INVESTIGATIONS OF THE STRUCTURAL PROPERTIES OF THE ACETYLCHOLINE RECEPTOR AND ITS POLYPEPTIDE SUBUNITS FROM *TORPEDO CALIFORNICA*

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Catherine Devine Strader

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ABSTRACT: CHAPTER I

Microsequence analysis of the four polypeptide subunits of the acetylcholine receptor from Torpedo californica electroplax was performed and demonstrated a striking amino acid homology among these four chains. Further sequence analysis of both membrane-bound and Triton-solubilized, chromatographically purified receptor was used to measure the stoichiometry of the subunits. The values determined for the two preparations of receptor were the same: the ratio of the four subunits (40,000: 50,000:60,000:65,000 daltons) is 2:1:1:1, indicating a value of 255,000 daltons for the molecular weight of the receptor, in agreement with experimentally determined values. These results demonstrate that the acetylcholine receptor is a pentamer composed of five subunits, two being identical and the other three being structurally related to them by sequence homology. Genealogical analysis suggests that divergence from a common ancestral gene occurred early in the evolution of the receptor. This shared ancestry argues compellingly that each of the four subunits plays a functional role in the receptor's physiological action.

ABSTRACT: CHAPTER II

Antibodies raised in rabbits to Triton-solubilized, purified acetylcholine receptor from *Torpedo californica* were used to immunospecifically label intact *T. californica* electroplaque membrane vesicles attached to coverslips and oriented with the extracellular face of the synaptic membrane facing outward. Hemocyanin conjugated to Protein A was then used as a marker, making the antibody binding visible at the electron microscopic level. Parallel labeling experiments were performed on vesicles attached to coverslips and sheared by sonication, leaving their cytoplasmic faces fully exposed to the labeling solution. While differences in antibody populations among different rabbits were observed, antigenic determinants of the receptor were present on both faces of the membrane, with those on the extracellular side more numerous than those on the cytoplasmic side, demonstrating the transmembrane nature of the receptor molecule.

ABSTRACT: CHAPTER III

The exposure of the four subunits of the acetylcholine receptor from Torpedo californica on both the extracellular and cytoplasmic faces of the postsynaptic membranes of the electroplaque cells has been investigated. Sealed membrane vesicles containing no protein components other than the receptor were isolated and were shown to have 95% of their synaptic surfaces facing the medium. The susceptibility of the four receptor subunits in these preparations to hydrolysis by trypsin both from the external and from the internal medium was used to investigate the exposure of the subunits on the synaptic and cytoplasmic surfaces of the membrane. It was shown by sodium dodecylsulfate gel electrophoresis of the tryptic products that all four subunits are exposed on the extracellular surface to a similar degree. All four subunits are also exposed on the internal surface of the membrane but the apparent degree of exposure varies with the subunit molecular weight, the larger subunits being more exposed. The results are discussed in terms of a possible topographic model of the receptor as a transmembrane protein complex.

ABSTRACT: CHAPTER IV

SDS-polyacrylamide gel electrophoresis of acetylcholine receptor from Torpedo californica electroplax membrane fragments shows, in addition to the four receptor subunits of 40,000, 50,000, 60,000, and 65,000 daltons, other components of apparent molecular weights 43,000, 47,000, and 90,000 daltons. One of those has previously been identified as actin. In this study, the deoxyribonuclease I inhibitory activity of actin has been used to identify actin in Torpedo californica receptor-enriched membranes, and affinity chromatography on a deoxyribonuclease I agarose column has been used to purify actin from the membrane preparations. In addition the protein components have been analyzed by electrophoresis on a series of SDS-polyacrylamide gels of varying acrylamide concentrations. Evidence is presented that actin is a component of most preparations of receptor-enriched membrane fragments, but is separate from the 43,000 dalton protein.

ABSTRACTS OF PROPOSITIONS

Proposition I:

Experiments are proposed for the identification of the domains of the four transmembrane acetylcholine receptor subunits exposed on the extracellular face of the post-synaptic membrane, traversing the membrane interior, and exposed on the cytoplasmic membrane face.

Proposition II:

Topographic studies on the distribution in the erythocyte membrane of the three protein components involved in the cellular reponse to adrenergic agonists, the β-adrenergic receptor, the guanine nucleotide regulatory protein, and the adenylate cyclase, with the system locked in various stages of the response mechanism, would provide information on the interactions among these three proteins responsible for the adrenergic response.

Proposition III:

Ligatin is a protein thought to be involved in the attachment of soluble proteins to cell membranes. Structural investigations, involving electron microscopic examina-

tion and copolymerization of ligatins from various sources would allow evaluation of the binding specificity(s) and general presence of this recently discovered protein.

Proposition IV:

The use of azido-phenols and -catechols as probes for the investigation of the nature of the substrate binding sites of the enzyme oxidase, tyrosinase, is proposed.

Proposition V:

Experiments are proposed for the elucidation of the mechanism(s) involved in the rescue from interferon inhibition of RNA viruses by DNA viruses.

ABBREVIATIONS

acetylcholine	AcCh
acetylcholine receptor	AcChR
amino acids:	
alanine	Ala (A)
arginine	Arg (R)
asparagine	Asn (N)
aspartic acid	Asp (D)
cystine	Cys (C)
glutamic acid	Glu (E)
glutamine	Gln (Q)
glycine	Gly (G)
histidine	His (H)
isoleucine	Ile (I)
leucine	Leu (L)
lysine	Lys (K)
methionine	Met (M)
phenylalanine	Phe (F)
proline	Pro (P)
threonine	Thr (T)
tryptophan	Trp (W)
tyrosine	Tyr (Y)
valine	Val (V)

buffers:

NPE	10 mM Na phosphate, 400 mM NaCl, 1 mM EDTA; pH 7.4
NTC	50 mM Tris, 350 mM NaCl, 5 mM $CaCl_2$; pH 7.4
PBS	10 mM Na phosphate, 150 mM NaCl; pH 7.4

α-bungarotoxin	α-BuTx
bovine serum albumin	BSA
carbamylcholine	Carb
Coomassie brilliant blue	CBB
deoxyribonuclease I	DNase I
experimental autoimmune myasthenia gravis	EAMG
hemocyanin-protein A	Hcy/A
high performance liquid chromatography	HPLC
histrionicotoxin	HTX
immunoglobulin	Ig
4-(N-maleimido)benzyl-trimethylammonium iodide	MBTA
membrane fragments, solubilized in Triton	MFTS

membrane fragments, solubilized in Triton, depleted in AcChR	MFTS-R
normal rabbit immunoglobulin	NRG
phenylthiohydantoin	Pth
sodium dodecyl sulfate	SDS
transglutaminase	TGase
d-tubocurarine	dTC
vesicular stomatitis virus	VSV

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INTRODUCTION

The conversion of a nerve impulse into a muscular contraction occurs at the neuromuscular junction, where it is mediated by the diffusion of acetylcholine (AcCh) across the synaptic space between the nerve and the muscle (a distance of 200-500 Å). When an electrical impulse reaches the nerve terminal, it causes depolarization, or lowering of the membrane potential, of the terminal membrane and subsequent release of acetylcholine into the synaptic space. Upon reaching the postsynaptic membrane of the muscle fiber, AcCh binds to the protein acetylcholine receptor (AcChR), causing ion channels in the postsynaptic membrane to open. This leads to an influx of sodium ions, resulting in depolarization of the postsynaptic membrane and the generation of a large ($\sim$ 50 mV) end plate potential, thus propagating the impulse in the muscle (Katz, 1966; Thesleff, 1975).

The presence of calcium ions outside the membrane is necessary for the release of acetylcholine; the depolarization of the presynaptic membrane leads to an influx of Ca^{++} which, once inside the nerve terminal, causes the discharge of acetylcholine from its presynaptic vesicles into the synaptic gap (Katz, 1971). The genera-

tion of an end plate potential large enough to cause firing of the muscle results from the simultaneous expulsion of the contents of many presynaptic vesicles through the membrane. When the nerve is at rest, individual vesicles, each containing 10^3-10^5 AcCh molecules, release their contents into the synaptic space infrequently at random, each generating a miniature end plate potential of approximately 0.5 mV, well below the firing threshold of the muscle (Katz, 1971; Gage, 1976). When the nerve impulse invades the presynaptic terminal, the frequency of these quantal releases of AcCh increases tremendously; the simultaneous release of AcCh from a large number of vesicles generates an end plate potential large enough to transmit an impulse down the muscle fiber.

The mechanism by which the binding of acetylcholine to its receptor causes the opening of ion channels and resultant depolarization of the postsynaptic membrane has not yet been determined; a clear understanding of the structural and functional properties of the AcChR molecule is necessary before these questions can be answered. Advances in this field have followed the development, in the past decade, of techniques for the isolation of purified AcChR in both its membrane-bound

and detergent solubilized forms. For biochemical and electrophysiological studies, the electric organs from Torpedo and Electrophorus provide a rich source of receptor; the electric tissue shows morphological similarity to highly innervated muscle, with large numbers of synaptic connections (Bennett, 1971). The large electroplaques of Electrophorus are ideally suited to electrophysiological studies, while the high density of synapses in the Torpedo electric organ (covering up to 50% of the innervated face of the electroplaque) permits the isolation of large quantities of AcChR from a single electric organ.

Acetylcholine receptor has been purified in both its native membrane environment and in detergent solubilized form for biochemical characterization. Isolated membrane fragments from Torpedo electroplax appear in the form of membrane vesicles; the preparation can be enriched in AcChR-containing vesicles by sucrose density gradient centrifugation (Cohen, et al, 1972; Duguid and Raftery, 1973). Application of the technique of treatment of membrane preparations with base (Steck and Yu, 1973) removes peripheral proteins, giving preparations containing no proteins other than the AcChR, in its membrane-bound state (Neubig, et al, 1979; Elliott, et al, 1980). Such preparations are invaluable in studying

the interactions of the AcChR with agonists and antagonists and in measuring in vitro flux of inorganic cations through receptor channels.

Detergent solubilization of the AcChR from the membrane and subsequent purification to homogeneity has permitted determination of structural parameters and biochemical characterization of the molecule itself. AcChR has been solubilized in detergent and purified by affinity chromatography from several species of Torpedo (Cohen, et al, 1972; Schmidt and Raftery, 1973; Eldefrawi and Eldefrawi, 1973; Weill, et al, 1974; Gordon, et al. 1974; Deutsch and Raftery, 1979) from species of the Elasmobranch Narcine (Chang, et al, 1977; Deutsch and Raftery, 1979), and from the eel Electrophorus electricus (Olsen, et al, 1972; Biesecker, 1973; Lindstrom and Patrick, 1974; Chang, 1974). Purified Torpedo AcChR sediments in sucrose gradient centrifugation as two peaks at 9S and 13.7S, with the 13.7S component shown to be a dimer of the 9S form (Raftery, et al, 1972; Carroll, et al, 1973; Potter, 1973; McNamee, et al, 1975). Molecular weight determinations for the AcChR purified from Torpedo californica have ranged from 250,000 to 330,000 daltons (Martinez-Carrion, et al, 1975; Edelstein, et al, 1975; Reynolds and Karlin, 1978; Hucho, et al, 1978); diffi-

culties in calculating the amount of detergent bound to the protein have complicated these determinations. Using membrane osmometry, the molecular weight of the receptor was determined to be 270,000 ± 30,000 daltons (Martinez-Carrion, et al, 1975). In sedimentation equilibrium studies with [^{3}H]-Triton-solubilized AcChR, molecular weights of 330,000 and 660,000 daltons were found for the monomer and dimer, respectively (Edelstein, et al, 1975). More recent measurements in D_2O and H_2O gave molecular weights of 250,000 daltons for the 9S form and 500,000 daltons for the 13.7S form (Reynolds and Karlin, 1978). A molecular weight of 270,000 daltons was also found using suberimidate crosslinking prior to SDS gel electrophoresis (Hucho, et al, 1978). The isoelectric point of the AcChR is approximately 5 (Raftery, 1974). A glycoprotein, composed of approximately 5% neutral sugars, primarily mannose, galactose, glucose, and N-acetyl-D-glucosamine, (Raftery, et al, 1975b; Vandlen, et al, 1979), the receptor binds quantitatively to concanavalin A (Raftery, 1974). The AcChR monomer is composed of four subunits with molecular weights of 40,000, 50,000, 60,000 and 65,000 daltons, which will be discussed in more detail below (Raftery, et al, 1974; Weill, et al, 1974; Raftery,

et al, 1975a; Chang and Bock, 1977; Lindstrom, et al, 1978; Vandlen, et al, 1979; Froehner and Rafto, 1979; Elliott, et al, 1980).

Perhaps the single most important development leading to the isolation and characterization of the AcChR was the discovery of α-neurotoxins which form a stable complex with the AcChR, specifically blocking the binding of AcCh (Lee and Chang, 1966; Lee, et al, 1967). α-Bungarotoxin (α-BuTx), purified from the venom of the krait Bungarus multicinctus and radiolabeled with ^{125}I, has been widely used in biochemical studies on the AcChR, and has recently been thoroughly characterized (Blanchard, et al, 1979). The binding of [^{125}I]-α-BuTx to the membrane-bound AcChR has been found to show linear kinetics (Weber and Changeux, 1974; Colquhoun and Rang, 1976; Weiland, et al, 1976; Blanchard, et al, 1979) with one exception (Bulger, et al, 1977). The linear kinetics are described by a simple bimolecular mechanism for α-BuTx binding, indicating that the toxin binding to the membrane-bound receptor is to one class of homogeneous sites. Bulger, et al (1977), studying the binding of α-BuTx to Electrophorus, observed more complicated biphasic kinetics which could be explained by an allosteric model. The basis for these differing results is unclear at the present time.

The binding of [^{125}I]-α-BuTx to Triton-solubilized, chromatographically purified AcChR showed biphasic kinetics in the case where toxin was present in excess over receptor (Blanchard, et al, 1979a). Two mechanisms were proposed to fit these data, one of which involved two separate classes of independent toxin binding sites, and the other of which involved negative cooperativity for toxin binding to the receptor (Blanchard et al, 1979a). The correctness of the two models or of more complicated models that might fit the data cannot yet be determined. An examination of the second-order rate constants for α-BuTx binding to membrane-bound and to Triton-solubilized receptor shows that toxin binding to the solubilized protein is also much faster (Blanchard, et al, 1979a).

From the work that has been done to date, it is clear that toxin binding to the membrane-bound and detergent-solubilized AcChR proceed quite differently. The reason for these effects of detergent solubilization are not known, although it seems likely that conformational changes in the receptor upon removal from its native membrane environment are responsible.

In competition with α-toxins, the AcChR binds cholinergic ligands in both its membrane-bound and detergent-solubilized forms. In vivo, prolonged exposure of

the receptor to agonists (ligands that cause the depolarization response) results in the phenomenon known as desensitization, wherein the response to agonists is attenuated as the time of agonist application is increased (Katz and Thesleff, 1957). This results from a change in the state of the AcChR from one of low affinity for ligands to one of high affinity (Katz and Thesleff, 1967). Incubation with agonists can also induce an increase in the affinity of the AcChR for antagonists (Rang and Ritter, 1969; 1970a; 1970b); so the high-affinity state appears not to be ligand-specific. The transition has also been observed to a lesser extent during pre-incubation with antagonists (Quast, et al, 1978b). The desensitization phenomenon has been observed for the membrane-bound receptor in vitro, as well (Weber, et al, 1976; Colquhoun and Rang, 1976; Weiland, et al, 1976; Lee, et al, 1977; Quast, et al, 1978b). Traditionally, the desensitization in vitro has been explained in terms of the simple two-state model (high- and low-affinity states) originally proposed by Katz and Thesleff (1957) for the in vivo results. Kinetics studies on the effects of the agonist carbamylcholine (Carb) on α-BuTx binding to the receptor do not fit the two-state model, however, and more complex models, involving several conformational changes in the

receptor, have been proposed to explain the data (Quast, et al, 1978a; 1979; Dunn, et al, 1980).

