. The tissue was frozen at -75°C and thawed before used.

B. CHEMICALS

Lyophilized venom of Bungarus multicinctus was purchased from Miami Serpentarium, Miami, Florida and was purified by ionic exchange chromatography using Sephadex CM-50 and CM-32 columns (20). Radioiodination of α-bungarotoxin was done by the method of Hunter and Greenwood (21) to a specific activity of approximately 1×10^{5} Ci/mole and 95% of radioactivity was in the form of monoiodo-bungarotoxin.

Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were isolated from chicken egg yolks, and purified by chromatography on silicic acid (22). Phosphatidylinositol (PI), sphingomyelin and cholesterol were purchased from Sigma and used without further purification. Unless otherwise stated, most of radioactive chemicals and other compounds were purchased from New England Nuclear and Sigma, respectively. Sodium cholate was twice recrystallized in 70% ethanol.

C. SOLUBILIZATION OF ACETYLCHOLINE RECEPTOR

The method of Schiebler and Hucho (23) was modified and summarized in Figure 1. One hundred and twenty grams of electric tissue from Torpedo californica was thawed and cut into small pieces and homogenized in a Virtis homogenizer for 3 min at maximum speed in 120 ml of 2mM ethylenediamine tetraacetate (EDTA), 0.1mM phenylmethylsulfonylfluoride (PMSF), 0.4M NaCl and 0.02M Tris, pH 7.4. This and all subsequent steps were performed at $4^{o}C$ unless otherwise stated. The homogenate was sieved through a nylon screen, centrifuged 90 min at 27,000xg and the supernatant was discarded. The pellet was resuspended in 120 ml of 2mM EDTA, 0.1mM PMSF and 0.02M Tris, pH 7.4 and centrifuged 90 min at 39,000xg. The pellet was resuspended and centrifuged again. After this second wash, acetylcholine receptor was extracted by adding 25 ml sodium cholate (2% w/v in the above buffer). The mixture was stirred at room temperature for 1 hr and centrifuged 60 min at 100,000xg. The pellet was discarded.

D. PURIFICATION OF ACETYLCHOLINE RECEPTOR

Purification was performed by dividing the supernatant from part C into 6 equal portions. One portion was incubated with 5 μl of ^{125}I-α-bungarotoxin (^{125}I-α-Bgt, $3x10^{5}$cpm/μl) for 30 min, $37^{o}C$. If the lipid distribution was to be studied, 2 μl of solvent-free ^{3}H-dioleoyl lecithin (DOL)

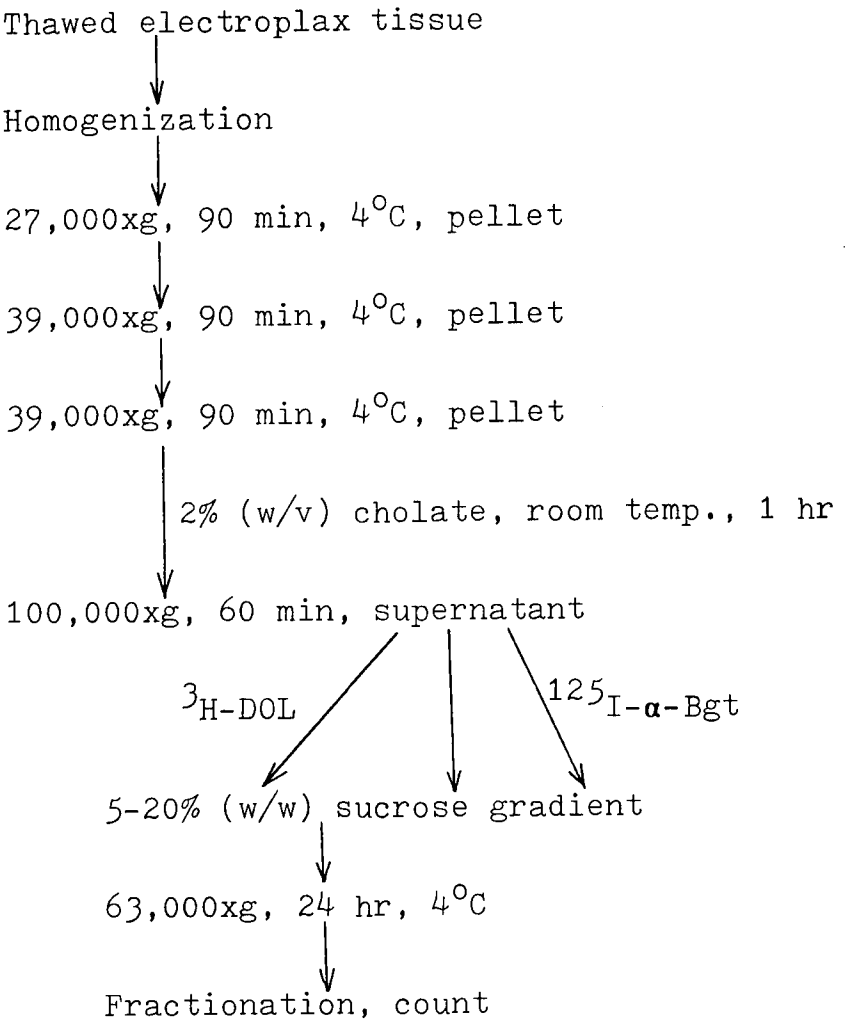


Figure 1. Solubilization and partial purification of acetylcholine receptor.

was added to another supernatant portion. Each portion was layered on top of six 35 ml linear sucrose gradients (5-20% w/w in 0.1M NaCl, 0.02% NaN_3, 1% sodium cholate and 0.01M Tris, pH 7.4) centrifuged for 24 hr at 63,000xg, 4°C in a Beckman SW 27 rotor. The gradients were fractionated and counted for the radioactivity. ^{125}I was counted in a γ-counter whereas ^{3}H was counted in a liquid scintillation counter.

E. SPECIFIC TOXIN BINDING ASSAY

One hundred microlitres of crude or gradient purified acetylcholine receptor (0.17-1.3 mg protein) was labeled with saturation amount of α-Bgt, composed of ^{125}I-α-Bgt ($5x10^5$cpm, 0.29 μg) and unlabeled α-Bgt (38.4 μg). The labeling was performed at 37°C for 30 min. The mixture was then passed through a Biogel P-60 column (1x22 cm) to separate the toxin-receptor complexes and free toxin. The fractions were collected and the radioactive peak eluted out in the void volume represented the total amount of toxin-receptor complexes. The recovery of the column elution was greater than 90%.

From the void volume radioactive peak, the activity of the acetylcholine receptor was calculated and expressed in term of nmole toxin (α-Bgt) binding sites/mg protein. All calculations were based on the value of 8,000 daltons for the molecular weight of the α-Bgt (12). Protein was determined by Lowry method(24) with bovine serum albumin as standard.

F. RECONSTITUTION PROCEDURE

The fractions of the sucrose gradients yielding the lipid-depleted receptor were concentrated to 6 ml and collected into a test tube containing 10 mg of a solvent free lipid mixture composed of 46% PC, 31% PE, 14% phosphatidylserine (PS), 7% sphingomyelin, 2% PI and 15% cholesterol. Trace amount of ^{3}H-DOL (1.2×10^{6}cpm total) was added to mark the lipids. To this suspension, sodium cholate was added to a final concentration of 1.5% (w/v). The mixture was stirred for 10 min at room temperature to dissolve all the lipids, applied to a Sephadex G-50 (coarse particle size) column (35x1.5 cm) previously equilibrated with 0.1M NaCl, 0.02% NaN_3, 0.01M Tris, pH 7.4 and eluted with the same buffer at 4°C with a flow rate of 7-8 ml/hr as described by Brunner et al. (19). The lipid peak that came out in the void volume was collected and dialysed for 12 hr against 0.16M NaCl, 5mM KCl, 2mM $MgCl_2$, 2mM $CaCl_2$, 0.02% NaN_3 and 3mM sodium phosphate, pH 7.4 (Ringer's solution).

G. $^{22}Na^{+}$-INFLUX MEASUREMENT

Twenty millilitres of the reconstituted vesicle suspension was concentrated to 5 ml by ultrafiltration on Amicon XM-100 filter. This concentrated receptor vesicles was used throughout the $^{22}Na^{+}$-influx experiment.

^{22}NaCl (final concentration 30 μCi/ml) was added to receptor vesicle suspension at room temperature. At 0, 30,

100, 180 min of incubation time, 100 μl of the incubation mixture was applied to a Biogel P-60 column pre-equilibrated with Ringer's solution. The column was packed to 1 ml bed volume in a Pasteur pipette. Fractions of six drops were collected directly into scintillation vial and counted for both ^{22}Na and ^{3}H in liquid scintillation counter. It took approximately 5 min to elute the void volume which contained the receptor vesicles.

H. ELECTRON MICROSCOPY

Negative staining preparation was performed by placing a drop of the suspension of reconstituted acetylcholine receptor vesicles on a formvar coated grid and dried. No fixation procedures were used. Staining was performed with 1% potassium phosphotungstate, pH 7.2. The specimen was viewed in a JEM 6C electron microscope, operating at 50 kV.

I. SODIUM DODECYL SULPHATE GEL ELECTROPHORESIS

The method described by Davies and Stark (25) was modified. The protein (typically 5-10 μl, containing 80-100 μg of protein) was diluted 1:5 with 1% sodium dodecyl sulphate (SDS), 1% β-mercaptoethanol, 0.01% bromphenol blue in 50% glycerol. The mixture was boiled for 3 min to denature the protein. Electrophoresis was done at 8mA/gel. The gels (6x0.6 cm) contained 5% acrylamide, 0.135% methylenebisacrylamide in running buffer (0.1M sodium borate, 0.1M sodium acetate and 0.1% sodium dodecyl sulphate,

pH 8.5) and were polymerized with 0.075% ammonium persulphate and 0.033% N,N,N',N'-tetramethylethylenediamine. The gels were cut at the dye marker and stained with Coomassie brilliant blue for 45 min (26). Destaining was performed in 7.5% acetic acid and 10% trichloroacetic acid solutions. The gels were stored in distilled water with 0.02% NaN_3.

Destained gels were scanned at 660 nm with a Gilford gel scanner and mobilities estimated from distance of migration relative to the tracking dye (bromphenol blue). Standard proteins and their subunit molecular weights were: β-galactosidase (130,000), bovine serum albumin (68,000), α-aldolase (40,000), chymotrypsinogen (25,000) and cytochrome C (13,000).

J. ORIENTATION OF THE RECONSTITUTED RECEPTOR

Two parallel experiments were done to determine the orientation of the reconstituted acetylcholine receptor in vesicle membranes. The total amount of receptor incorporated into lipid vesicles was determined by solubilization of receptor vesicles with 1% sodium cholate. The receptor (0.3 μg protein) was then labeled with saturation amount of ^{125}I-α-Bgt (4.4×10^5 cpm, 0.012 μg) at 37°C for 1 hr. Unlabeled α-Bgt (12 μg) was added and the sample was passed through a Biogel P-60 column at 4°C to separate the toxin-receptor complexes and free toxin. The fractions were collected and the peak which was eluted in the void volume represented

the total amount of receptor-toxin complexes.

To determine the amount of right-side-out receptors, the vesicles were first labeled with ^{125}I-α-Bgt for 1 hr at 37°C in the absence of detergent. By this way, only the receptors that have the toxin binding sites on the outside of lipid bilayer will be labeled. After 1 hr, large amount of unlabeled α-Bgt (12 μg) was added and vesicles were then solubilized with 1% sodium cholate. The receptors which faced inside lipid bilayer will be marked only by the unlabeled α-Bgt because ^{125}I-α-Bgt was diluted 1,000 times with the unlabeled α-Bgt. The sample was passed through Biogel P-60 column at 4°C. The fractions were collected and the void volume peak represented the amount of bound receptors oriented "right-side-out." A flow chart summarizes these procedures is shown in Figure 2.

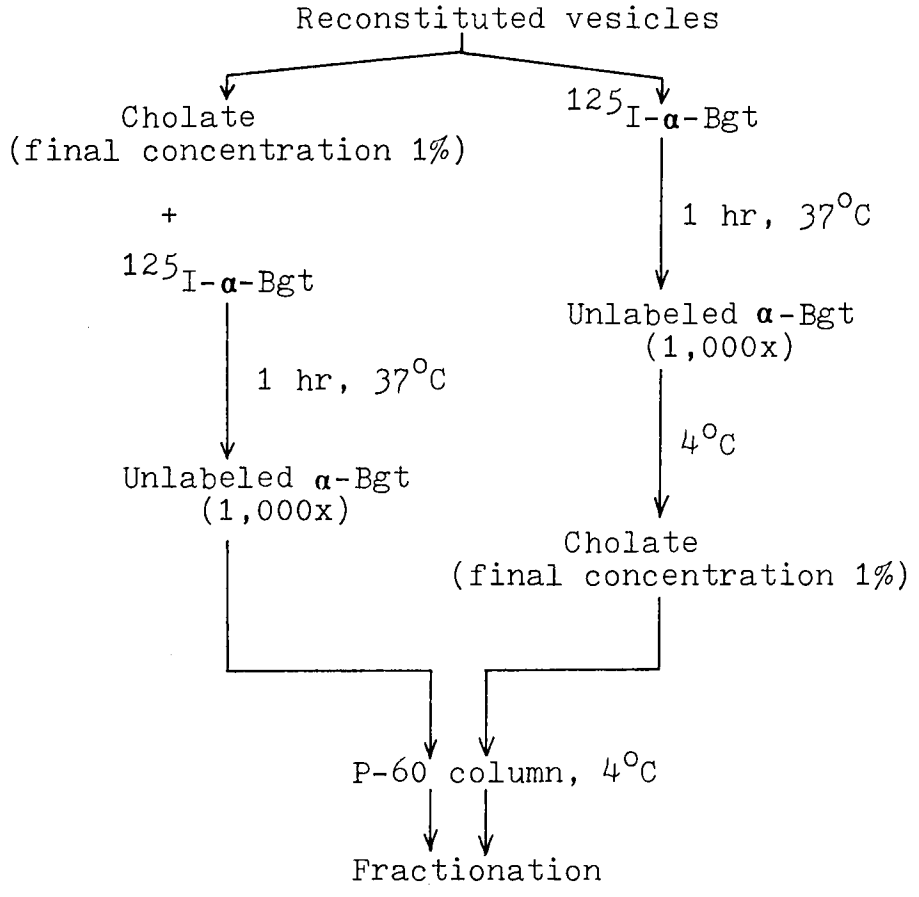


Figure 2. Orientation assay of the reconstituted acetylcholine receptor.

CHAPTER III

RESULTS

A. PARTIAL PURIFICATION OF ACETYLCHOLINE RECEPTOR

Acetylcholine receptor was extracted from electroplax tissue as described in the previous chapter. Figure 3 shows the fractionation of the receptor by sucrose gradient centrifugation after extraction with sodium cholate. The acetylcholine receptor sedimented between 6.5 and 11 S whereas the endogeneous lipids floated at the top of the gradient. The fractions rich in receptors and poor in lipids were pooled for further experiments.

The toxin binding activity of the solubilized receptor was determined by incubating receptors with saturation amount of ^{125}I-α-Bgt and fractionating on a Biogel P-60 column (Figure 4). The toxin-receptor complexes eluted in the void volume were well separated from the unbound toxin. The specific toxin binding activity of the receptor was determined for both the crude receptor preparation and after purification by sucrose gradient sedimentation. Table I shows these values. There was approximately 4-fold purification of the receptor by the centrifugation step.

The partially purified receptor showed four major polypeptide bands on SDS-gel electrophoresis in the presence of a reducing agent, dithiothreitol (Figure 5). Their

Figure 3. Fractionation of the acetylcholine receptor by sucrose density gradient. The ^{125}I-α-Bgt-receptor complexes (o—o), lipid (●—●), and protein (■—■) contents are plotted.