Studies of the binding of agonists and antagonists to the membrane-bound AcChR in vitro have involved the monitoring of changes in intrinsic protein fluorescence (Bonner, et al, 1976; Barrantes, 1976), in extrinsic fluorescence probes (Grunhagen and Changeux, 1976; Grunhagen, et al, 1976; 1977; Schimerlik et al, 1979; Quast, et al, 1978a; 1979; Dunn, et al, 1980), and competition with the binding of radiolabeled neurotoxins (Weber, et al, 1975; Weiland, et al, 1976; 1977; Lee, et al, 1977; Quast, et al, 1978a). Ligand binding to the detergent-solubilized receptor has been less intensively studied, since solubilized AcChR does not exhibit the ligand-induced conformational change from low- to high-affinity state (Raftery, et al, 1975a; Vandlen, et al, 1976; Blanchard, et al, 1979). There is disagreement among researchers as to the stoichiometry of AcCh binding sites to α-neurotoxin sites in membrane-bound AcChR, with some reports of a 1:1 ratio (Sugiyama and Changeux, 1975; Damle, et al, 1976; Dunn, et al, 1980) and others of AcCh:toxin of 0.5:1 (Raftery, et al, 1975; Schimerlik, et al, 1979). This disagreement may result from variations in membrane preparations or from differences inherent in the various experimental techniques (Neubig and Cohen,

1979; Dunn, et al, 1980). In a study on the binding of the antagonist, d-tubocurarine (dTC) to membrane-bound receptor, Neubig and Cohen (1979) found the ratio dTC:α-BuTx to be 1:1, with evidence that dTC bound to the same sites as AcCh. The two dTC sites were non-identical, however, with one high-affinity site (K_d = 34 nM) and one of much lower affinity (K_d = 7 μM).

Little is known about the actual structure of the ligand binding site(s) on the receptor. It has been determined that a reactive disulfide bond lies near the binding site (Karlin and Bartels, 1966; Lindstrom, et al, 1973; Ben-Haim, et al, 1973), the reduction and alkylation of which prevents depolarization upon agonist binding in vivo. This inhibition of depolarization was found to occur as a result of a reduction in the conductance of individual ion channels as well as from a lowering of the affinity of AcCh binding to the receptor (Ben-Haim, et al, 1975; Schiebler, et al, 1977). Treatment of membrane-bound receptor with reducing agents destroyed the ability of AcChR to undergo the Carb-induced conformational change from the low- to high-affinity state (Moore and Raftery, 1979b). Upon removal of the reducing agent, the ability to undergo this affinity change was restored, and alkylation following the reduction resulted in a permanent loss of the ability

to undergo this interconversion, locking the receptor in a high-affinity state. Treatment of the receptor with p-chloromercuribenzoate was found to lock it in its resting, low-affinity form or, if already in the high-affinity form due to pre-treatment with Carb, to lock it in the high-affinity state (Moore and Raftery, 1979b); this was interpreted in terms of a tentative model for the interconversion of high- and low-affinity states based on the interchanging of a disulfide bond among three sulfhydryls near the agonist binding site.

Alkylation of reduced AcChR with the antagonist-site directed affinity agent 4-(N-maleimido)benzyltrimethylammonium iodide (MBTA) (Weill, et al, 1974), or with the agonist bromoacetylcholine (Chang, et al, 1977; Damle, et al,, 1978; Moore and Raftery, 1979a) resulted in the labeling of the 40,000 dalton subunit, implicating it as the site of ligand binding. Both MBTA and bromoacetylcholine bound to half of the α-BuTx binding sites on the receptor.

Binding of agonists to the AcChR leads to the flux of inorganic cations through the ion channel and to depolarization of the postsynaptic membrane in vivo. The depolarization response to agonists is inhibited by the action of local

anesthetics, a proposed mechanism for which is the blocking of the receptor in its open-channel form (Ruff, 1976; Neher and Sakmann, 1976; Steinbach, 1977). Local anesthetics also increase the rate of the desensitization response to agonists (Magazanik and Vyskocil, 1973; Magazanik, 1976). The alkaloid histrionicotoxin (HTX) also blocks depolarization and inhibits response to agonists (Albuquerque, et al, 1973a; 1973b; Kato and Changeux, 1976), indicating that HTX interacts with the ion channel, as well. HTX binding in vitro has been found to be non-competitive with agonists or α-BuTx (Kato and Changeux, 1976; Dolly, et al, 1977), although the sites seem to be conformationally related: the binding of HTX causes an increase in the affinity for AcCh of membrane-bound AcChR (Kato and Changeux, 1976), while the affinity of the AcChR for HTX is increased with Carb binding (Elliott and Raftery, 1977). The rate of in vitro "desensitization", i.e. the rate of interconversion of the high- and low-affinity ligand binding states of the AcChR, is unaffected by HTX binding (Elliott and Raftery, 1977). It has been proposed that HTX acts by blocking the receptor in the open-channel state (Albuquerque, et al, 1973a; 1973b). HTX binds to the receptor in a ratio of one HTX per four

α-BuTx sites; i.e. one HTX bound per AcChR dimer (Elliott and Raftery, 1977; 1979). The actual binding site of HTX has not yet been identified, but the most recent work with highly purified membrane-bound AcChR indicates that the site resides on one of the four AcChR subunits (Neubig, et al, 1979; Elliott, et al, 1979) and not on another membrane component of 43,000 daltons as had previously been postulated (Sobel, et al, 1978).

The binding of local anesthetics to the AcChR is weakly competitive with α-BuTx binding (Weber and Changeux, 1974; Weiland, et al, 1977; Blanchard, et al, 1979b) and inhibits the binding of HTX to the receptor (Blanchard, et al, 1979b). From the magnitudes of the interactions, it was concluded that two distinct local anesthetic binding sites were responsible for these two effects (Blanchard, et al, 1979b). Local anesthetics were found to affect the rate of conversion of the AcChR from its low-affinity to high-affinity state for ligand binding in the presence of Carb, with the magnitude of the effect varying widely with the local anesthetic used. These effects correlated roughly with the binding of a given local anesthetic to the high- or low-affinity states of the AcChR; this correlation could be explained by a direct effect of local anesthetics

on the interconversion of states or by a non-specific perturbation of the protein environment by local anesthetic binding (Blanchard, et al, 1979b). The nature of local anesthetic binding sites on the AcChR is not known. Affinity labeling of AcChR-enriched membrane fragments with the local anesthetic analog procaine amide azide showed labeling of the 40,000 dalton subunit of the AcChR, which was specifically blocked by agonists, of a membrane component at 43,000 daltons, and of one at 90,000 daltons, this latter of which was shown to be unrelated to the AcChR protein (Blanchard and Raftery, 1979b). Recently, it has been determined that the 43,000 dalton component can be separated from the membrane-bound receptor (Neubig, et al, 1979; Elliott, et al, 1980) and is not necessary for AcChR function (Moore, et al, 1979). In light of the fact that local anesthetics cause a wide range of effects in a large number of different systems (Levy, et al, 1977), the significance in terms of the AcChR of the labeling of the 43,000 dalton protein with a local anesthetic analog is unclear (Blanchard, 1980).

In response to the binding of agonists, the ion channel in the AcChR molecule opens, allowing cation translocation through the membrane. Agonist-induced

flux has been measured *in vitro* using $^{22}Na^{+}$ and membrane-bound AcChR (Kasai and Changeux, 1971; Michaelson and Raftery, 1974; Hazelbauer and Changeux, 1974; Popot, *et al*, 1976; Hess and Andrews, 1977; Miller, *et al*, 1978; Hess, *et al*., 1978; Moore, *et al*., 1979a; 1979b) with a filtration assay system. Because of the time required to perform the measurements, the fast flux process could not be measured by this method, the flux being complete by the time ($\sim$ 10 sec) of the first data point in even the fastest assays. The amplitude of the flux was determined by this method to vary with agonist concentration and appears to reflect the desensitization process (Miller, *et al*, 1978; Bernhardt and Neumann, 1978; Neubig, *et al*, 1979; Moore, *et al*, 1979a; 1979b; Elliott, *et al*, 1979). Although the time courses of the ion flux observed by the filtration assay method and *in vivo* are dissimilar, other characteristics correlate more closely. In both cases, the agonist-induced flux is blocked by α-BuTx and HTX and is inactivated by increased incubation with agonist, which is thought to reflect *in vitro* desensitization (Miller, *et al*, 1978). Flux measurements on purified membrane-bound AcChR partially inactivated by treatment with α-BuTx demonstrated that the removal from the membrane of a large percentage of a com-

ponent necessary for flux would result in a detectable decrease in flux amplitude, leading to the conclusion that the AcChR, composed of four subunits, is the only element necessary for the agonist-induced flux response (Moore, et al, 1979b). Recent technological improvements have allowed the measurement of in vitro flux on the time scales observed in vivo: using a quench-flow technique with $^{86}Rb^{+}$, measurements could be made at 20 milliseconds (Cash and Hess, 1980), while the use of Tl^{+} to quench the fluorescence of a vesicle-entrapped fluorescent probe allowed continuous flux measurements after 2.5 milliseconds (Moore and Raftery, 1980). The rate of flux and ion transport number per single channel determined by this latter method approximate those observed in vivo, demonstrating that the purified membrane preparations contain all the components of the physiologically active AcChR.

The physiological relevance of the isolated receptor protein has been further demonstrated by reconstitution studies, in which detergent-solubilized AcChR was reconstituted into lipid vesicles to make functional, excitable membranes. Initial studies of the reconstitution of detergent-solubilized, chromatographically purified AcChR yielded conflicting results, with some reports of

some functionally active (measured by agonist-induced $^{22}Na^{+}$ flux) preparations (Michaelson and Raftery, 1974) and other reports of only inactive preparations (Karlin, et al, 1975; McNamee, et al, 1975; Howell, et al, 1978). Functional preparations were obtained when solubilized membrane preparations were used without further purification (Hazelbauer and Changeux, 1974) or when exogenous lipids were added to these membrane extracts (Epstein and Racker, 1978). Functional reconstituted vesicles from highly purified membrane preparations have recently been reported (Wu and Raftery, 1979; Changeux, et al, 1979) and extensively characterized (Wu and Raftery, 1980). After solubilization in sodium cholate, determined to be complete by the sedimentation of the proteins as the 9S and 13.7S receptor monomer and dimer on sucrose gradients, the AcChR was reconstituted with exogenous phospholipids into spherical, sealed vesicles. These vesicles, which contained only the AcChR protein, exhibited agonist-induced flux which was specifically blocked by α-BuTx or HTX, with quantitive pharmacological responses approximating those of the native membrane (Wu and Raftery, 1980).

Functional studies on the AcChR, then, have proceeded rapidly in the past few years with the develop-

ment of highly purified membrane-bound receptor preparations (Neubig, et al, 1979; Elliott, et al, 1980). Structural information on the disposition of the receptor protein in the membrane has been obtained through electron microscopic studies on Torpedo and Electrophorus electroplaques and on the AcChR-enriched membrane fragments. In the Torpedo, the electic organs are located in lobes on either side of the animal's head. The organ consists of stacks of electroplaques, large disc-like cells five to seven millimeters in diameter, each cell being 10-30 μ thick. Individual cells within the stacks are separated by approximately 3 μ of nerve fibers and connective tissue (Sheridan, 1965). The dorsal face of each electroplaque is non-innervated and is characterized morphologically by deep, narrow infoldings along its surface; the ventral face is richly innervated and is relatively smooth, with widely separated infoldings (Sheridan, 1965). Both cell surfaces are covered with a basement membrane. The membrane structure of the innervated face of the electroplaque (the postsynaptic membrane) has a trilaminar appearance when viewed under the electron microscope (Rosenbluth, 1975). The inner dense lamina measures 25-30 Å and has a distinctive amorphous cytoplasmic coating (Rosenbluth, 1975);

it is separated from the outer lamina by a space of 20-30 Å. The thick (approximately 60 Å) outer dense lamina appears studded with granules 65-70 Å in diameter, thought to be acetylcholine receptors, which at times appear to be regularly spaced, but, in general, show no apparent lattice structure (Rosenbluth, 1975).

In Electrophorus electroplax, Bourgeois, et al (1971) used high resolution indirect immunofluorescence to study the distribution of AcChR in the cell. Using a rabbit antiserum directed against Naja nigricollis α-toxin and fluorescent sheep anti-rabbit immunoglobulins, they found the toxin binding sites dispersed, but not uniformly, over the innervated face of the electroplaque. In an attempt to more precisely localize the AcChR, Bourgeois, et al (1972) used high resolution autoradiography with tritiated toxin. Using light microscopy they found that, as expected, all the toxin sites were on the innervated face of the electroplaque and the pre-incubation with p-(trimethylammonium)benzenediazonium fluoroborate (an irreversible cholinergic antagonist) blocked all toxin binding. Under the electron microscope, a statistical distribution of the positions of α-toxin sites was compiled; it was found that the density of toxin sites was 100 times higher

in the postsynaptic membrane than on the remainder of the innervated face, between synapses (Bourgeois, et al, 1972).

Electron microscopic studies of isolated membrane fragments from both Torpedo and Electrophorus enriched in AcChR have shown the presence of circular rosette-like structures, measuring 80-90 Å in diameter (Nickel and Potter, 1973; Cartaud, et al, 1973; Raftery, et al, 1974). Upon higher magnification, each such structure was seen to be composed of five to six round subunits, 20-25 Å in diameter, around a 15 Å electron-opaque core, giving it a doughnut-shaped appearance (Nickel and Potter, 1973). Negative staining of purified, Triton-solubilized receptor reveals these same structures (Cartaud, et al, 1973; Eldefrawi and Eldefrawi, 1975; Ross, et al, 1977; Sobel, et al, 1977a; Chang, et al, 1977). In membrane fragments, as the preparations become more enriched in AcChR, these structures become more prevalent; hence, they are generally thought to be AcChR molecules. This hypothesis was confirmed by the immunospecific labeling of these rosettes in negatively stained membrane fragments with anti-receptor antibodies (Klymkowsky and Stroud, 1979).

Membrane-bound receptor molecules are densely packed, with a center-to-center distance of 80-90 Å, at approxi-

mately 10,000-15,000 per μ^2, and often form ordered patches of hexagonal arrays (Nickel and Potter, 1973; Cartaud, et al, 1973; Heuser and Salpeter, 1979). X-ray diffraction on AcChR-enriched membrane fragments supports the presence of a hexagonal lattice organization of receptor molecules, which collapses when the water content dips below 35% (Dupont, et al, 1974; Ross, et al, 1977). Freeze-fracture of the membrane into cytoplasmic and synaptic halves reveals the same hexagonal lattice. In fixed membrane preparations and whole electroplax tissue, the AcChR particles appear more strongly anchored to the cytoplasmic half of the membrane, with the fracture plane leaving protruding spheres on the cytoplasmic side and complementary hollows on the synaptic side (Cartaud, 1974; Allen, et al, 1977); in unfixed tissue, the AcChR particles were found evenly distributed between the membrane halves (Allen, et al, 1977). Electron microscopic evidence relating to the transmembrane nature of the AcChR has been obtained in several laboratories and will be discussed in Chapter II.