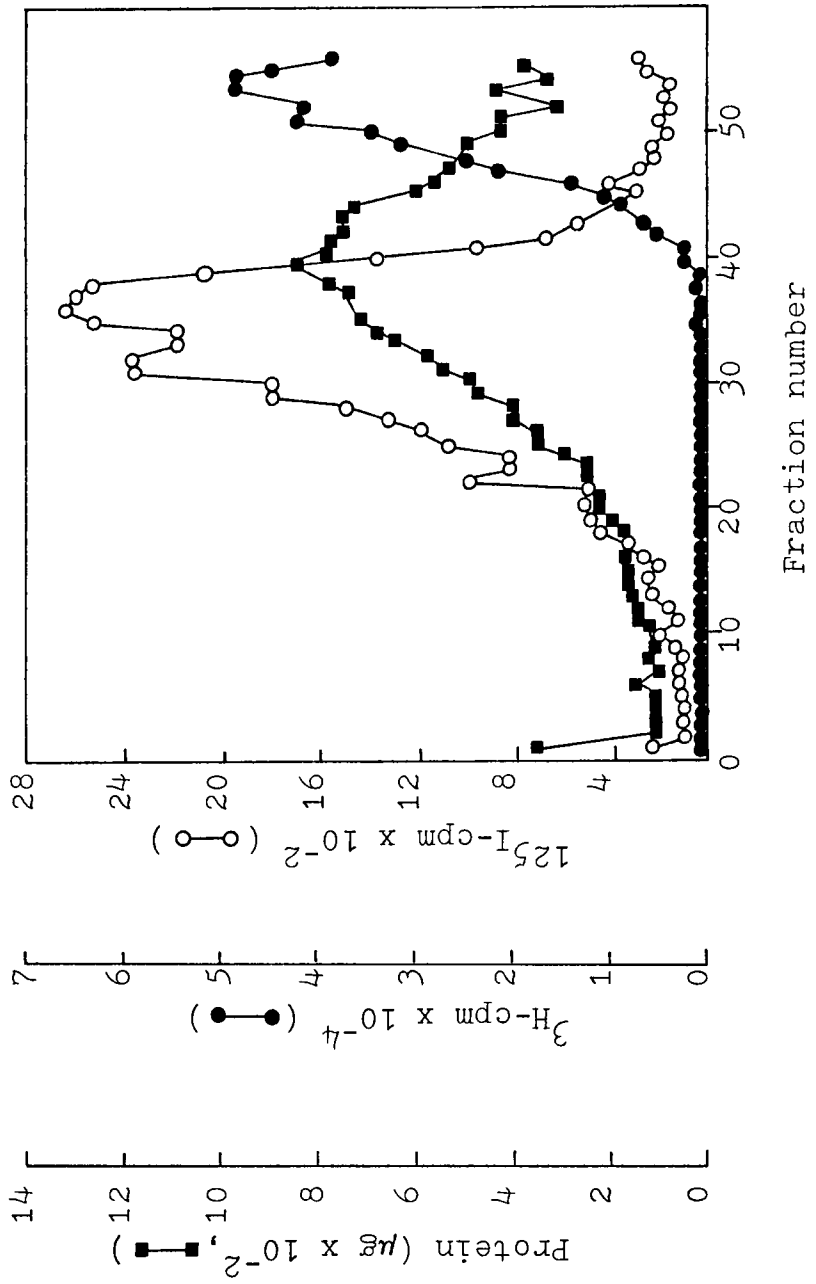
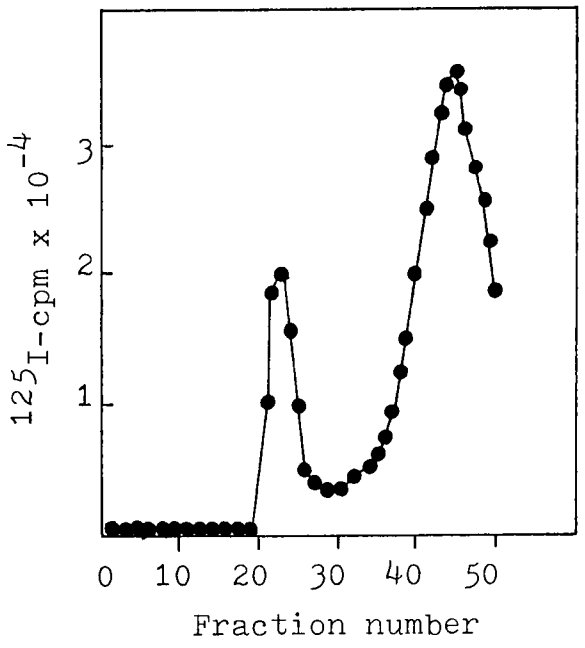
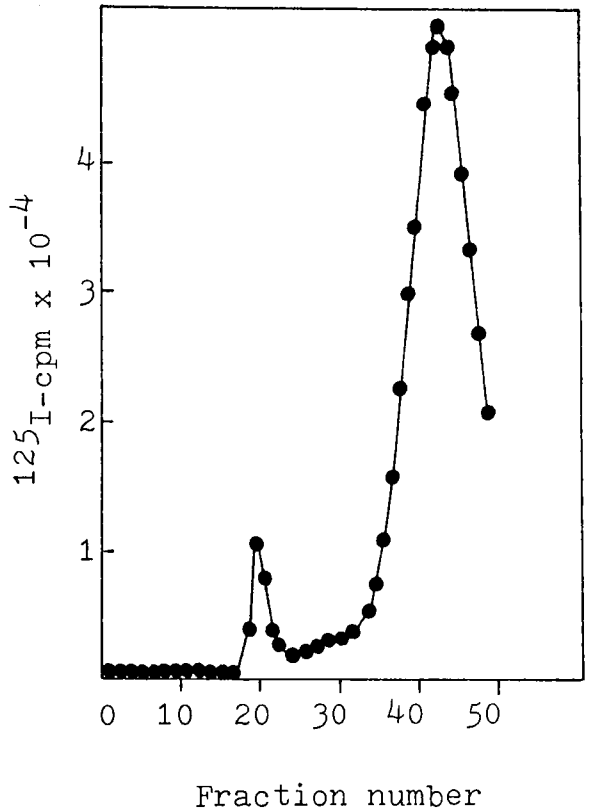

Protein (μg x 10⁻², ■—■)
0
2
4
6
8
10
12
14
³H-cpm x 10⁻⁴ (●—●)
0
1
2
3
4
5
6
7
¹²⁵I-cpm x 10⁻² (o—o)
4
8
12
16
20
24
28
0
10
20
30
40
50
Fraction number

Figure 3

Figure 4. Elution profiles of the acetylcholine receptor on Biogel P-60 columns. Receptor before (a) and after (b) purification by sucrose density gradient sedimentation were incubated with ^{125}I-α-Bgt.


^{125}I-cpm x 10^{-4}
1
2
3
0
10
20
30
40
50
Fraction number

(a)


^{125}I-cpm x 10^{-4}
1
2
3
4
0
10
20
30
40
50
Fraction number

(b)

Figure 4

TABLE I

SUCROSE DENSITY GRADIENT PURIFICATION
OF ACETYLCHOLINE RECEPTOR

Preparation	Protein			Receptor		
	ml	mg	%	n mole	n mole per mg protein	%
Crude receptor	10	137	100	72.6	0.53	100
Purified receptor	6	62	45	20.3	2.03	28

Figure 5. Sodium dodecyl sulphate gel electrophoresis of acetylcholine receptor. The gels of purified receptor before (left) and after (center) reconstitution are shown. The standards (right) are β-galactosidase, 130,000; bovine serum albumin, 68,000; α-aldolase, 40,000; chymotrypsinogen, 25,000; cytochrome C, 13,000.

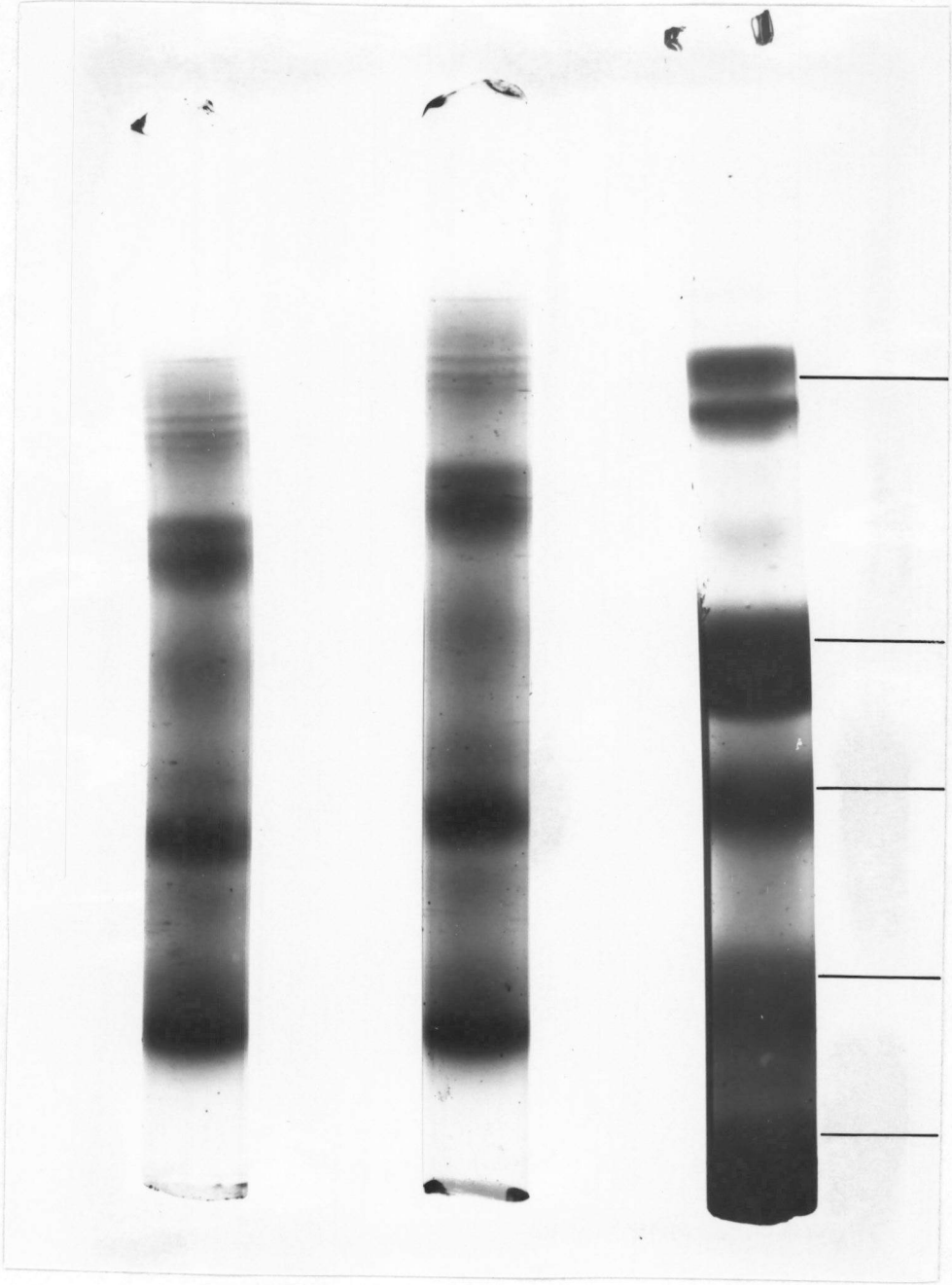


Figure 5

apparent molecular weights were 17,000, 40,000, 60,000 and 90,000 daltons. Densitometric scans of the gels stained with Coomassie brilliant blue (Figure 6) showed that the 40,000-dalton band was the most abundant one and the 60,000-dalton band appeared in the least amount.

B. RECONSTITUTION OF ACETYLCHOLINE RECEPTOR VESICLES

The phospholipid composition of the microsac membranes of Torpedo electroplaxes highly enriched with the acetylcholine receptor was reported by Schiebler and Hucho (23). Identical composition of the phospholipid was chosen for the reconstitution studies in the attempt to mimic the lipid environment of the receptor in the native membrane. The amount of cholesterol used (15%), however, was lower than what it is in the native membrane (20%), because complete solubilization of the lipid-receptor mixture in 1.5% sodium cholate could not be achieved with 20% cholesterol.

Reconstitution was carried out by Sephadex gel filtration. Figure 7a shows the elution profiles of the receptor and the lipids from column chromatography. Approximately 65% of the receptor, pre-labeled with ^{125}I-α-Bgt, was eluted in the void volume together with 95% of the ^{3}H-lipids. In a separate experiment in which the ^{14}C-cholate was used, only 0.9% of the total cholate was eluted in the void volume; the elution of the bulk cholate was well retarded (Figure 7b). The reconstituted acetylcholine

Figure 6. Densitometric scan of gel from gel electrophoresis. The gel was stained with Coomassie blue and the absorbance was measured at 660 nm. The gels of purified acetylcholine receptor either before or after reconstitution showed the same pattern.

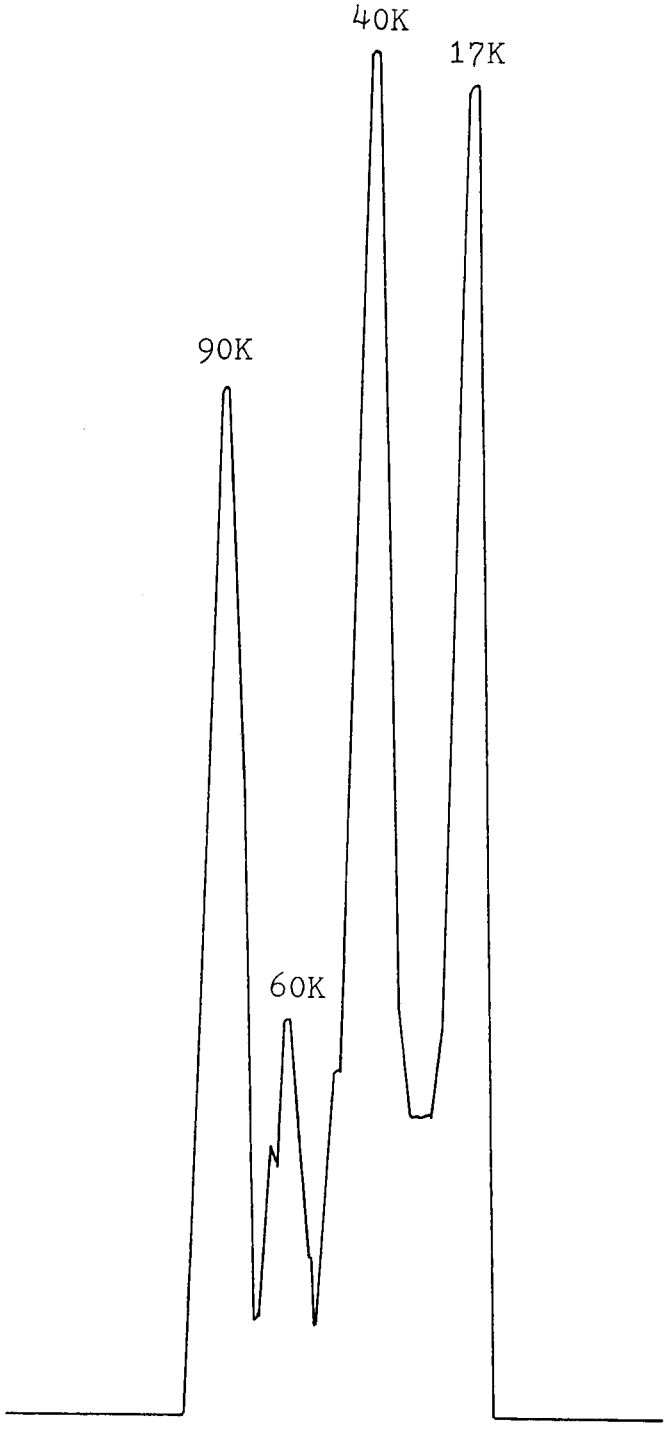
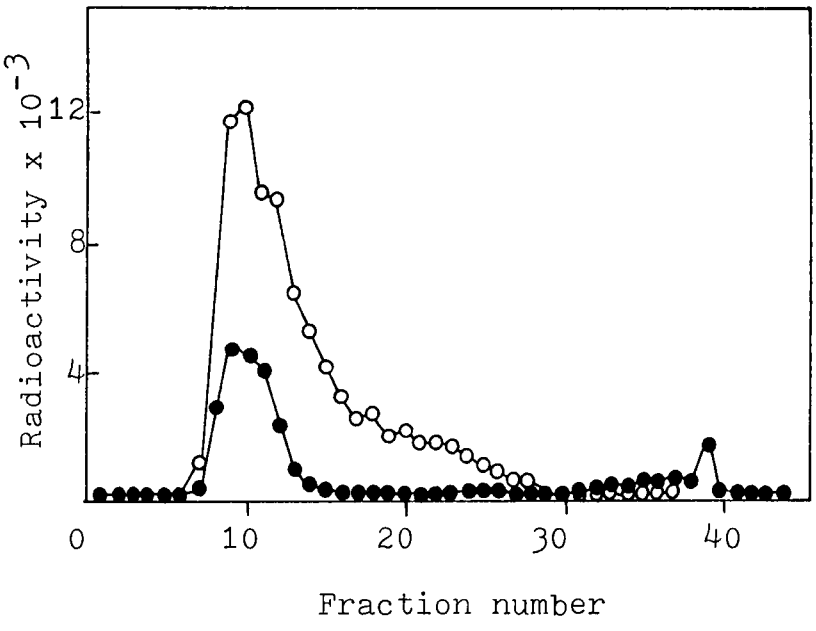
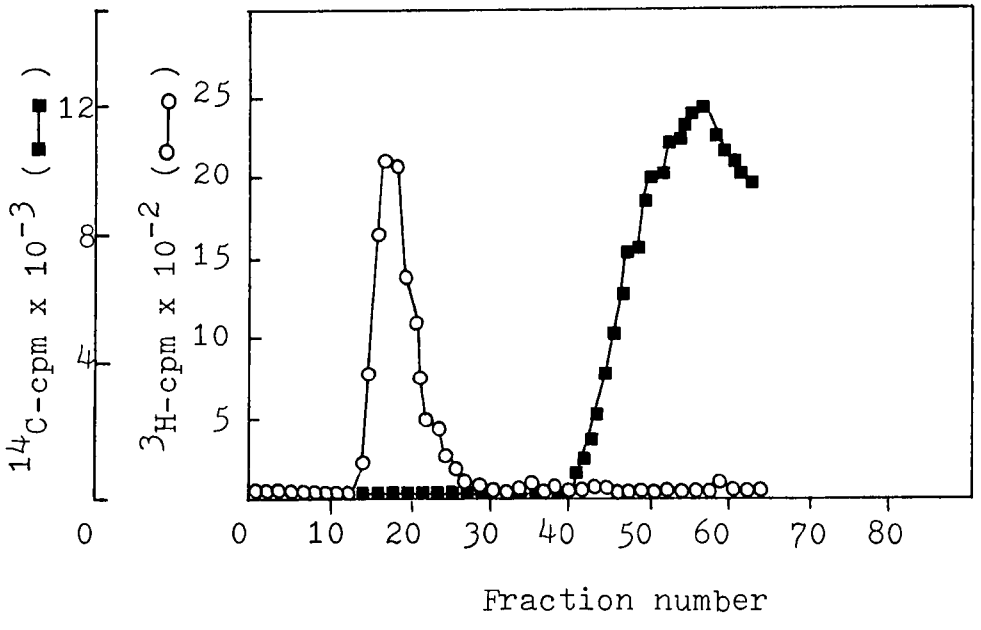


Figure 6

Figure 7. Reintegration of the lipid-depleted acetylcholine receptor complexes into lipid vesicles by gel filtration, showing the amount of receptor incorporated into vesicles (a), and the amount of sodium cholate remained in the receptor vesicle suspension (b). o—o, ^{3}H-DOL; ●—●, ^{125}I-α-Bgt labeled acetylcholine receptor; □—□, ^{14}C-cholate.


Radioactivity x 10^{-3}
12
8
4
0
10
20
30
40
Fraction number

(a)


^{14}C-cpm x 10^{-3} (■—■)
^{3}H-cpm x 10^{-2} (o—o)
12
8
4
0
25
20
15
10
5
0
10
20
30
40
50
60
70
80
Fraction number

(b)

Figure 7

receptor vesicles were dialysed for 12 hours and the residual amount of the cholate in the vesicle preparation was further reduced to 0.2% of the original amount. The lipid to protein ratio of these vesicles was 0.31 by weight.

C. PROPERTIES OF THE RECONSTITUTED ACETYLCHOLINE RECEPTORS

The reconstituted receptor vesicles were examined by the negative-staining electron microscopy. These vesicles appeared fairly heterogeneous in size, ranging from 0.15 to 0.90 μm (Figure 8). Not all of the vesicles had the stain penetrated. For those vesicles did have the stain inside, they appeared to be unilamellar. Aggregated vesicles were also found. The size distribution of the vesicles were further analysed by directly measuring from the electron micrographs. Figure 9 shows a histogram of such an analysis. The mean diameter of the vesicles was 0.39 ± 0.18 μm. This conclusion was further substantiated by the elution profile of the reconstituted vesicles in a Sepharose 4B column (Figure 10). Most of the vesicles eluted in the void volume. Since Sepharose 4B has a particle exclusion size of 800 A^{o}, this result strongly supports the direct measurement from the electron micrographs.