Little is known about the morphology of the 25 Å subunits of the AcChR complex observed with negative-stain electron microscopy, but it is thought that they may correspond to the polypeptide subunits of the AcChR.

Except for one account (Sobel, et al, 1977b), researchers have reported that three (Neubig et al, 1979) or, in most cases, four subunits comprise the AcChR (Raftery, et al, 1974; Weill, et al, 1974; Raftery, et al, 1975a; Chang and Bock, 1977; Lindstrom, et al, 1978; Vandlen, et al, 1979; Froehner and Rafto, 1979; Elliott, et al, 1980; Raftery, et al, 1980; Strader, et al, 1980), with molecular weights of 40,000, 50,000, 60,000, and 65,000 daltons. As mentioned above, affinity labeling with MBTA and with bromoacetylcholine have implicated the 40,000 dalton subunit in ligand binding (Weill, et al, 1974; Chang, et al, 1977; Damle, et al, 1978; Moore and Raftery, 1979a); the functional significance of the other three subunits has not yet been determined. Several lines of evidence, however, indicate that all four subunits are components of the receptor molecule.

First, is the appearance of the four subunits as the only components of both detergent-solubilized, chromatographically purified AcChR (Raftery, et al, 1974; Weill, et al, 1974) and highly purified membrane-bound AcChR (Elliott, et al, 1980). These same four subunits were found as the only components of AcChR purified by affinity chromatography from four species of the Elasmobranch family Torpedinidae (Deutsch and Raftery, 1979). Individual

subunits of the same molecular weight from different species were found to cross-react immunologically, but the four subunits did not cross-react among themselves, demonstrating that the four were not derived from one another by degradation (Claudio and Raftery, 1977). Each of the four subunits isolated from Torpedo was able to cause symptoms of the disease experimental autoimmune myasthenia gravis (EAMG) in rats (Lindstrom, et al, 1978). Since EAMG is characterized by the formation of antibodies to the AcChR, and subsequent degradation of this protein, this indicates that all four subunits are components of the AcChR. In addition, antibodies to each of the four subunits were effective in precipitating detergent-solubilized receptor from solution (Lindstrom, et al, 1978).

It has been demonstrated that the 13.7S dimer of the AcChR is formed by a disulfide bridge between 65,000 dalton subunits, one from each monomer (Suarez-Isla and Hucho, 1977; Chang and Bock, 1977; Hamilton, et al, 1977; Witzemann and Raftery, 1978a). Membrane-bound receptor monomers, after reduction, can be oxidized by diamide to dimers and higher oligomers linked by a disulfide bridge between a 50,000 dalton subunit from

each monomer; copper phenanthroline causes the formation of similar receptor dimers, these linked through the 65,000 dalton subunit (Hamilton, et al, 1979). After cross-linking, oligomers can be extracted from the membrane with detergent and separated on sucrose gradients, where the 9S monomer and 13.7S dimer, as well as higher oligomers, are observed, indicating that the 50,000 and 65,000 dalton subunits are an integral part of the receptor molecule.

Photoaffinity labeling of detergent-solubilized, chromatographically purified AcChR with the cholinergic antagonist analog, bis(3-azidopyridinium)-1,10-decane perchlorate showed labeling of the 40,000 and 60,000 dalton subunits, while, with the membrane-bound AcChR, the 40,000 and 50,000 dalton subunits were labeled (Witzemann and Raftery, 1977). This labeling was competitive with the binding of agonists and antagonists and was blocked by α-BuTx. Photoaffinity labeling with bis(azido)ethidium chloride showed that, in solubilized AcChR, all four subunits were labeled, with the labeling of the 40,000 dalton subunit being partially blocked by α-BuTx (Witzemann and Raftery, 1978b). In membrane-bound AcChR, this affinity reagent labeled the 40,000 dalton subunit preferentially, with only minor incorpora-

tion into the other three subunits; α-BuTx blocked some, but not all, of this labeling. Pre-treatment with cholinergic ligands caused increased labeling of the 40,000 dalton subunit and significant labeling of the 50,000 and 60,000 dalton subunits, as well; pretreatment with HTX had no direct effect on incorporation of label, but was able to prevent the enhancement of labeling of the 40,000 and 65,000 dalton subunits by Carb (Witzemann and Raftery, 1978b). In another photoaffinity labeling experiment, a photolabile derivative of α-BuTx was used to label membrane-bound AcChR, where it was found cross-linked to both the 40,000 and the 65,000 dalton subunits (Witzemann and Raftery, 1978c; Witzemann, et al, 1979). The specificity of these photoaffinity labeling experiments for the various receptor subunits, taken together, provides strong evidence that all four polypeptide chains are part of the receptor complex, with the 40,000 dalton subunit containing ligand binding sites and the other three related, either by containing further binding sites or by conformational linkage to the binding sites.

Further evidence for the relationship of all four polypeptide subunits as part of the AcChR complex is provided by the discovery of a considerable degree of sequence

homology among the amino-termini of the four chains (Raftery, et al, 1980). This result, discussed in detail in Chapter I, indicates that all four evolved from a common ancestral gene as components of the receptor protein.

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Chapter I: The Subunits of the Acetylcholine Receptor: Amino-Terminal Sequence Homology and Stoichiometry

INTRODUCTION

In response to the binding of acetylcholine, the acetylcholine receptor-associated ion channels open, leading to ion flux and subsequent depolarization of the postsynaptic membrane. The receptor complex of four polypeptide subunits alone is responsible for this function (Moore, et al, 1979); the process by which it is accomplished has not yet been elucidated. Structural studies on the acetylcholine receptor (AcChR) subunits and their properties should provide a means for understanding their interrelationships and their roles in the response to acetylcholine.

The acetylcholine receptor, isolated from the electric organs of Torpedo californica, and chromatographically purified after detergent extraction from the postsynaptic membrane, sediments in sucrose gradient centrifugation as a monomer at 9S and a 13.7S dimer (Raftery, et al, 1972; Carroll, et al, 1973; Potter, 1973; McNamee, et al, 1975). The molecular weight of the monomer has been determined by several techniques to be 250,000-270,000 daltons (Martinez-Carrion, et al, 1975; Edelstein, et al, 1975; Reynolds and Karlin, 1978; Hucho, et al, 1978). The receptor is a glycoprotein, containing approximately 5% neutral sugars (Raftery, et

al, 1975b; Vandlen, et al, 1979), and has an isoelectric point of 4.9 (Raftery, 1974). These and other properties of the AcChR protein complexare summarized in Table I.

The subunit composition of the AcChR has been the subject of controversy. It has been established that sites for the binding of snake α-neurotoxins and for agonists and antagonists reside on a polypeptide of molecular weight ∿ 40,000 daltons. Except for one account (Sobel, et al, 1977), researchers have found preparations of purified Torpedo AcChR to contain two (Neubig, et al, 1979) or, in most cases three other polypeptide subunits, with molecular weights of 50,000, 60,000, and 65,000 daltons (Raftery, et al, 1974; Weill, et al, 1974; Raftery, et al, 1975a; Chang and Bock, 1977; Lindstrom, et al, 1978; Vandlen, et al,, 1979; Froehner and Rafto, 1979; Elliott, et al, 1980). Although the functional roles of these other three subunits have not yet been determined, several lines of evidence, outlined briefly below and discussed in more detail in the Introduction to this thesis, indicate that all four subunits are integral components of the receptor molecule. (i) These four subunits comprise the only components of both detergent-solubilized, chromatographically purified AcChR (Raftery, et al, 1974;

Weill, et al, 1974) and of highly purified membrane bound AcChR (Elliott, et al, 1980) in Torpedo californica as well as of solubilized, purified AcChR from four different species of Elasmobranch (Deutsch and Raftery, 1979). (ii) Immunologically, each of these four subunits cross-reacts interspecifically but the four do not cross-react among themselves, demonstrating that the lower molecular weight subunits are not degradation products of the larger ones (Claudio and Raftery, 1977). In addition, the ability of each of the four subunits to cause symptoms of experimental autoimmune myasthenia gravis demonstrates that all are components of the AcChR (Lindstrom, et al, 1978). (iii) The naturally occurring 13.7S dimer of the AcChR is formed by a disulfide bridge linking the 65,000 dalton subunit from each monomer (Suarez-Isla and Hucho, 1977; Chang and Bock, 1977; Hamilton, et al, 1977; Witzemann and Raftery, 1978a). Under specific experimental conditions, 13.7S receptor dimers can also be formed by disulfide bonds between a 50,000 dalton subunit from each monomer (Hamilton, et al, 1979). (iv) Affinity labeling with derivatives of α-bungarotoxin (α-BuTx) (Witzemann and Raftery, 1978c; Witzemann, et al, 1979), and of receptor-binding ligands (Weill, et al,

1974; Chang, et al, 1977; Witzemann and Raftery, 1977; 1978b; Damle, et al, 1978; Moore and Raftery, 1979) has implicated, under various conditions, all four subunits in the ligand binding process, either by direct binding or through conformational involvement.

The stoichiometry of the four AcChR subunits has also been a source of controversy in recent years. Measuring the protein concentrations of subunits extracted from SDS polyacrylamide gels on detergent-solubilized AcChR, Lindstrom et al (1979a) found the AcChR subunit stoichiometry to vary from 40,000:50,000:60,000:65,000 = 4.4:2:1:1.3 to 2.1:1.1:1:1.1 and concluded that 2:1:1:1 was the correct ratio, since this stoichiometry resulted from the highest yields of protein from the gel. Reynolds and Karlin (1978) have proposed a stoichiometry for these four subunits of 2:1:1:1 on the basis of its compatability with a measured molecular weight of 250,000 daltons for detergent-solubilized AcChR.

In this Chapter and elsewhere (Hunkapiller, et al, 1979; Raftery, et al, 1980; Strader, et al, 1980) evidence is presented for the existence of a considerable degree of sequence homology among the four AcChR subunits; the sequence data are further utilized to

determine unequivocally the stoichiometry of these four subunits in both membrane-bound and detergent-solubilized, chromatographically purified AcChR. These results are discussed in terms of evolutionary and structural relationships among the four subunits and their possible functional implications.

MATERIALS AND METHODS

Membrane fragments enriched in AcChR were prepared from Torpedo californica electric organs by the method of Elliott, et al (1980). Briefly, this procedure involved the centrifugation of crude membrane homogenates on discontinuous sucrose density gradients in a Beckman VTi50 vertical rotor at 45,000 rpm for one hour. The band sedimenting in the middle of the gradient was collected, separated from the sucrose solution by dilution and centrifugation, and all protein except for the AcChR removed by extraction with base (Neubig, et al, 1979; Elliott, et al, 1980). Membrane fragments were diluted to 1-2 mg/ml in H_2O and titrated to pH 11 with NaOH. After stirring for one hr at 4°C, the membranes were centrifuged at 45,000 x g for 45 min. The supernatant and the loose, soft pellet were removed and the small, hard pellet resuspended in 1 mM Tris, pH 7.4. After

readjustment to pH 11, the centrifugation step was repeated and the hard pellet resuspended in a small volume of 10 mM Tris, pH 7.4, giving a preparation containing approximately 4 nmol α-BuTx sites per mg protein. Purified detergent-solubilized AcChR was prepared according to Schmidt and Raftery (1973a); Triton X-100 extracts of Torpedo membrane fragments were purified by affinity chromatography on a resin containing the quaternary ammonium ligand $NH(CH_2)_5CONH(CH_2)_3N^+(CH_3)_3$.

The concentration of α-BuTx sites was determined according to Schmidt and Raftery (1973b), using DEAE-cellulose filter discs and [^{125}I]-α-BuTx (Clark, et al, 1972; Blanchard, et al, 1979). Protein concentrations were determined either by the method of Lowry, et al. (1951), with BSA as the standard, or by amino acid analysis, using the AcChR amino acid compositions determined by Vandlen, et al. (1979). Samples for amino acid analysis were hydrolyzed at 100°C for 20-24 hrs in 6N HCl in vacuo, dried under N_2, and analyzed on a Durrum D-500 amino acid analyzer. Analytical SDS-polyacrylamide gel electrophoresis was done according to Laemmli (1970) with a 10% acrylamide,

0.27% bisacrylamide separating gel and a 3% acrylamide, 0.08% bisacrylamide stacking gel. The total gel dimensions were 13 cm x 11 cm x 0.1 cm for a slab accommodating 17 samples. After staining with Coomassie brilliant blue (CBB) gels were scanned with a Gilford linear transport unit at 550 nm.

Purification of Individual AcChR Subunits: The base-treated membrane fragments were heated in gel sample buffer (5% β-mercaptoethanol, 3% SDS, 10% glycerol in 0.125 M Tris, pH 6.8) and ∿ 5 mg electrophoresed according to Laemmli (1970) on a preparative slab gel measuring 13.5 cm x 11 cm x 0.6 cm, with acrylamide concentrations as described above. Electrophoresis was at 20 mA for 18 hrs, after which the gel was stained in 0.25% CBB, 25% methanol, 10% acetic acid for 5-10 min. A narrow horizontal band at the position of each subunit was cut from the gel and the protein removed by electroelution into a dialysis bag in Tris-glycine buffer containing 0.1% SDS at 5 mA/tube for 18 hrs. The protein solutions were dialyzed against several changes of 0.15M NaCl, 0.02% SDS and then against 0.02% SDS in water for 48 hrs, after which they were lyophilized and their purity confirmed by re-electrophoresis.

Amino Terminal Amino Acid Sequence Analysis:

Automated Edman degradation of protein samples was performed by Dr. M. W. Hunkapiller with the spinning cup sequenator designed and built at Caltech according to Hunkapiller and Hood (1980), using a program previously described (Hunkapiller and Hood, 1978). This program involved a coupling step for 25 min in 0.17 M Quadrol buffer, a cleavage step for 5 min, and treatment for 40 min with 25% trifluoroacetic acid for the automated conversion of the amilinothiazolinones to the phenylthiohydantoin (Pth) derivatives of amino acids. All procedures were performed at 52°C. Before sequencing each protein, a solution containing 6 mg polybrene and 100 nmol glycylglycine in 0.5 ml H_2O were subjected to eight complete cycles on the sequenator. Pth amino acids were identified by high performance liquid chromatography (HPLC) on a DuPont Zorbax CN column, using the procedures and standard chromatograms previously described (Johnson, et al, 1979; Hunkapiller and Hood, 1980).

For amino-terminal sequence analysis of the four isolated AcChR subunits, 1-10 nmol was dissolved in 0.5 ml H_2O, loaded on the sequenator, and the automated degradation begun with the coupling step. Fifty-six residues on each subunit were identified in this manner.