The reassociation of the solubilized acetylcholine receptor with exogenous lipids resulted in an insertion of the receptor proteins into the lipid bilayer of the vesicle membranes. The orientation of the receptor in the vesicle

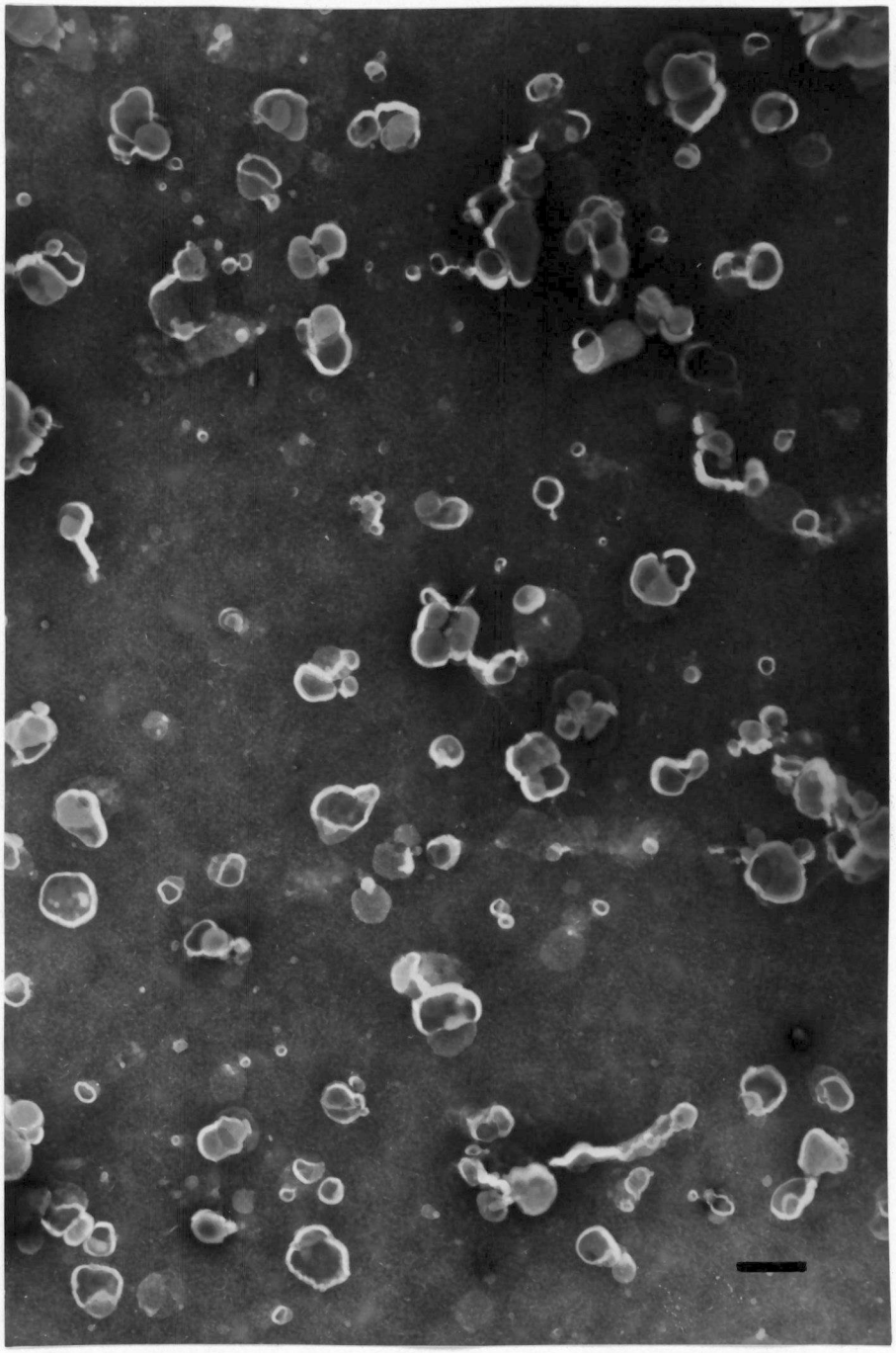


Figure 8. Negatively stained preparation of reconstituted receptor vesicles. The bar represents 1 μm.

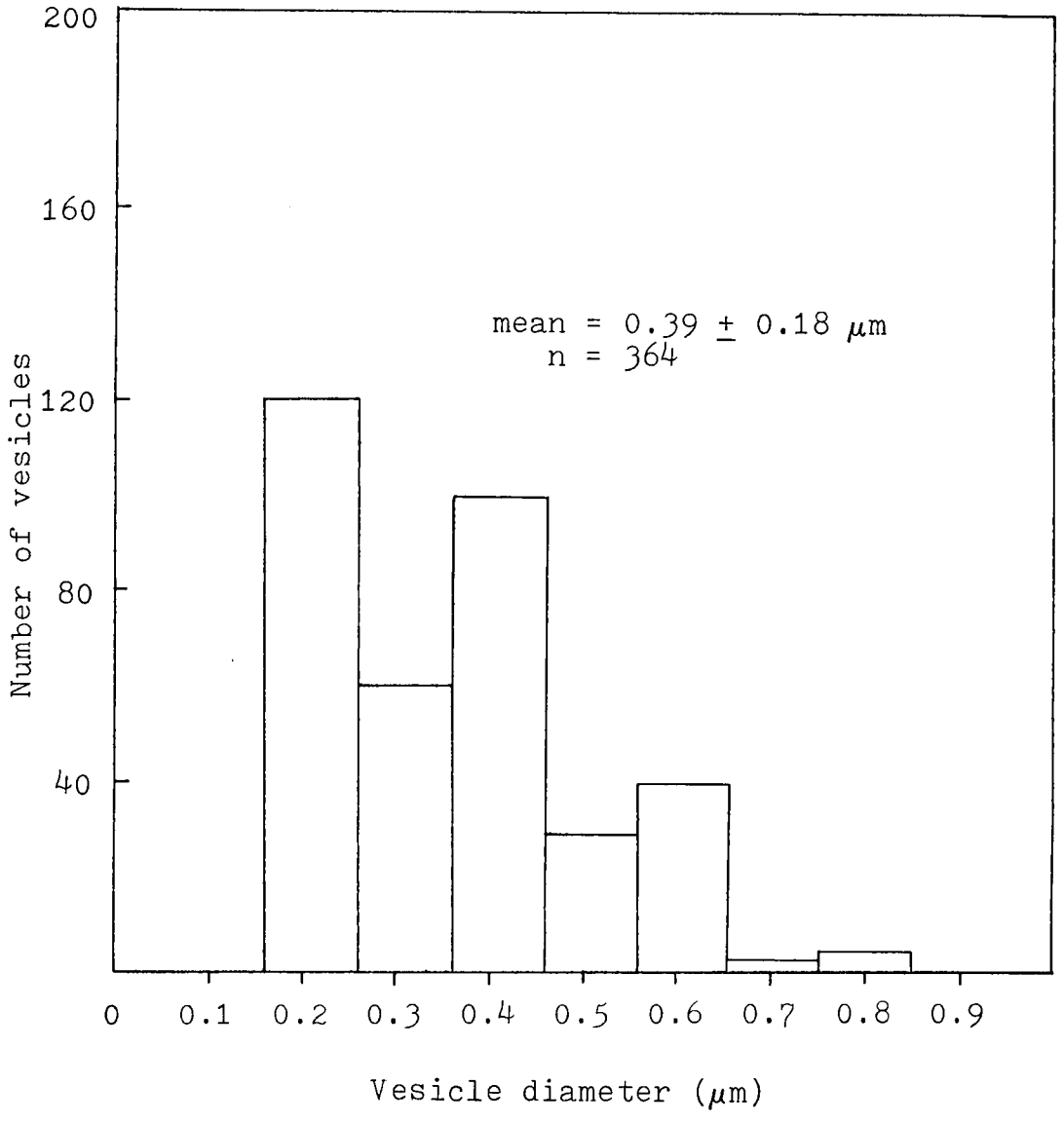


Figure 9. Size distribution of reconstituted vesicles, as determined by direct measurements from electron micrographs of the negatively stained vesicles.

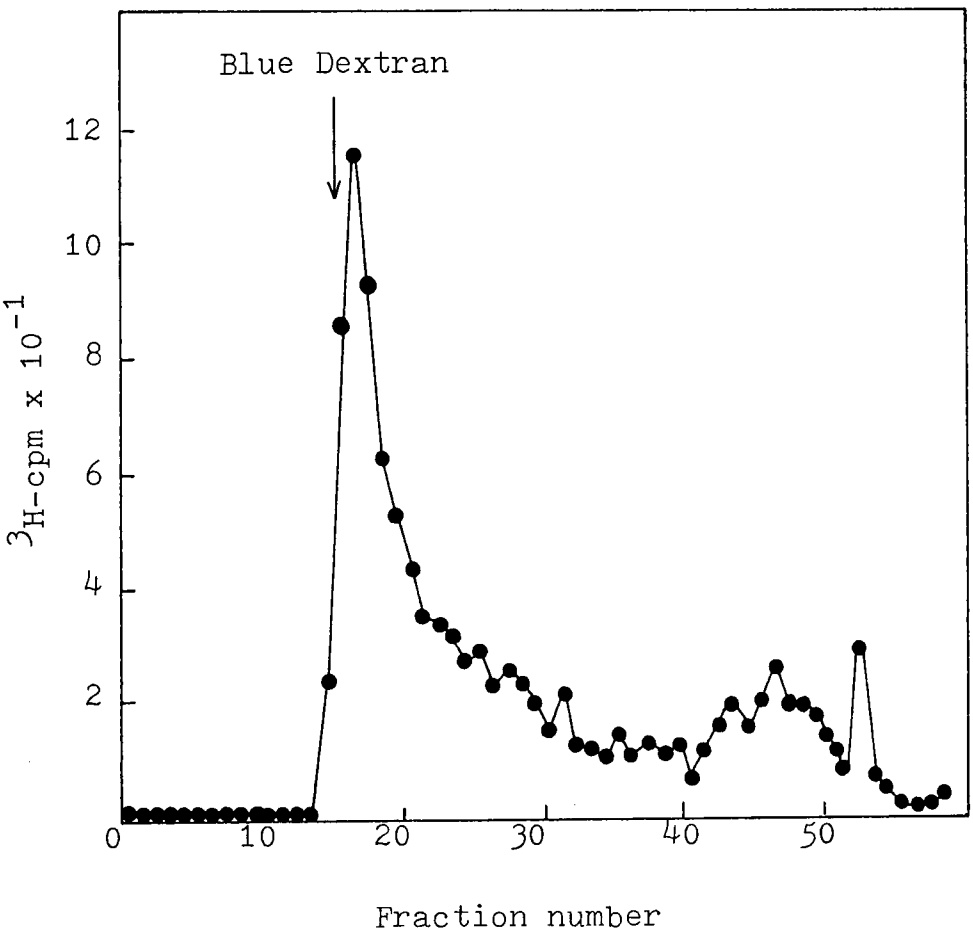
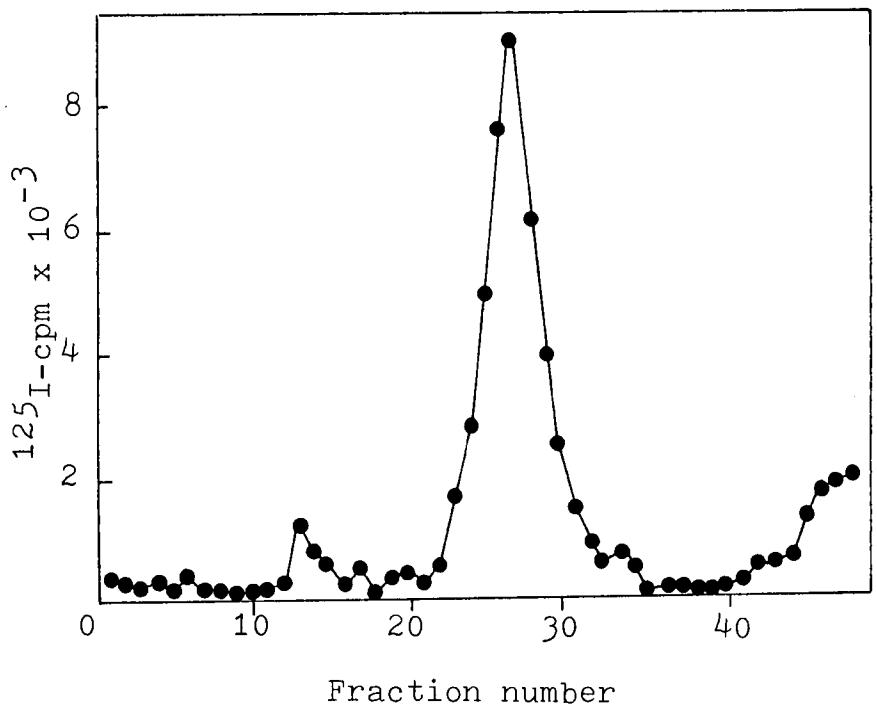


Figure 10. Elution profile of reconstituted vesicles labeled with ^{3}H-DOL from a Sepharose 4B column (1x22 cm). Blue Dextran was used as the void volume indicator.

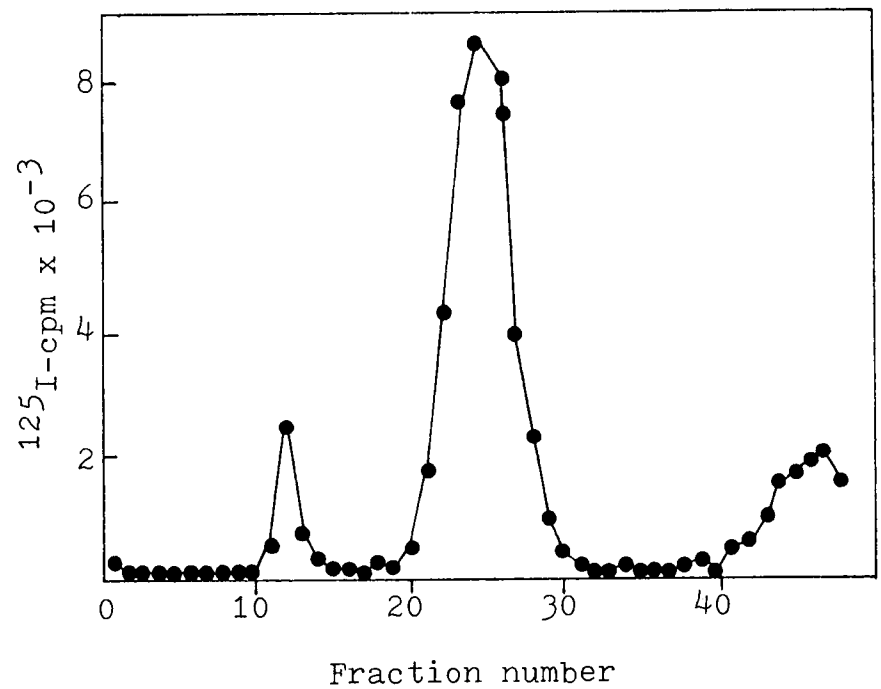
was examined by the use of radiolabeled bungarotoxin. If saturation amount of ^{125}I-α-Bgt was incubated with the reconstituted receptor vesicles, only those receptors having their toxin binding sites exposed on the outer surfaces of the vesicles could bind, since toxin could not penetrate the vesicle membranes. After incubation, 1,000 fold excess of unlabeled toxin was added, and the vesicles were solubilized with sodium cholate. The latent toxin binding sites should only be tagged by the unlabeled toxin. This mixture was then chromatographed on a Biogel P-60 column to measure the amount of the radioactive toxin-receptor complexes (Figure 11a). In a parallel experiment, vesicles were solubilized before the addition of the ^{125}I-α-Bgt. All receptors should be tagged regardless of their orientation. Large excess of unlabeled toxin was still added before the gel filtration for the sake of comparison (Figure 11b). It was found that the amount of the receptors labeled with ^{125}I-α-Bgt without vesicle solubilization was approximately 55% of the total receptors available for toxin binding. This result was interpreted as the receptors were randomly oriented in the reconstituted vesicles.

The SDS-polyacrylamide gel electrophoresis pattern of the receptors after the reconstitution was identical to those before the reconstitution (Figure 5, page 23). The scan of the gel (Figure 6, page 26) still revealed four major polypeptide bands of the molecular weights of 90,000, 60,000,

Figure 11. Orientation of incorporated acetylcholine receptors. The receptors exposed on the outer surfaces of vesicles (a) or the total toxin-receptor complexes (b), regardless of the orientation in the lipid bilayer, were chromatographed on Biogel P-60 column.


^{125}I-cpm x 10^{-3}
8
6
4
2
0
10
20
30
40
Fraction number

(a)


^{125}I-cpm x 10^{-3}
8
6
4
2
0
10
20
30
40
Fraction number

(b)

Figure 11

40,000 and 17,000 daltons. The relative abundance of the polypeptide chains did not change during the reconstitution.

D. $^{22}Na^{+}$-INFLUX OF THE RECONSTITUTED RECEPTOR VESICLES

In order to test the functional properties of the reconstituted receptor vesicles, influx of a monovalent cation, $^{22}Na^{+}$, was examined. A millipore filtration method was adapted to separate the vesicle-associated and the free $^{22}Na^{+}$, similar to what was reported in the literature (23), no reproducible results could be obtained. The reconstituted vesicles must be fairly fragile; it might be collapsed on the filter paper upon suction. Therefore, a slower method was used to separate the vesicle-associated and the free $^{22}Na^{+}$, i.e. gel filtration. Figure 12 shows the elution profile of the vesicles incubated with $^{22}NaCl$. The separation of associated and the free $^{22}Na^{+}$ was complete, although the elution of the vesicles from the column took about 5 min. Labeling vesicle lipid with ^{3}H and express the result in terms of $^{22}Na/^{3}H$ greatly improved the reproducibility of the data.