Determination of Subunit Stoichiometry: Two preparations of membrane-bound AcChR (3.5 and 3.8 nmol) were sequenced, each being dissolved in 10% SDS before loading into the spinning cup; two different preparations of detergent-solubilized, chromatographically purified AcChR were each loaded directly into the cup in the buffer (0.1% Triton, 10 mM phosphate, 1 mM EDTA, pH 7.4) used in the affinity chromatography. The subunit stoichiometry was calculated by comparison of the yields of the Pth amino acids at positions 9, 10, and 14 assigned to each of the four subunits; these were positions at which each of the four was previously determined (see Results) to have a different amino acid residue. The yields of the Pth amino acids were calculated by subtracting the background Pth amino acid levels and correcting for the differing recoveries of the various Pth amino acids as determined by sequenator analysis of the isolated subunits (Ala 95%, Ser 35%, Glu 80%, Asn 65%, Val 95%, Lys 50%, Asp 60%, Thr 40%).

RESULTS

The isolated AcChR subunits, purified from membrane-bound AcChR by preparative gel electrophoresis and shown in Figure 1, were subjected to amino-terminal sequence

analysis, as described above, for 54 amino acid residues apiece. Typical yields of the various amino acids are shown in Figures 2 and 3. Figure 2 gives relative yields for the residue in each of the first 25 steps for the 40,000 dalton subunit, while, in Figure 3, the yields of two amino acids are plotted through all 54 steps for the 50,000 and 60,000 dalton subunits. It can be seen from these Figures that the signal-to-noise ratio of these data is high and clear identification of the residue at each step may be made.

The percentage of total protein applied to the sequenator that accounted for the major sequence of each subunit was calculated by assuming 95% recovery of aliphatic Pth-amino acids (Hunkapiller and Hood, 1980) and extrapolating the observed yields of the aliphatic residues in the sequenator runs back to cycle zero. Comparison of the protein quantities determined by this method with those determined by amino acid analysis of separate aliquots of each subunit gave the following initial yields of the subunits: 80% of the 40,000 dalton subunit (from 9.6 nmol total), 80% of the 50,000 dalton subunit (from 3.1 nmol total), 80% of the 60,000 dalton subunit (from 2.9 nmol total), 91% of the 65,000

dalton subunit (from 11.0 nmol total).[1] These high initial yields eliminate the possibility of contaminating sequences (even one with a blocked amino terminus) being present in appreciable amounts. No contaminating sequences were present in the individual subunits up to the detection limit of 3%, other than a slight (∿ 15%) contamination of the 60,000 dalton subunit by the 50,000 dalton chain.

The sequences of the amino-terminal 54 amino acids for the four subunits are given in Figure 4. As can be seen there is appreciable homology among the subunits (solid boxes) with additional "pseudo-homology" where amino acids that are structurally very similar occupy the same position in different subunits, although the four chains are clearly distinct proteins. At 11 of the 54 positions (20%), the residues on all four subunits are identical; three of the four are identical at 13

[1]The protein concentrations for individual subunits were determined by amino acid analysis because CBB interfered with the Lowry protein assay. For Triton-solubilized, purified AcChR or for membrane-bound AcChR, however, the Lowry assay was often used. Due to differences in amino acid composition between the BSA standard and the AcChR, a conversion factor had to be used to compare protein concentrations determined by the Lowry assay with those determined by amino acid analysis. From comparison of AcChR concentrations measured by both methods on the same samples, these factors were experimentally determined to be (Lowry assay:amino acid analysis) 1.20 for Triton-solubilized AcChR and 1.25 for membrane-bound AcChR.

more positions (24%), meaning that three or more subunits have identical residues at 44% of the amino-terminal 54 amino acids. The sequence homology between two chains ranges from 35% (40,000 and 50,000 dalton subunits) to 50% (60,000 and 65,000 dalton subunits), and is summarized in Table II.

These four subunit sequences were compared in a homology search with all other protein sequences in the data bank of the National Biomedical Research Foundation by Dr. M. O. Dayhoff. No significant homology was detected between any of the subunits and these other proteins.

A genealogical tree, according to Fitch and Margoliash (1967), was generated for the four subunits and is shown in Figure 5. This gives the evolutionary pathway by which the sequences of these four polypeptides could arise from a single ancestral sequence with the minimum nucleotide substitution. It is interesting to note that the point of initial divergence of the four subunits from their common ancestral polypeptide was early in their evolution; subsequent differentiation generated the contemporary subunits, presumably for the purpose of their different functions in the receptor complex.

Determination of the stoichiometry of the four AcChR subunits was first attempted by densitometry scanning of stained gels on membrane-bound and Triton-solubilized receptor (representative gels of these two preparations are shown in Figure 6). This method failed when it became apparent that for none of the four subunits is staining density a linear function of protein concentration. Thus, the apparent stoichiometry determined by this method varies with the amount of protein electrophoresed on the gel. This is shown in Figure 7, where the apparent stoichiometry of the four subunits can be varied from 2:1:0:1 to 4:2:1:2 to 4:1:0.5:1, merely by loading different volumes of the same protein solution on the same slab gel. It is interesting to note that, at low protein concentrations, some preparations appear by densitometry to be composed of only three subunits, with the 60,000 dalton subunit becoming apparent only at higher protein concentrations (see Figure 7B). This may perhaps account for some of the discrepancies noticed in similar preparations from different laboratories (compare Neubig, et al, 1979 to Elliott, et al, 1980). It is also clear from Figure 7 that equivalent amounts of membrane-bound AcChR and Triton-solubilized AcChR stain quite differently with CBB.

The higher molecular weight subunits appear by this method to be present in smaller amounts in the Triton-solubilized preparation, although the subunit stoichiometries of the two preparations are in fact identical. While it is possible, using 30-40 μg AcChR, to obtain the correct stoichiometry with the membrane-bound AcChR by this method, the correct value is never observed with the Triton-solubilized protein, suggesting that Triton may interfere with the staining process.

A second problem with the determination of the stoichiometry of the AcChR subunits by densitometry scanning of gels is the considerable tendency of the 40,000 dalton subunit in Triton-solubilized AcChR to undergo degradation from 40,000 daltons to ∿ 35,000 daltons with prolonged heating in gel sample buffer. As can be seen in Figure 8, the appearance on the gel of the band at 35,000 daltons corresponded to the disappearance of the 40,000 dalton subunit from the gel; none of the other three subunit bands was affected. When the 40,000 dalton subunit, isolated from a preparative slab gel, was similarly heated, the 35,000 dalton band appeared with concomitant disappearance of the 40,000 dalton band, again implicating a degradation product of the 40,000 dalton subunit as the source.

The method used in the present study to determine the stoichiometery of the AcChR subunits avoids the use of densitometry scanning by measuring the concentration of each subunit independently while the AcChR complex remains intact. The first 14 amino acids from the amino termini of the four AcChR subunits were determined to be (see Figure 4):

	5	10
40,000:	Ser-Glu-His-Glu-Thr-Arg-Leu-Val-Ala-Asn-Leu-Leu-Glu-Asn-	
50,000:	Ser-Val-Met-Glu-Asp-Thr-Leu-Leu-Ser-Val-Leu-Phe-Glu-Thr-	
60,000:	Glu-Asn-Glu-Glu-Gly-Arg-Leu-Ile-Glu-Lys-Leu-Leu-Gly-Asp-	
65,000:	Val-Asn-Glu-Glu-Glu-Arg-Leu-Ile-Asn-Asp-Leu-Leu-Ile-Val-	

In spite of the high incidence of homology between two, three, or all four of the subunits at several positions, four of the first 14 positions (#5, #9, #10, and #14) contain a distinct amino acid for each subunit. When the amino-terminal amino acid sequence of the whole AcChR complex was determined, four simultaneous sequences were observed; the ratios of the amino acids at positions 9, 10, and 14 gave the stoichiometries of the four subunits, as shown in Table III. Position 5 was not used since residual Glu from the previous step for all four chains contaminated this step to a slight extent. HPLC chromatograms obtained for the

Pth amino acids from cycles 9 and 10 of an automated Edman degradation of the base-treated membrane AcChR preparation are shown in Figure 9 to illustrate the quality of the data.

DISCUSSION

The stoichiometry of the AcChR subunits was determined by amino-terminal sequence analysis on both base-extracted, membrane-bound AcChR and on Triton-solubilized, chromatographically purified AcChR, each from two different preparations, with the same results (shown in Table III); the stoichiometry of the subunits (40,000: 50,000:60,000:65,000) is 2:1:1:1. This would dictate a molecular weight for the AcChR monomer of 255,000 daltons, consistent with the experimental determinations of 250,000-270,000 daltons (Martinez-Carrion, et al, 1975; Edelstein, et al, 1975; Reynolds and Karlin, 1978; Hucho, et al, 1978). The fact that the stoichiometry of subunits determined for the two different types of preparations was identical provides strong evidence that this is the ratio found in the native electroplax membrane environment: any selective loss of subunits would have had to have occurred during both preparations. Since

one procedure involves day-long affinity chromatography after the protein is solubilized by detergent from its membrane environment and the other involves treating the receptor-containing membrane fragments at high pH and low ionic strength for one hour, any effects of the two treatments on the integrity of the receptor molecule would not be expected to be identical. Thus the determination of the same subunit stoichiometry for both preparations indicates strongly that this is the true stoichiometry within the native receptor molecule.

This stoichiometry demonstrates that the AcChR monomer is a pentamer of molecular weight 255,000 daltons, composed of two 40,000 dalton polypeptides and one each of 50,000, 60,000 and 65,000 daltons. This pentameric structure is consistent with observations reported from studies of negatively stained membrane preparations visualized by electron microscopy (Nickel and Potter, 1973; Cartaud, et al, 1973; Raftery, et al, 1975b; Allen, et al, 1977); AcChR molecules were identified as cylindrical rosettes 90 Å in diameter, where "subunits" were clustered around a central depression 25 Å in diameter. X-ray diffraction studies have shown this AcChR cylinder to be 110 Å long (perpendicular to the membrane), giving a particle with dimensions that generally agree with a molecular weight of approximately

250,000 daltons (Ross, et al, 1977).

Amino-terminal amino acid sequence analysis has revealed a considerable degree of sequence homology among the four AcChR subunits, with just a single two residue insertion into the 65,000 dalton subunit to allow the alignment of homologous amino acids. The homology is spread consistently along the chains in this region; i.e. the non-homologous amino acids are interspersed evenly among the homologous residues, with the result that there are no long stretches of 100% homology among the subunits. For this reason, it is not surprising that the subunit homology has to date been detected neither by comparisons of peptide maps of individual subunits (Froehner and Rafto, 1979; Nathanson and Hall, 1979; Lindstrom, et al, 1979a) nor by immunological cross-reactivity among the subunits (Claudio and Raftery, 1977; Lindstrom, et al, 1979a, b; Claudio and Raftery, 1980). Both methods would require longer stretches of homologous residues for similarities among the subunits to be detected.

The evolutionary relationships among the four subunits are clear from the extensive homology shared among their sequences; this homology provides very strong evidence that these four polypeptide chains

are indeed all part of the same AcChR molecule. From both the degree of homology and the closeness of the homology alignment among the chains, it seems likely that the genes encoding each of the four subunits descended from a single ancestral coding sequence. The genealogical tree (Figure 5) generated upon this premise, indicates that the gene duplication leading to the formation of four distinct subunits and their subsequent divergence occurred early in receptor evolution. In this respect, some of the variations in data on the subunit structure of AcChR from various species can perhaps be understood. In general, purification of AcChR from various electric fish in the presence of protease inhibitors yields preparations containing subunits of similar, but not necessarily identical, molecular weights and functional behavior as those observed for Torpedo californica (Deutsch and Raftery, 1979). The early gene duplication calculated from the degree of homology among the four T. californica AcChR subunits presumably occurred in an early ancestor to all these species of electric fish; such an early event would account for the strong similarity, but lack of identity, of the subunits for the various species.

The differentiation of the four Torpedo californica AcChR subunit chains included changes in length as well as amino acid substitutions, resulting in the contemporary subunits of 40,000, 50,000, 60,000, and 65,000 daltons. It is assumed that these amino acid substitutions and additions or deletions are in some way related to the functions of the four subunits, which have not yet been determined. In the future, it will be interesting to relate the subunit functions to their amino acid sequences.

The amino-terminal amino acid sequence homology among the four AcChR subunits could have implications for the interactions of ligands with the receptor. As reviewed in the Introduction to this thesis and mentioned briefly in this chapter, affinity labeling experiments have, under various conditions, shown evidence of ligand interactions with all four AcChR subunits. In all cases, the 40,000 dalton subunit is labeled by these ligands and it is generally accepted as containing the binding site(s) for α-toxins and cholinergic ligands. With the discovery of the appreciable degree of homology among the four subunits, these labeling results may perhaps be interpreted in terms of putative binding sites on the other three

subunits. Such sites may be functionally active or, alternatively, may be weaker, residual binding sites, not completely lost during the process of evolution, but with their functional significance diminished in favor of new roles for these subunits. It will now be necessary to conduct careful studies in order to conclusively determine the location of the agonist binding site that leads to membrane depolarization on the physiological time scale. It will also be important to determine whether other effects such as desensitization, blockage by antagonists, or the effects of local anesthetics or histrionicotoxin on the properties of the ion channel are due to binding to sites on the homologous subunits of the receptor.

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TABLE I

PROPERTIES OF PURIFIED ACETYLCHOLINE RECEPTOR FROM T. CALIFORNICA

STRUCTURE

SUBUNIT COMPOSITION ..... 40, 50, 60, 65 $\times 10^3$ DALTONS

MOLECULAR WEIGHT ..... 250,000 - 270,000 DALTONS

PHYSICAL PROPERTIES

S VALUE 9S

..... 13.7S AS DIMER

STOKES RADIUS ..... 72 Å

pI 4.9

SPECIFIC ACTIVITY ..... ONE α-BUTX PER 110 ± 15 x 10^3 DALTONS

COMPOSITION (RESIDUES)

$D_{37}T_{25}S_{27}E_{35}P_{21}G_{19}A_{16}C_3V_{26}M_{10}I_{28}L_{30}Y_{13}F_{16}W_4K_{21}H_{11}R_{14}$ 40K

$D_{49}T_{27}S_{34}E_{50}P_{31}G_{28}A_{27}C_4V_{31}M_7I_{26}L_{40}Y_{15}F_{18}W_4K_{23}H_{10}R_{19}$ 50K

$D_{61}T_{28}S_{38}E_{58}P_{33}G_{34}A_{29}C_6V_{32}M_7I_{35}L_{50}Y_{19}F_{23}W_8K_{29}H_{11}R_{21}$ 60K

$D_{68}T_{30}S_{42}E_{60}P_{36}G_{35}A_{30}C_6V_{34}M_{10}I_{38}L_{53}Y_{20}F_{25}W_{12}K_{29}H_{12}R_{23}$ 65K

CARBOHYDRATE ..... ~75 RESIDUES/MOLECULE

O-SUBSTITUTED SER ..... ~22 RESIDUES/ MOLECULE

O-SUBSTITUTED THR ..... ~23 RESIDUES/MOLECULE

PHOSPHOSERINE ..... ~7 RESIDUES/MOLECULE

} OF AcChR

Table II

SEQUENCE HOMOLOGY

	40K	50K	60K	65K
40K	-	35%	39%	41%
50K	35%	-	39%	35%
60K	39%	39%	-	50%
65K	41%	35%	50%	-

Table III

SUBUNIT STOICHIOMETRY

	Triton-Solubilized AcChR		Membrane-bound AcChR	
	PREP. #1	PREP. #2	PREP. #3	PREP. #4
40,000	1.93 ± .13	1.92 ± .14	1.96 ± .04	2.05 ± .16
50,000	1.02 ± .08	1.07 ± .09	1.03 ± .04	1.02 ± .01
60,000	1.00 ± .10	1.02 ± .21	1.01 ± .03	1.00 ± .07
65,000	1.04 ± .07	1.00 ± .13	1.01 ± .08	0.93 ± .08

Average

40,000	1.97 ± .12
50,000	1.03 ± .06
60,000	1.01 ± .10
65,000	0.99 ± .09

Figure 1

SDS polyacrylamide gel of purified AcChR-enriched membrane preparation (right) and the four isolated subunits used for amino terminal sequence analysis. The subunits of molecular weights 40, 50, 60, and 65 x 10^3 daltons are marked 1, 2, 3, and 4, respectively. Proteins were stained with Coomassie Brilliant Blue.

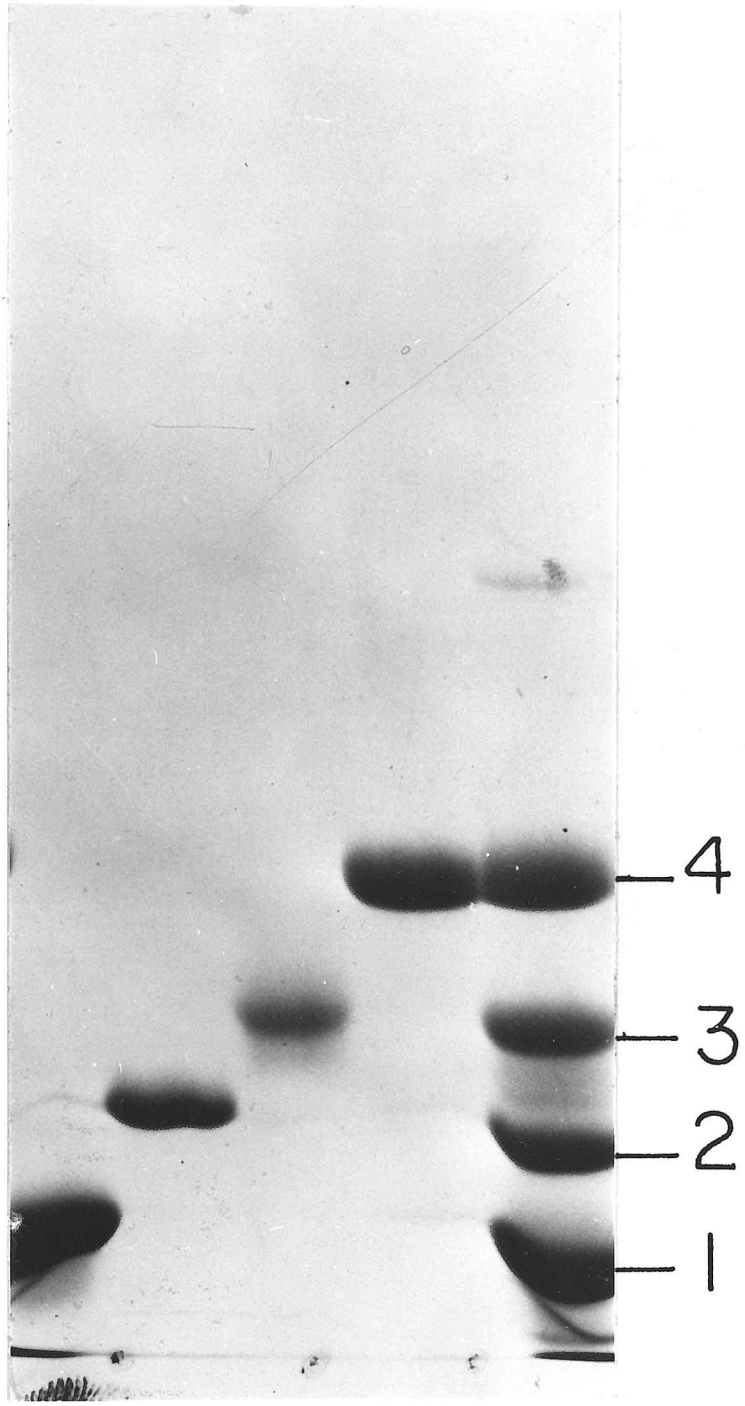

4
3
2
1

Figure 2

Yields of Pth amino acids identified in the amino terminal sequenator analysis of 9.6 nmol of the 40,000 dalton subunit. The yields were calculated by comparison of HPLC peak heights with those of a standard Pth amino acid mixture. They are not corrected for the different recoveries of the various Pth amino acids. A successive cycle efficiency of 96% can be calculated from the yields of the aliphatic Pth amino acids.

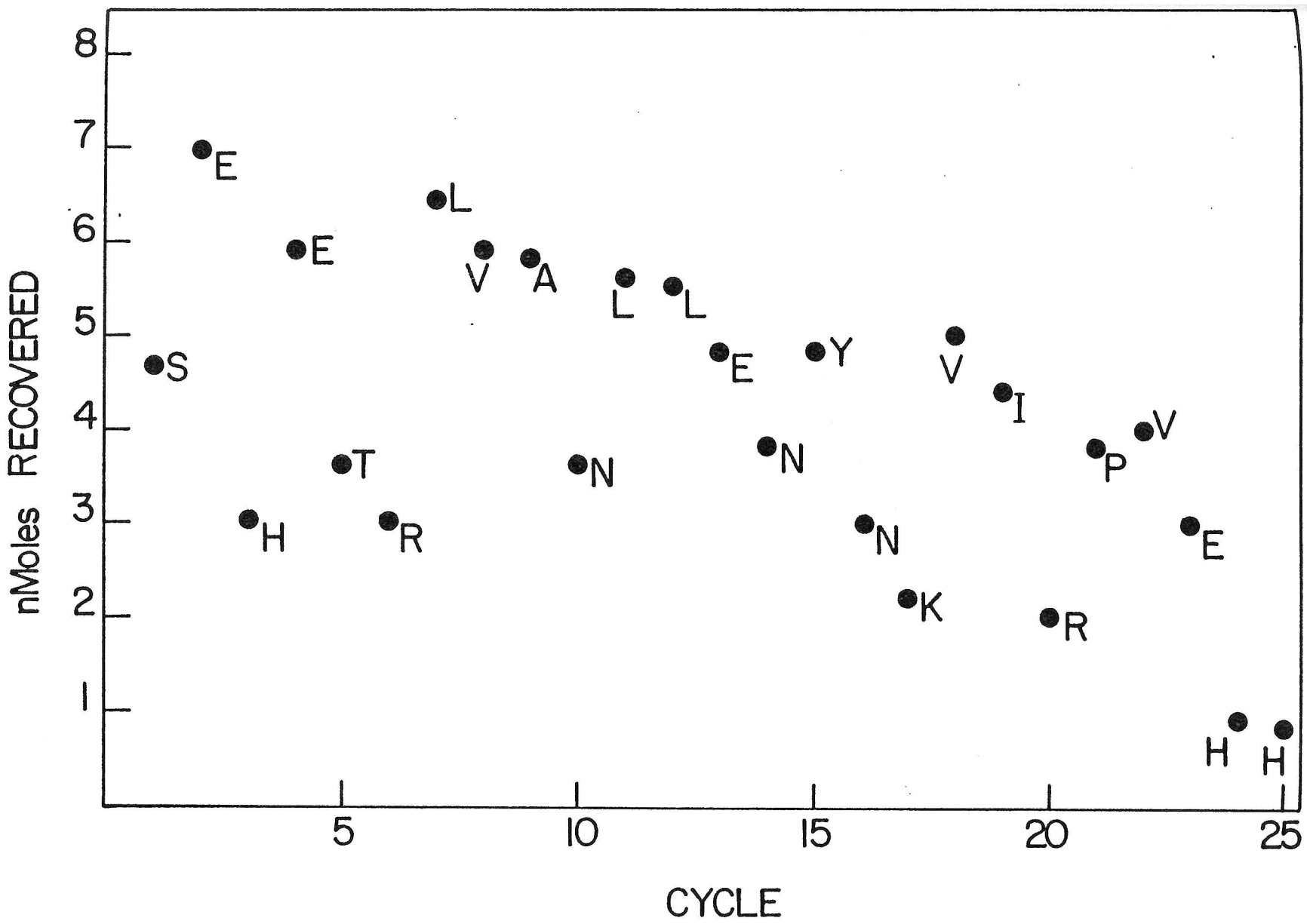

nMoles RECOVERED
8
7
6
5
4
3
2
1
E
L
E
V
A
L
L
S
E
Y
V
I
V
T
N
N
P
H
R
N
E
K
R
H
H
5
10
15
20
25
CYCLE

Figure 3

Yields of Pth-Thr and Pth-Val at each cycle from automated amino terminal sequence analyses of the purified 50 and 60 x 10^3 dalton AcChR subunits. High performance liquid chromatography (HPLC) analysis of the phenylthiohydantoin (Pth) amino acids from the sequenator was performed using DuPont Zorbax CN columns as described. The Pth yields were calculated by comparison of HPLC peak heights with those of a standard Pth amino acid mixture. They are not corrected for the different recoveries of the two Pth amino acids (∿30% for Pth-Thr and ∿95% for Pth-Val) from the sequenator.

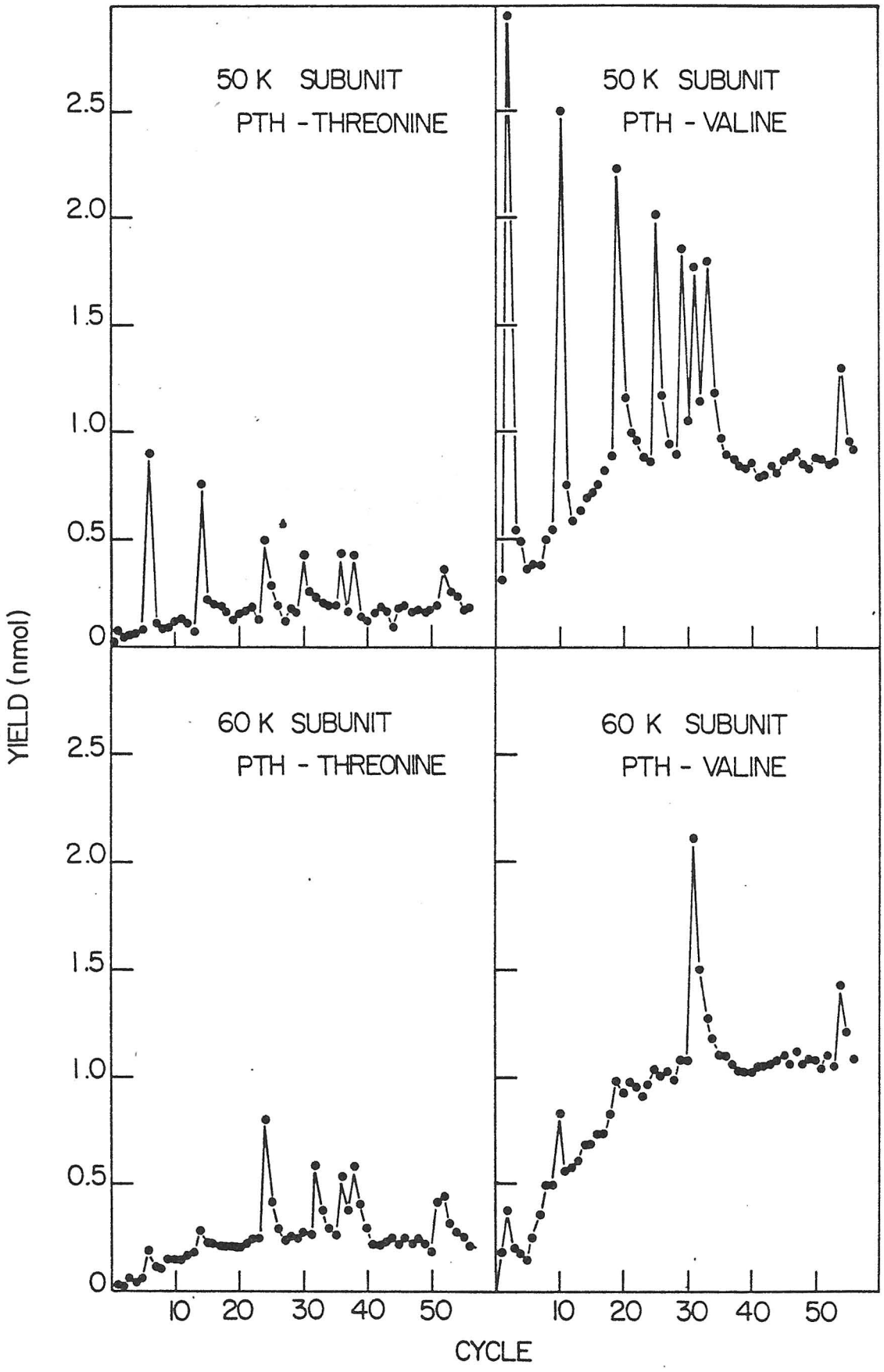

50 K SUBUNIT
PTH - THREONINE
50 K SUBUNIT
PTH - VALINE
60 K SUBUNIT
PTH - THREONINE
60 K SUBUNIT
PTH - VALINE
YIELD (nmol)
0
0.5
1.0
1.5
2.0
2.5
10
20
30
40
50
CYCLE

Figure 4

Sequence homology at the amino termini of AcChR subunits. The one letter code described in J. Biol. Chem. 243: 3357 (1968) is used to denote the amino acids. A solid box around the letters denotes identity between at least two subunits. A dotted box denotes the presence of structurally similar amino acids in the subunits.

SEQUENCE HOMOLOGY AT N-TERMINII OF ACETYLCHOLINE RECEPTOR SUBUNITS

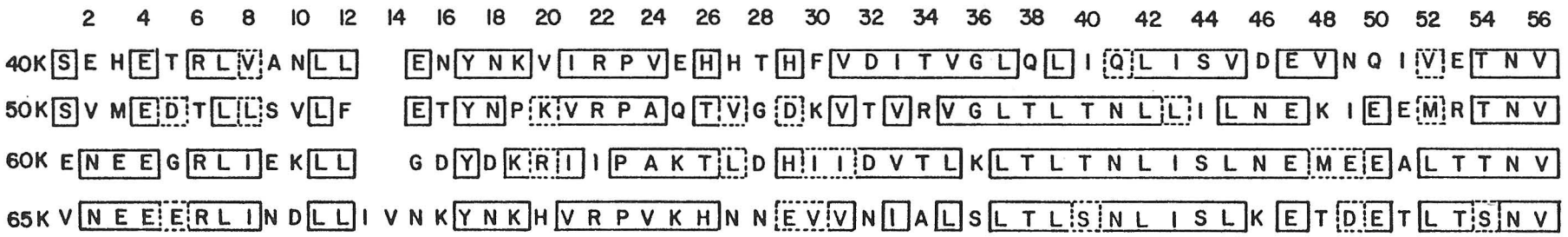


Figure 5

A phylogenetic tree generated from the amino terminal regions of the four AcChR subunits. Each branch point represents a nodal or ancestral sequence. The four subunits are represented as the terminal twigs of the tree. The minimum number of single base substitutions separating two nodal sequences is indicated by numbers in the center of the line joining two corresponding branch points on the tree.