When $^{22}NaCl$ was added to the reconstituted vesicles, there was a rapid influx of $^{22}Na^{+}$ into vesicles followed by a slow increase at a constant rate (Figure 13). When 0.02mM carbamylcholine was added at the same time as $^{22}Na^{+}$, the initial influx rate was significantly enhanced, followed by a slower increase. The rate of slow influx was identical

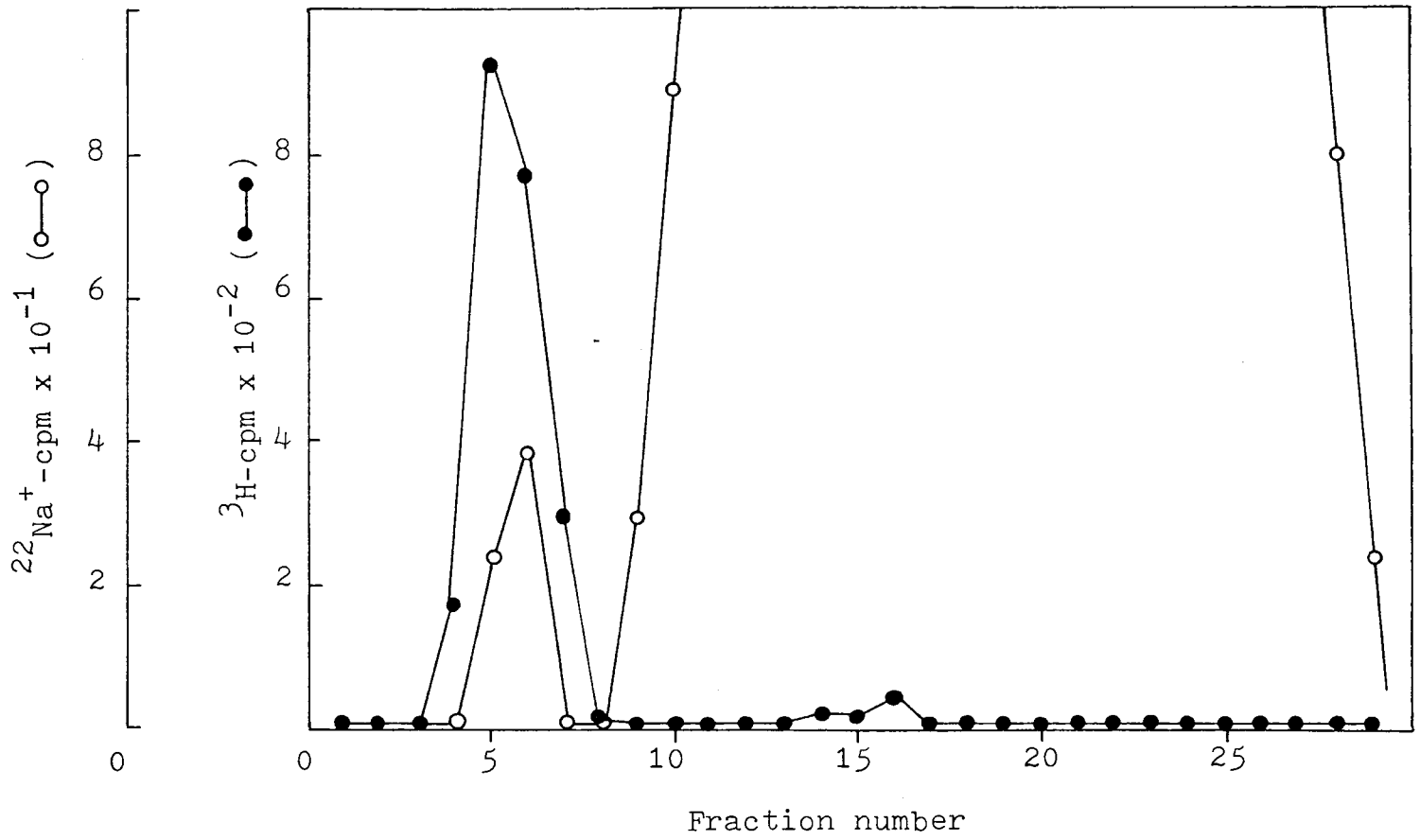


Figure 12. Biogel P-60 column elution profile of the reconstituted vesicles incubated with $^{22}NaCl$. o—o , $^{22}NaCl$; ●—● , ^{3}H-DOL labeled reconstituted vesicles.

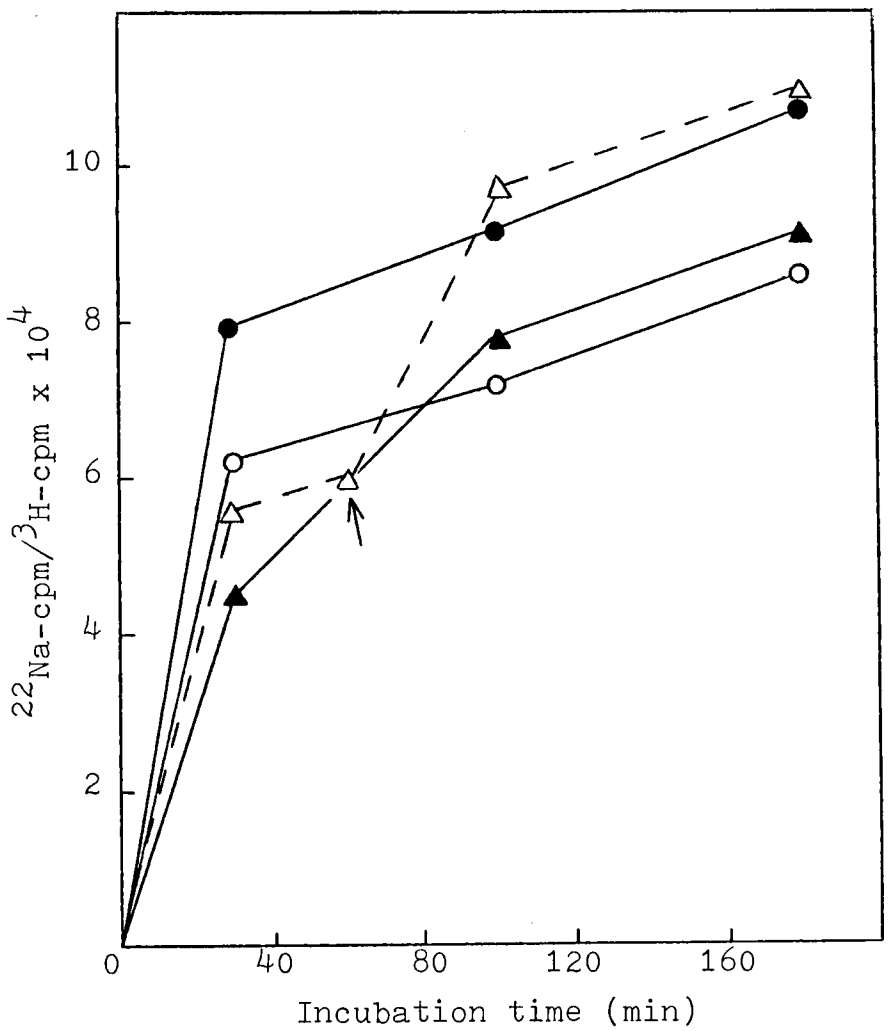


Figure 13. The uptake of $^{22}Na^{+}$ into acetylcholine receptor vesicles. The uptake was measured in the absence of carbamylcholine (o—o); in the presence of 0.02mM carbamylcholine (●—●). The carbamylcholine enhanced influx was also shown by adding 0.02mM carbamylcholine (arrow) at 60 min of incubation time (Δ—Δ). The carbamylcholine enhanced influx was blocked by 30 min preincubation with 2mM d-tubocurarine (▲—▲).

to that without carbamylcholine. The enhancement of the initial influx by carbamylcholine was further confirmed by adding carbamylcholine 60 min after the addition of $^{22}Na^{+}$. The influx jumped to the elevated level at the next time interval examined (100 min). The effect of carbamylcholine could completely be blocked by a pre-incubation of vesicles with 2mM d-tubocurarine, a potent cholinergic antagonist. The ability of carbamylcholine to enhance the initial influx of $^{22}Na^{+}$ is therefore indicative of the chemical excitability of the reconstituted receptor vesicles. The $^{22}Na/^{3}H$ obtained in this experiment was about 50% of the equilibrium value.

In control experiments, vesicles of the identical lipid composition without receptor proteins were used for the $^{22}Na^{+}$ influx measurement. As shown in Figure 14, no increase of the $^{22}Na^{+}$ influx was detected after the addition of carbamylcholine.