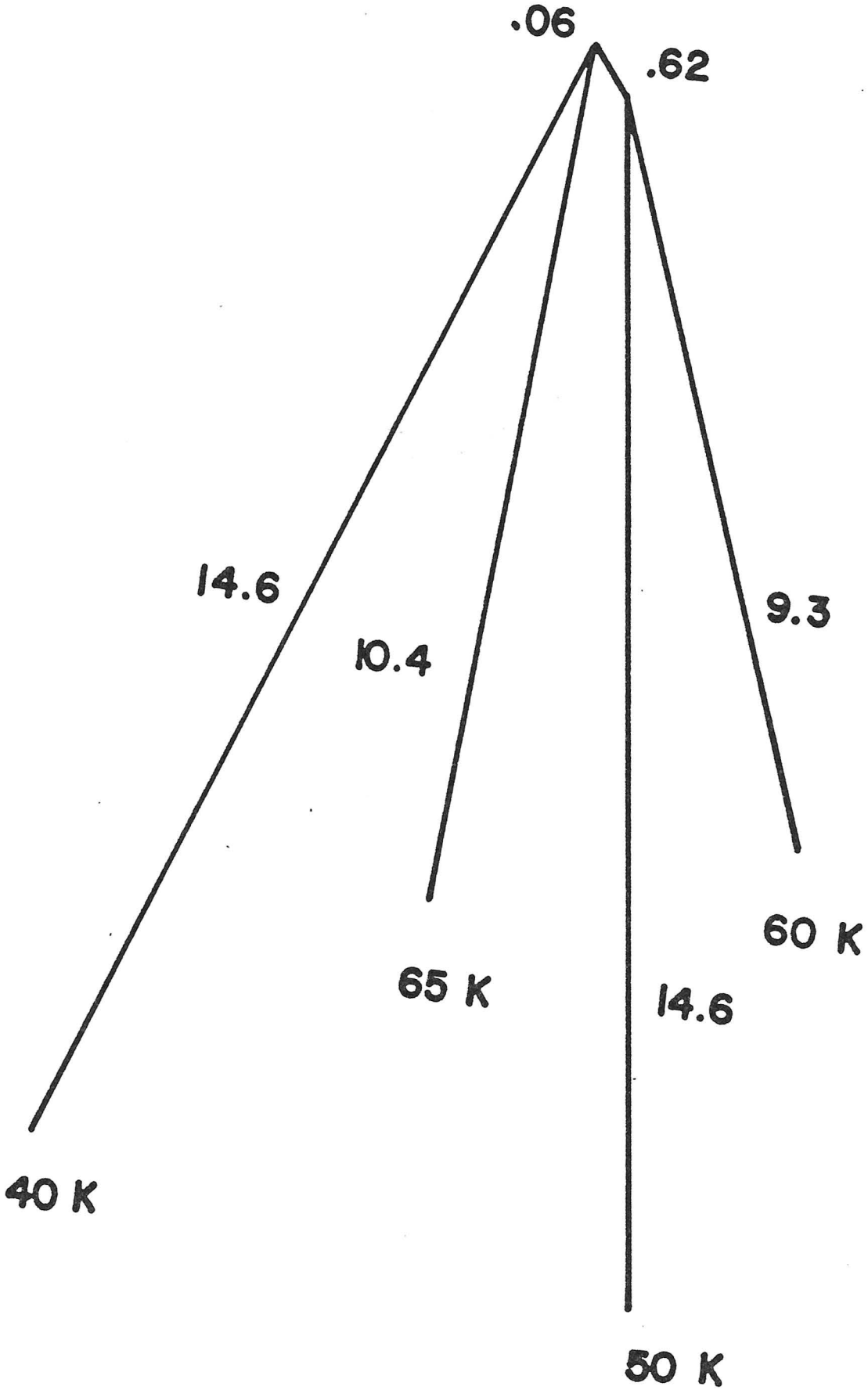

.06
.62
14.6
10.4
9.3
40 K
65 K
14.6
60 K
50 K

Figure 6

SDS polyacrylamide gels on (A) base-treated membrane fragments and (B) Triton-solubilized AcChR. The four subunits are marked.

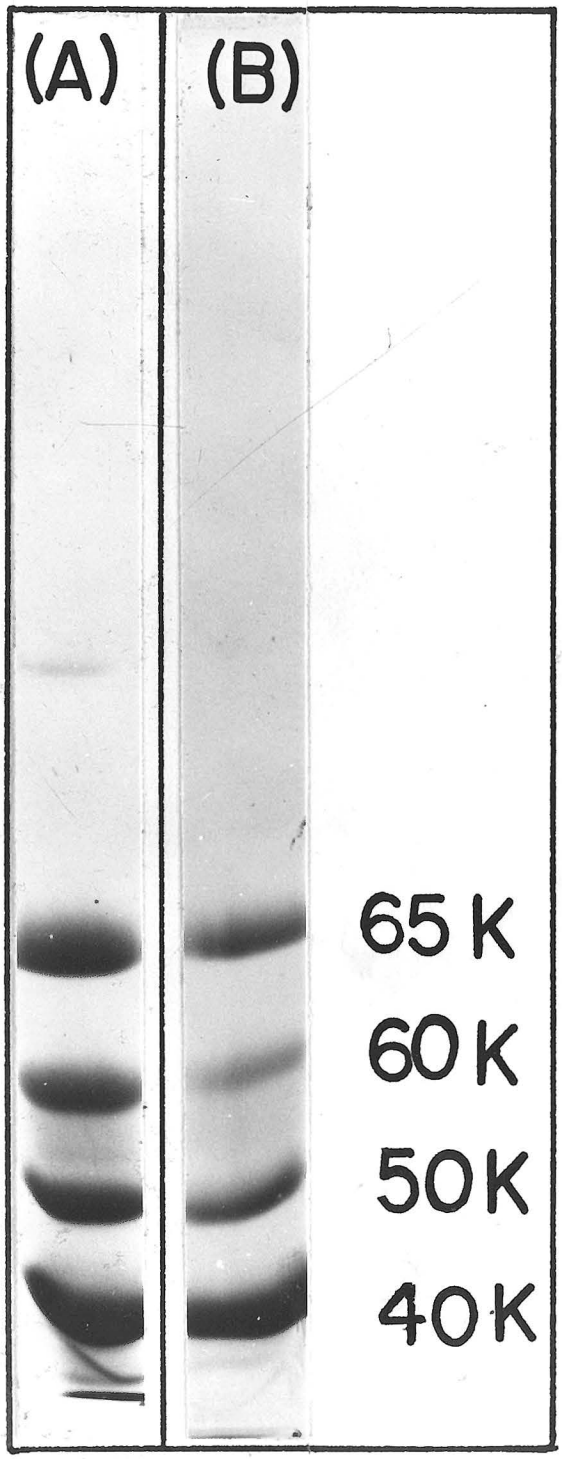

(A)
(B)
65 K
60 K
50K
40K

Figure 7

Intensity of CBB staining vs. protein concentration for gels on (A) base-treated membrane fragments and (B) Triton-solubilized AcChR, measuring the staining intensity for the 40,000 (●), 50,000 (○), 60,000 (▲), and 65,000 (△) dalton subunits. The apparent stoichiometries (rouned to integers) at various protein concentrations are bracketed [40,000:50,000:60,000: 65,000] at the top of the graph.

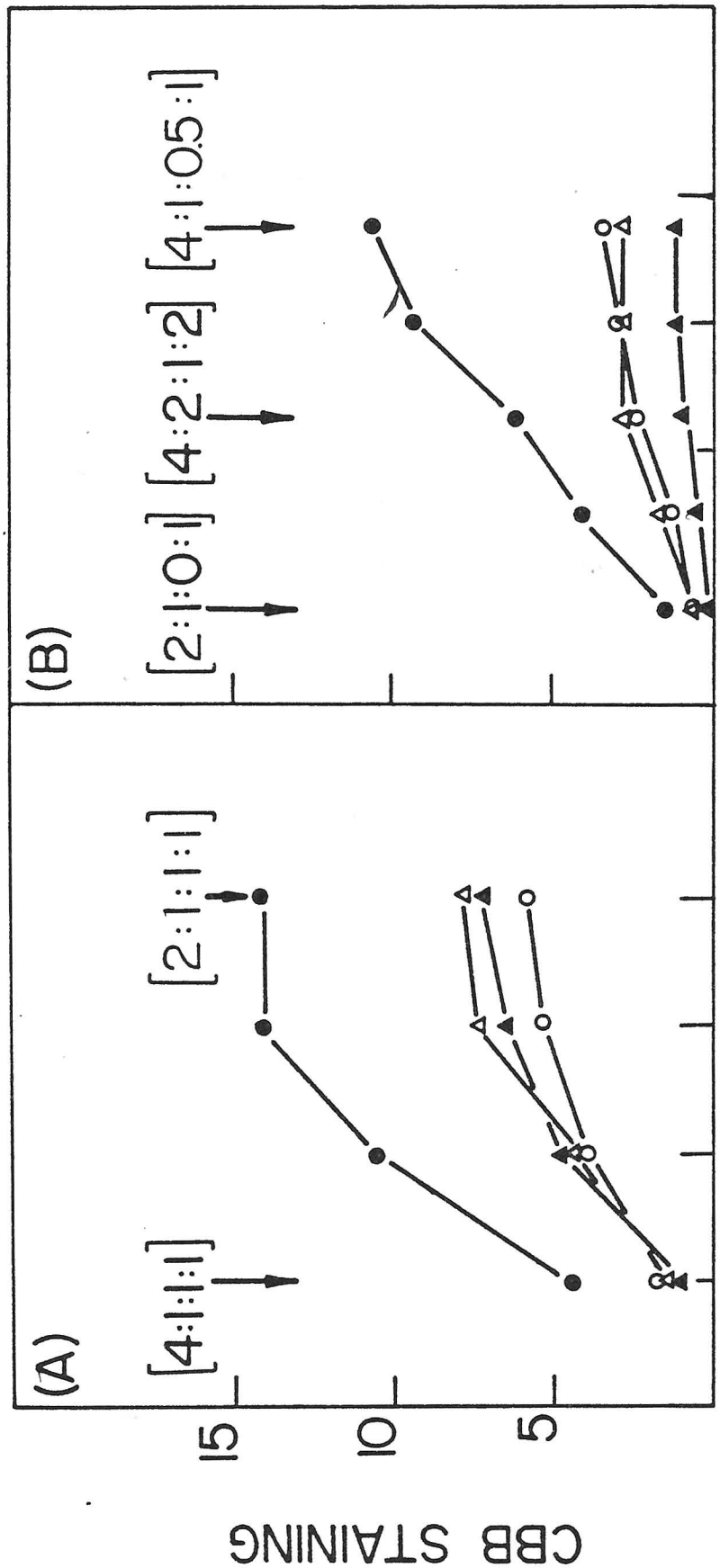

(A)
(B)
[4:1:1:1]
[2:1:1:1]
[2:1:0:1]
[4:2:1:2]
[4:1:0.5:1]
CBB STAINING
15
10
5
0
10
20
30
40
μg AcChR
μg AcChR

Figure 8

Staining with CBB of AcChR subunits with heating in gel sample buffer. The 50,000 (□), 60,000 (▽), and 65,000 (△) dalton subunit staining intensities remain constant. That of the 40,000 (●) decreases with prolonged heating, with a corresponding increase in the intensity of staining of the band at 35,000 daltons (○).

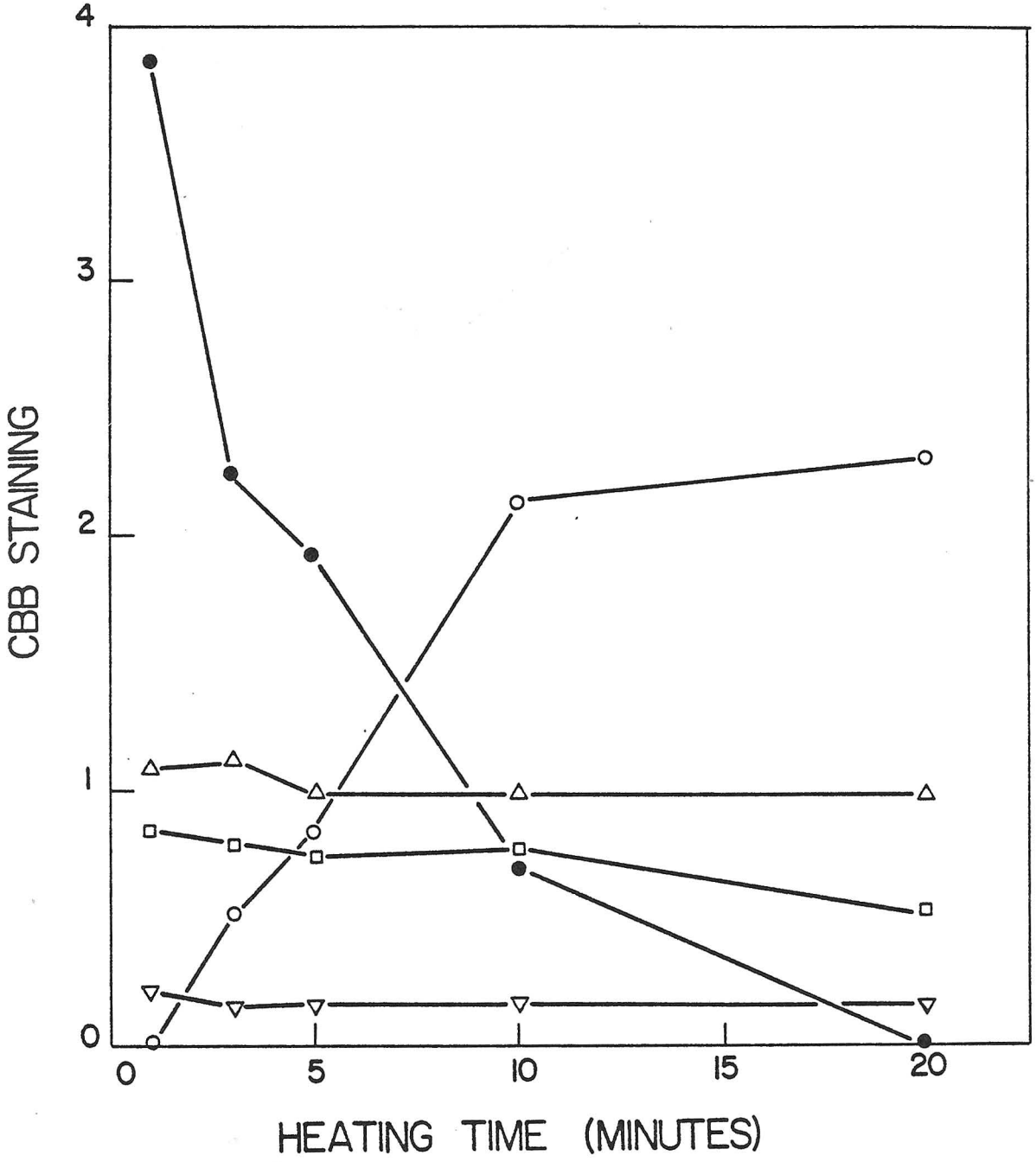
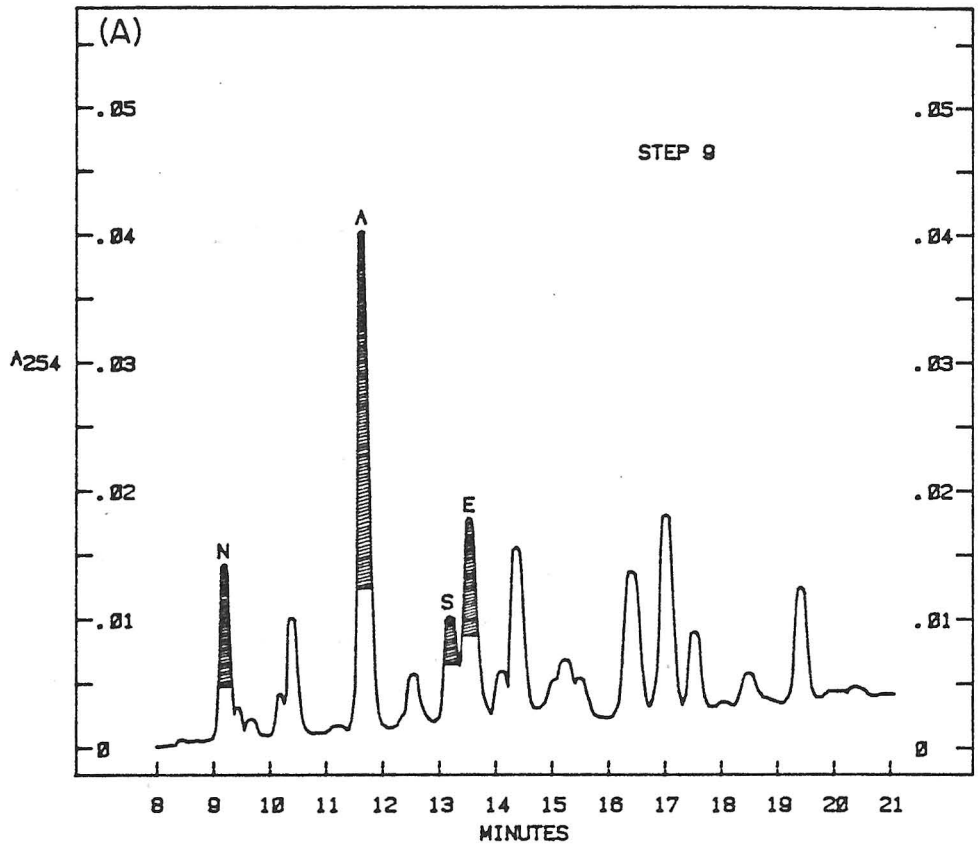
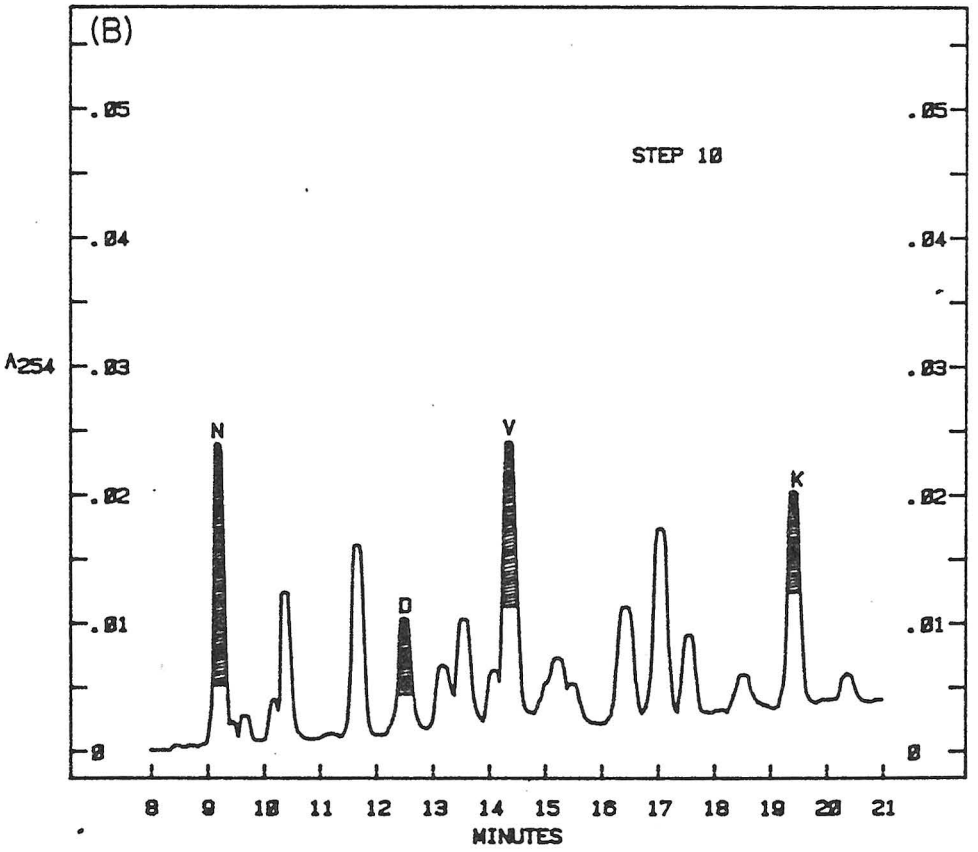

4
3
2
1
0
CBB STAINING
0
5
10
15
20
HEATING TIME (MINUTES)

Figure 9

HPLC chromatograms of the Pth amino acids from cycles 9 and 10 in a sequenator analysis of 3.5 nmol of base-extracted, membrane-bound AcChR. The four shaded areas in each chromatogram represent the area above background for the four amino acids present in the AcChR subunits at those steps. The amino acids are denoted by the one letter amino acid code (A, alanine; D, aspartic acid; E, glutamic acid; K, lysine; N, asparagine; S, serine; V, valine). 10% aliquots from the two cycles were injected into the HPLC system. The Pth amino acid mixtures from the sequenator cycles were treated with 1N HCl in methanol prior to injection to esterify the side chain carboxyls of Pth-Asp and Pth-Glu.


(A)
STEP 9
A254
.05
.04
.03
.02
.01
0
N
A
S
E
8 9 10 11 12 13 14 15 16 17 18 19 20 21
MINUTES


(B)
STEP 10
A254
.05
.04
.03
.02
.01
0
N
D
V
K
8 9 10 11 12 13 14 15 16 17 18 19 20 21
MINUTES

Chapter II: Demonstration of the Transmembrane Nature of the Acetylcholine Receptor by Labeling with Anti-Receptor Antibodies

INTRODUCTION

The acetylcholine receptor (AcChR) in its membrane-bound state can be isolated in the form of AcChR-enriched membrane vesicles from the electroplax of Torpedo californica (Cohen, et al, 1972; Duguid and Raftery, 1973; Elliott, et al, 1980). Such membranes have most, if not all, of the molecular properties of the postsynaptic membrane of the electroplaques; they bind α-neurotoxins (Cohen, et al, 1973; Duguid and Raftery, 1973) and cholinergic ligands (Raftery, et al, 1974a; Weber and Changeux, 1974) at structurally related sites and possess distinct binding sites for local anesthetics (Cohen, et al., 1974; Schimerlik, et al, 1976) and the alkaloid histrionicotoxin (Elliott and Raftery, 1977; Eldefrawi, et al, 1977). Cholinergic agonists binding to the AcChR cause the flux of inorganic cations through the membrane (Kasai and Changeux, 1971; Hess and Andrews, 1977; Miller, et al, 1978), suggesting that the AcChR is all or part of a transmembrane complex. The question of the disposition of the AcChR molecule in the membrane, however, remains a controversial issue.

When examined at the electron microscopic level, AcChR enriched membrane fragments are observed to be mostly in the form of vesicles, with densely packed cylindrical rosettes 60-80 Å in diameter (Nickel and Potter, 1973; Cartaud, et al, 1973; Raftery, et al, 1974b) that become more prevalent as the membrane preparations become more enriched in AcChR. Negative staining of Triton-solubilized purified AcChR reveals these same rosettes (Cartaud, et al, 1973; Eldefrawi and Eldefrawi, 1975; Ross, et al, 1977; Sobel, et al, 1977). This correlation of the presence of the rosettes with that of the AcChR as well as the recent immunospecific labeling of negatively stained membrane fragments with antibodies to AcChR by Klymkowsky and Stroud (1979), has led to the identification of this rosette structure as the AcChR molecule.

X-ray diffraction studies of AcChR enriched T. californica membrane fragments by Ross, et al (1977) have shown that the protein present spanned the membrane, extending 55 Å on one side of the bilayer and 15 Å on the other. Likewise, Rash et al (1978) observed transmembrane staining with $OsO_4/K_3Fe(CN)_6$ of the protein at mammalian neuromuscular junctions and in membrane preparations from Torpedo oscellata and

correlated this with anti-AcChR antibody binding they observed on one side of these same membranes. In neither study was it possible to directly determine the identities of the protein(s) responsible for these results. Using a more direct approach, with visualization of the binding of ferritin-conjugated anti-AcChR antibodies to Torpedo membrane vesicles by electron microscopy of thin sections, Karlin et al (1978) found antibody binding to the outside surface of the vesicles with some labeling on the inside surface which they were unable to distinguish from non-specific trapping that occurred. Using the same experimental approach, Tarrab-Hazdai et al (1978) observed some binding of anti-AcChR antibodies to both sides of the membrane of some open vesicles as evidence that the receptor spans the membrane.

A major problem in these studies has been in ascertaining the accessibility of both sides of the membrane to the antibody molecules. The recent isolation of intact, outside-out receptor-containing membrane vesicles provides a method for determining which side of the electroplaque synaptic membrane (cytoplasmic or extracellular) corresponds to which side of the isolated membrane vesicles being labeled in such studies

(Hartig and Raftery, 1979). In the present study, these intact vesicles were used with their extracellular faces exposed for immunospecific labeling of the AcChR molecule. In further studies the vesicles were sheared, exposing their cytoplasmic faces to the immunospecific labeling reagents. This method provided a preparation in which the orientation of the membrane vesicles was well-defined and in which both sides of the membrane were completely accessible to the labeling reagents. In this chapter and elsewhere (Strader, et al, 1979) evidence obtained by this technique is presented which demonstrates that the AcChR is a transmembrane protein.

MATERIALS AND METHODS

Crude membrane fragments were prepared from Torpedo californica electroplax by the method of Reed, et al (1975). Purified AcChR, solubilized in Triton, was prepared from crude membrane fragments according to Schmidt and Raftery (1973a) using affinity chromatography. Intact, right-side out vesicles enriched in AcChR (0.5 - 1.5 nmol α-BuTx binding sites per mg of total protein) were made in NPE buffer (10 mM Na phosphate, 0.4 M NaCl, 1 mM EDTA, pH 7.4) by the method of

Hartig and Raftery (1979). Briefly, crude membrane fragments were prepared in isotonic buffer and were then fractionated on a 4-20% sucrose gradient at 195,700 xg for one hour. The osmotically intact AcChR enriched vesicles banded at the top of the gradient. The "sidedness" of the vesicles was determined by the α-bungarotoxin (α-BuTx) sites assay described by Hartig and Raftery (1979). Intact vesicles were first incubated with an excess of [^{125}I] α-BuTx (Clark, et al, 1972; Blanchard, et al, 1979) in NPE. After 30 minutes, identical aliquots were diluted into (a) 10 mM Na-phosphate, 50 mM NaCl, 0.1% Triton (wash buffer) (total sites sample) and (b) wash buffer containing an excess of unlabeled α-BuTx (outside sites sample). After 30 minutes further incubation, 0.1 ml aliquots were applied to Whatman DE-81 discs and washed for 30 minutes in wash buffer, with three changes before counting in a Beckman Gamma-4000 gamma counter. The fraction of outside-out (extracellular side out) vesicles was determined from the ratio [outside sites counts]/[total sites counts].

α-Bungarotoxin was purified from the venom of Bungarus multicinctus by the method of Clark, et al (1972). Triton-solubilized membrane fragment proteins

depleted in AcChR (MFTS-R) were prepared by removing the AcChR from Triton-solubilized membrane fragments on an α-BuTx affinity column and were the gift of Dr. T. Claudio (Caltech).

Protein concentrations were determined by the method of Lowry, et al (1951) using bovine serum albumin as the standard. The concentration of α-BuTx sites was determined according to Schmidt and Raftery (1973b) using DEAE-cellulose filter discs and [^{125}I] α-BuTx. SDS-polyacrylamide gel electrophoresis was done according to Laemmli (1970), with a 12.5% acrylamide, 0.1% methylene-bis-acrylamide separating gel and a 3% acrylamide, 0.08% bis-acrylamide stacking gel. Immunodiffiusion assays were conducted according to Ouchterlony (1949).

Individual AcChR subunits were isolated by preparative SDS-polyacrylamide gel electrophoresis. Approximately 5 mg of Triton-solubilized, chromatographically purified AcChR was electrophoresed on a slab gel measuring 13.5 cm x 11 cm x 0.6 cm for 18 hrs at 20 mA. After brief staining in 0.25% Coomassie blue in 25% methanol, 10% acetic acid, a narrow horizontal band was cut from the gel at the position of each subunit, and the protein removed by electrodialysis at 5 mA/

tube for 16 hrs. After dialysis into saline, the purified subunits were used to immunize rabbits, as described below.

Preparation of Antibodies:

Antibodies to Triton-solubilized, purified AcChR (anti-AcChR) from Torpedo californica were raised in New Zealand white rabbits according to Claudio and Raftery (1977). The purity of the AcChR was checked on SDS gels before injection, with pure AcChR defined as the complex consisting of only the four polypeptide chains previously described (Raftery, et al, 1974b; Weill, et al, 1974; Raftery, et al, 1975; Chang and Bock, 1977; Lindstrom, et al, 1978, Vandlen, et al, 1979; Froehner and Rafto, 1979; Elliott, et al, 1980; Raftery, et al, 1980). Over a period of two years, six different rabbits were injected with several different AcChR preparations (see Table I); only two of the rabbits (#5 and #6) received the same AcChR preparation. 0.5-1 mg of AcChR emulsified in Freunds complete adjuvant was injected subcutaneously at multiple sites along the back. One to two weeks after boosting, all but one of the rabbits displayed the paralysis symptomatic of experimental autoimmune myasthenia gravis

(EAMG) (Patrick and Lindstrom, 1973) and were bled to death. Rabbit #2, despite repeated injections, never showed symptoms of the disease; 40 ml of blood were obtained from an ear vein weekly for two weeks following each challenge of this rabbit. Antibodies to α-BuTx or to isolated subunits were prepared following the same injection schedule, using 0.1 mg α-BuTx or 0.5-1 mg of each subunit emulsified in Freunds complete adjuvant per injection. The immunoglobulin fraction of antisera (Ig) was prepared by two fractionations with ammonium sulfate (0-33%) and the pellets dialyzed against phosphate buffered saline. This immunoglobulin fraction was clarified by centrifugation at 15,000 xg for 30 min before dilution into NPE for the labeling experiments. Normal rabbit Ig (NRG) were prepared by ammonium sulfate fractionation of pre-immune serum.

Antibody titers were determined by the method of Claudio and Raftery (1980). Triton-solubilized, purified AcChR, bound to [^{125}I] α-BuTx, was incubated with the Ig fraction of the anti-AcChR serum at room temperature. After 1 hr, enough Staphylococcus aureus to precipitate all the IgG was added and incubated for 30 min. The solution was centrifuged at 15,000 xg for five min and the pellet washed in 10 mM Na phosphate, 0.1% Triton, and counted on a Beckman Gamma-4000 gamma counter.