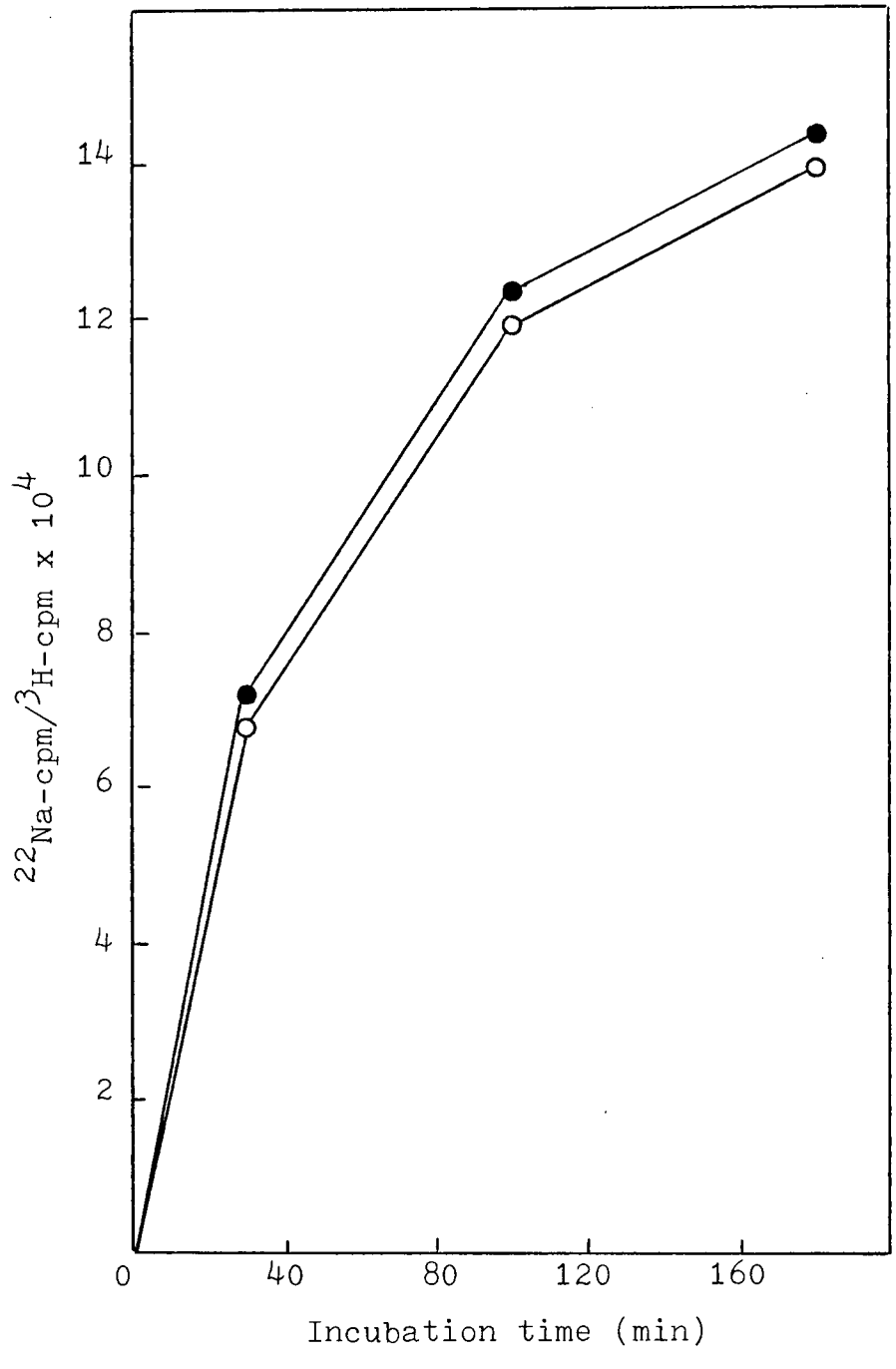


Figure 14. $^{22}Na^{+}$ uptake of lipid vesicles without acetylcholine receptor. The experiment was performed in the presence (●—●) and in the absence (o—o) of 0.02mM carbamylcholine.

CHAPTER IV

DISCUSSION

In the present study, the purification of the acetylcholine receptor and the reconstitution of a functional "excitable" membrane were attempted.

A. METHOD OF PURIFICATION

Acetylcholine receptor protein was extracted from electroplax tissue by a negatively charged detergent, sodium cholate. A nonionic detergent, i.e. Triton X-100 was not chosen due to the finding (11) that after solubilization of acetylcholine receptor with Triton X-100, all the ionic channel molecules are either dissociated or denatured. On the other hand, the cholate extract retains the proper binding activity for the local anesthetics and therefore probably contains the acetylcholine ionophore. For this reason, sodium cholate was chosen instead of Triton X-100.

After extraction, the receptor was purified by sucrose density gradient centrifugation instead of the affinity chromatography which was the commonly used method for purification.

In the affinity chromatography, acetylcholine receptor protein, but not the acetylcholine ionophore, was selectively purified. Karlin et al. (17) reported that the affinity purified receptor preparation had lost its ion translocation property after reconstitution. Recently Eldefrawi et al. (11)

used ^{3}H-perhydrohistrionicotoxin as a marker for the ionophore molecule. They suggested that the ionophore is a separate protein which can be separated from acetylcholine receptor complexes in the cholate extract by affinity adsorption of the receptor or by using the anti-receptor antibodies. Furthermore, the quarternary amino ligands used in the affinity column desensitize the ionophore function of the acetylcholine receptor in the native membranes. For these reasons, the sucrose density gradient technique was chosen in an attempt to retain the functional state of the receptor, although the degree of purification might not be as superior as that of the affinity chromatography.

The sucrose gradient purified receptor had a specific activity of 2.03 nmole toxin binding sites/mg protein and was 4 folds richer in the amount of acetylcholine receptor than in the crude extract. Schiebler and Hucho (23), whose purification method was modified and used in this study, reported the specific activity of purified receptor to be 2-4 nmole toxin binding sites/mg protein. Anyhow, the specific activity of the purified receptor, the affinity chromatography was used in some cases, was reported to range from 1 to 12 nmole toxin binding sites/mg protein (16,27, 28).

From SDS polyacrylamide gel, four major bands of apparent molecular weights of 17,000, 40,000, 60,000 and 90,000 daltons were observed from the purified acetylcholine

receptor in the presence of dithiothreitol. Schiebler and Hucho (23) also observed four bands of apparent molecular weights of 42,000, 48,000, 62,000 and 68,000 daltons from the receptors of T. marmorata. The 17,000-dalton peak found in this study, was probably not a degradation product of some polypeptide chains, since the receptor was prepared in the presence of a protease inhibitor (PMSF). It more likely represented an impurity in the preparation because the receptor used in this study had a lower specific toxin binding activity than that of Schiebler and Hucho. The 90,000-dalton peak, which occurred as a duplet, might be the result of aggregation of the major components caused by an oxidation process which led to the formation of intermolecular disulfide linkages (29,30). This explanation seems plausible, even though the electrophoresis was run in the presence of dithiothreitol, because in the absence of it (data not shown) a band of 150,000-200,000 daltons was observed. This indicated that dithiothreitol can only eliminate some, but not all, disulfide linkages under the employed condition.

A specific function, however, has been assigned only to the 40,000-dalton subunit, which apparently contains binding sites for small ligands (31) and α-neurotoxin (31, 32). Heidmann et al. (33) recently showed two bands from SDS gel electrophoresis of purified acetylcholine receptor from T. marmorata. The 40,000-dalton band, the cholinergic ligand

binding site, and another band of 43,000 daltons. The "43K protein" was believed to be a part of the acetylcholine ionophore (33,34). In the present work, the "43K protein" was either absent or obscured by the large 40,000 dalton band.

B. RECONSTITUTION OF ACETYLCHOLINE RECEPTOR

Purified receptors were reconstituted into lipid vesicles. The vesicles obtained were mostly unilamellar and heterogeneous in size with an average diameter of 0.39 ± 0.18 μm. Brunner et al (18), whose gel filtration technique for reconstitution was used in this study, earlier showed that the vesicles gained were unilamellar and had uniform size of diameter = 0.03 μm. The differences in size and heterogeneity of vesicles might occur from the fact that Brunner prepared vesicles from the protein free lecithin, whereas this study used high level of acetylcholine receptor proteins. The size of receptor lipid vesicles, therefore, should be larger since acetylcholine receptors were incorporated into the lipid bilayers. Heterogeneity of the vesicle size could also arise, depending on the amount of acetylcholine receptor incorporated into each vesicle.

The orientation of receptor incorporated into vesicles was determined. It was found that about 55% of the receptors exposed their toxin binding sites on the outside of the vesicles. This finding was in contrast with that of

Michaelson and Raftery (35) which they found that all the neurotoxin binding sites facing the outside of the vesicles. The result, however, was in agreement with those of Briley and Changeux (36), and Potter (37). The receptors were, therefore, inserted in a random manner and not in an asymmetrical one as in the case of the native subsynaptic membrane.

C. $^{22}Na^{+}$ INFLUX OF ACETYLCHOLINE RECEPTOR VESICLES

Functional reintegration of the receptor with exogenous lipids after removal of detergent was tested in this study by following $^{22}Na^{+}$ uptake of the vesicles. It was shown that $^{22}Na^{+}$ influx was accelerated in the presence of 0.02mM carbamylcholine, a compound known to enhance the selective cation permeability of the electroplax membrane (8). This effect was completely blocked when the vesicles were pre-incubated with 2mM d-tubocurarine. In the absence of cholinergic agonist, the untreated receptor vesicles exhibited only background influx as would be expected from closed vesicles. The receptor vesicles thus display chemical excitability in vitro.

The Biogel P-60 gel filtration technique was used in this study because of its reproducibility. The process was, however, rather slow; it took 5 min for one assay at any time point. By this method, therefore, only the slow phase of $^{22}Na^{+}$ influx was observed. Epstein and Racker (38)

recently showed the measurement of the rapid phase of $^{22}Na^{+}$ influx which lasted less than 1 min. Their ion-exchange column technique was adapted but no success was obtained. Since the present technique was severely limited to only the slow phase of $^{22}Na^{+}$ influx, no information on the initial rate of influx was obtained.

Another functional property of the acetylcholine regulator, which was not dealt with in the present study, is the phenomenon of receptor desensitization. Katz and Thesleff (39), and Del Castillo and Webb (40) showed that after about 1 sec of exposure to the agonist, the channels became unresponsive and did not open again unless the agonist was removed and reapplied. At 0.2mM carbamylcholine, the half-life for desensitization was about 3 sec. In the work of Epstein and Racker (38), a rapid desensitization of the reconstituted receptor was also observed.

D. CONCLUSION

Although the assay method used to detect the acetylcholine receptor throughout the purification procedures was by its specific binding to radioactive α-Bgt, our purified receptor preparation must contain sufficient amount of acetylcholine ionophore. The demonstrated sensitivity of the reconstituted receptor vesicles to a cholinergic agonist (carbamylcholine) and to an antagonist (d-tubocurarine) strongly supports this notion. In future experiments, it

may be possible to design methods to purify the ionophore molecule independent of the receptor protein. The use of the radioactive histrionicotoxin to assay for the ionophore molecule may facilitate the purification. Only until the ionophore and the receptor proteins are purified separately and recombined to restore the acetylcholine sensitivity, the mechanism of ion translocation of the acetylcholine regulator will be understood at the molecular level. The present work represents only the first step of this long and difficult, but greatly important, endeavour.

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VITA

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