IgG was isolated from the immunoglobulin fraction by chromatography on DEAE-cellulose, according to Sober, et al (1956). F_{ab} fragments were prepared from the IgG by hydrolysis with papain (Porter, 1959; Putnam, 1962). IgG (10 mg/ml in 0.1 M Na acetate, pH 5.5) was incubated with mercuripapain (papain : IgG = 1:100) in 10 mM cysteine and 2 mM EDTA for five and one half hrs at 37°. After quenching with 1mM PCMB, the mixture was dialyzed against 0.1 M Na acetate, pH 5.5 before chromatography on a CM-52 column to separate the F_{ab} fragments from the F_c.

$F_{(ab)'_2}$ fragments were isolated from IgG by hydrolysis with pepsin (Nisonoff, et al, 1960; Utsumi and Karush, 1965). IgG (10 mg/ml in 0.2 M Na acetate, pH 4.5) was digested with pepsin (pepsin : IgG = 1:75) at 37° for 24 hrs. After dilution into 10 mM Na phosphate, 0.2 M NaCl, 2 mM EDTA, pH 8.2, the pH was re-adjusted to 8.2 with 0.5% NaOH, and the solution dialyzed against this same buffer. The $F_{(ab)'_2}$ fragments were purified by chromatography on Sephadex G-75.

Preparation of Markers for Electron Microscopy:

(a) Colloidal gold - Particles of colloidal gold 150 Å in diameter were prepared by the method of Frens (1973). To 10 mg $HAuCl_4 \cdot 3H_2O$ in 100 ml boiling H_2O was

added 2 ml 1% sodium citrate and boiling continued for five mins. 40 sec after the addition of Na citrate, the yellow solution became faintly blue, which deepened within the next 30 sec to purple, finally reaching a reddish-orange color. After passage through a millipore filter (0.45μ), this solution was coated with protein by the method of Horisberger, et al (1975). To determine the amount of protein needed to completely coat the colloidal gold and stabilize it against flocculation, 5 ml of the colloid was added to increasing amounts of protein in 5 mM NaCl, pH 7. After 1 min, uncoated gold was flocculated with 1 ml 10% NaCl. The extent of flocculation was determined by a decrease in absorbance at 515 nm. The correct amount of protein (determined as above) was then mixed with the gold solution with vigorous stirring. After one min, 0.25 ml of 1% polyethylene glycol (Carbowax 20 M) was added and the pH immediately adjusted to 7 with ∿ 1 drop of 0.2 M K_2CO_3. The resultant gold conjugate was stable for several months at 4°C.

(b) Ferritin - α-BuTx-ferritin conjugates were prepared by the method of Kishida, et al (1975). Ferritin (EM grade, clarified by centrifugation), and

α-BuTx were mixed in equimolar amounts (∿ 20 mg/ml) in PBS and coupled with glutaraldehyde (0.03% final concentration) at room temperature for 45 min. After quenching with an excess of lysine-HCl, the mixture was stirred at 4°C overnight, then dialyzed aginst PBS. The conjugate was separated from unreacted α-BuTx by chromatography on a small P-60 column. For use with antibodies, ferritin coupled to IgG (goat anti-rabbit IgG) was purchased from Cappell Laboratories.

(c) Hemocyanin-Protein A - Hemocyanin-Protein A conjugate (Hcy/A) was prepared by the method of Miller, et al (1980), using glutaraldehyde as the crosslinking reagent. One ml of hemocyanin (70 mg/ml), purified by centrifugation and gel filtration from the hemolymph of Busycon canaliculatum, and 1 ml of Protein A (Pharmacia, 3.6 mg/ml) were conjugated with 0.22 ml of 0.5% glutaraldehyde. After 45 min at room temperature, 0.2 ml of 2 M glycine was added to bind unreacted glutaraldehyde. After 15 min at room temperature, the solution was dialyzed for 24 hr at 4°C against PBS and the Hcy/A conjugate separated from unconjugated Protein A by passage over a Sepharose 2B column.

For determination of the molar ratio of hemocyanin to Protein A, [^{125}I]-Protein A (0.5 Ci/mole) was in-

cluded in the conjugation step. After purification, the concentration of hemocyanin, calculated from its absorbance at 280 nm (the extinction coefficient of hemocyanin at 280 nm is ∿ 9 times that of Protein A) was compared to the concentration of Protein A, determined by its radioactivity. The use of radioactively labeled Protein A also allowed determination of the elution pattern of unconjugated Protein A.

The extent to which crosslinking hemocyanin to Protein A might induce clustering of hemocyanin molecules was determined by coating Alcian blue treated coverslips (Sommer, 1977) with dilute solutions (∿ 10^{-8} gm/ml) of Hcy/A. Replicas of the surface of the coverslips were prepared as described below and the number of clustered hemocyanins determined. Similar preparations of pure hemocyanin and hemocyanin conjugated to goat anti-rabbit IgG (Miller, et al, 1979) were used for comparison.

Labeling of Membrane Vesicles with Antibodies - Thin Section Technique with Electron-dense Markers:

Membrane vesicles were labeled in solution with gold-conjugated antibodies or with gold-α-BuTx in Tris-buffered saline, containing 1% polyethylene glycol,

pH 7.4, at a protein concentration of 2-5 mg/ml. Incubation was at room temperature for one hr with a 2-10 times excess of protein-gold. The membrane fragments were then separated from the unbound conjugate by centrifugation and washed three times in buffer before fixation.

Labeling of intact vesicles with ferritin-α-BuTx was performed in NPE buffer at room temperature for one hr, using a 2-10 times excess of ferritin conjugate. The membranes were washed by centrifugation, as above, before fixation.

For double-labeling experiments, intact vesicles in NPE were broken by osmotic shock into a 10-fold excess of H_2O, then pelleted by centrifugation and resuspended in NPE buffer. The intact and broken membrane vesicles were then labeled in parallel experiments as follows. The vesicles were incubated with a 2-10 times excess of anti-AcChR antibody for one hr at room temperature. After thorough washing by three centrifugation steps, they were further labeled with ferritin coupled to goat anti-rabbit IgG. After one hr, the samples were again washed and prepared for fixation and sectioning.

Preparation of Samples for Thin-Section Electron Microscopy:

After the final washing step, membranes were pelleted and the pellets fixed in 2% glutaraldehyde in the appropriate buffer at 4°C for 1-24 hr, then washed and post-fixed in 1% OsO_4 in buffer at 4°C for one hr. After transfer into 0.2 M Na maleate, pH 5.15 and washing, the pellets were block stained in 1.5% uranyl acetate in maleate buffer for one hr at room temperature. The samples were washed in water, then dehydrated in a series of ethanol solutions (70-100%), rinsed twice in propylene oxide, and embedded in Epon for three days at 70°C. Sections 600-900 Å thick were cut on a Reichert microtome, deposited on 300 mesh copper grids, and further stained with lead citrate before examination with a Phillips 201 electron microscope.

Labeling of Intact and Broken Vesicles with Antibodies - Replica Technique with Hcy/A:

(a) Intact vesicle labeling - To attach intact vesicles to coverslips, the glass coverslips were first treated with 1% Alcian Blue (Sommer, 1977) for 15 min and washed three times in NPE. 50 µl of intact vesicles (2 mg/ml) were incubated on the coverslip for 15 min and the excess membranes rinsed off with NPE. For identification of AcChR antigenic determinants,

35 µl of anti-AcChR Ig from each of the six rabbits (0.5 mg/ml) were then incubated on the coverslip for 30 min at room temperature. The coverslip was then rinsed three times in NPE, and treated with Hcy/A as described below. Bungarotoxin binding sites were visualized by incubation of the coverslip in α-BuTx (0.02 mg/ml) for 45 min before treatment with anti-BuTx Ig and Hcy/A. Control experiments were done by substituting NRG for immune antibodies.

(b) Cytoplasmic membrane labeling - To expose the cytoplasmic face of the vesicles a modification of the method of Jacobson and Branton (1977) was used. The coverslip was clamped between two pieces of plexiglass with a hole exposing most of the surface of the coverslip. This apparatus was suspended in one liter of cold NPE and sonicated in a bath sonicator (Laboratory Supply Co. Inc.) at 80 kHz for five min. The coverslip was then removed and labeled with antibodies as described above.

(c) Hemocyanin/Protein A labeling of immunolabeled vesicles - After labeling with immune or pre-immune antibodies, the coverslips containing intact or sonicated vesicles were incubated with Hcy/A (1.5 mg/ml in NPE) for 30 min at room temperature and washed three times in NPE.

Preparation of Replicas for Electron Microscopy:

Immediately after labeling with Hcy/A, samples were fixed in 1% glutaraldehyde in NPE for several hours, followed by fixation in 1% OsO_4 in half-strength NPE for one hr at 4°C. Following dehydration in a graded series of ethanol solutions, the samples were dried at the critical point using CO_2 as the transition fluid in a Polaron apparatus. The coverslips were shadowed at a 45° angle with platinum/palladium followed by coating with carbon at 90° angle in a vacuum evaporator. Replicas were then separated from the glass coverslip by etching with 48% hydrofluoric acid and the membrane fragments removed with twice-filtered household bleach. After rinsing with water, the replicas were deposited on copper grids and examined in a Phillips 201 electron microscope.

RESULTS

Colloidal gold-protein conjugates were prepared with α-BuTx, with antibodies to the AcChR and to the 40,000 dalton subunit of the AcChR, and with nonspecific serum or IgG. When immunoglobulins were dialyzed into the 5 mM NaCl, pH 7 solution used for coating the colloidal gold, some precipitation of the protein was

observed; this did not occur when the unfractionated serum was used. In all cases, the protein was able to stabilize the gold colloid against flocculation by salt, as determined by the assay described previously and shown in Figure 1. When gold conjugates prepared by this method were used to label AcChR-enriched membrane preparations, however, aggregation of the gold spheres was frequently observed (Figure 2). Labeling of membrane vesicles was never demonstrated by this method. Gold particles could frequently be seen aligned along a membrane, but this appears to have been merely fortuitous; large-field views show that almost none of the membranes in a given field were labeled with this marker. The gold spheres themselves were extremely electron dense and fairly small; in these respects they were ideal markers for transmission electron microscopy. However, their tendency to aggregate rather than to label, which probably resulted in part from the extremely low ionic strength solutions necessary for their preparation and the much higher physiological ionic strength at which the labeling reactions were performed, made their use inappropriate for these studies.

The labeling of intact and osmotically shocked vesicles with anti-AcChR followed by ferritin coupled to goat anti-rabbit IgG is demonstrated in Figure 3. In

these and similar studies (Karlin, et al, 1978; Tarrab-Hazdai, et al, 1978), a very high background level of ferritin is observed between the membrane vesicles; specific labeling must be measured in areas where this background is lowest. In Figure 3A, intact vesicles appear as empty spheres, the largest of which are encircled by rings of ferritin. The low ferritin background labeling in the regions away from the vesicles and the total absence of ferritin around some vesicles (presumably non-receptor containing membranes) attest to the specificity of the labeling observed.

Specific labeling of the osmotically shocked vesicles was more difficult to see, since the absence of the osmotically intact hollow spheres of vesicles made the distribution of ferritin in the background more uniform (Figure 3B). Membrane sheets would almost certainly be hidden under this ferritin background. The membranes that could be distinguished from the background appeared primarily as circles or "U"-shaped structures which, on close examination, were usually seen to consist of a double membrane of two parallel bilayers, the entire width of which was $\sim$ 250 Å. As shown in Figure 3B, the U-shaped double vesicles frequently were labeled on both sides; no labeling was observed on the interior of any single-bilayer membrane vesicle.

Further investigation of this phenomenon of double bilayers gave the results depicted in Figure 4 and in Table II. Intact vesicles prepared in isotonic buffer contained only 11% of the membranes in the form of double bilayers. After osmotic shock by dilution into H_2O and resuspension in isotonic buffer, these preparations contained 72% of the membranes as double bilayers. It appears that, upon osmotic shock, the vesicles "reseal" into spheres, which may be either "leaky" or osmotically intact; this was not determined here. Upon resuspension in the isotonic buffer of higher ionic strength, the osmotic pressure outside the vesicles is much higher than that inside, causing them to collapse. These collapsed vesicles appear in thin sections as double bilayered membranes of various shapes, depending on the angle of sectioning (see Figure 4). Thus, it is impossible to label the interior of osmotically shocked vesicles by this method: the interior face of the membrane is enclosed within the double bilayer of the vesicles, leaving only the exterior surface of the membrane exposed to the labeling reagents in these preparations.

For this reason, the technique of shearing intact vesicles attached to coverslips was used for labeling of

the interior surface of the membrane. This method, discussed below in more detail, avoided the use of osmotic shock while leaving the entire cytoplasmic surface of the membrane accessible to the labeling reagents.

The non-specificity of labeling observed with these two electron-dense markers necessitated the development of a new marker for immuno-electron microscopy, Hcy/A. After preparation as described above, this label was purified by Sepharose 2B chromatography. A typical elution profile is shown in Figure 5. Unconjugated Protein A elutes from the column close behind Hcy/A but is not detected in the profile of absorbance at 280 nm because of its low extinction coefficient relative to hemocyanin. For this reason only the peak fractions of the Hcy/A peak were collected; the tailing edge of the peak was contaminated with free Protein A, as can be seen from the profiles in Figure 5. The small amount of free hemocyanin which may be present in the Hcy/A solution appears to have little effect on the specificity of labeling, as described below.

The ratio of hemocyanin to Protein A in Hcy/A was determined as described in the Methods section and is summarized in Table III. Protein A and hemocyanin were initially mixed in a molar ratio of 10:1; after purification, however, the conjugate contained Protein A and

hemocyanin in a ratio of 0.8:1. Determination of the extent of aggregation of the Hcy/A marker gave the results shown in Table IV. Hcy/A is 95% monomeric; this compares well with the results from purified hemocyanin, indicating that the conjugation reaction does not cause aggregation. Thus Hcy/A has been characterized as a monomeric marker for immuno-electron microscopy in which the molar ratio of the immunolabel to the marker approaches 1:1.

Labeling Studies with Hcy/A:

The intact vesicles described here were from 94% to 100% extracellular side out; i.e. 94-100% of the total α-BuTx sites were exposed on the outside surface of the vesicles, as determined by the α-BuTx binding assay described in Methods. When examined at the electron microscopic level, replicas of these preparations consisted of spherical vesicles ranging in diameter from 0.1-1 μ (see Figure 6a). Upon sonication for five mins, the vesicles appeared as flat circles with a rough texture, often with a thickening at the perimeter attributable to edges not attached to the Alcian blue coating (Figure 6e). A time course of the effects of sonication showed that after 2 mins most vesicles maintained intact their spherical shape, while by 5 mins approximately 95% of the vesicles had been sheared, leaving their inside

faces exposed. At times longer than 10 mins vesicles were frequently totally removed from the Alcian blue and after 30 mins the Alcian blue began to pucker and detach. Occasionally, vesicles could be found which had collapsed during sonication and were not sheared off. Such collapsed vesicles could be readily distinguished from sheared vesicles in both labeled and unlabeled preparations by the smoother and more three-dimensional appearance of the former (see Figure 6).

The binding of anti-AcChR and anti-BuTx Ig to intact and sheared vesicles is summarized in Tables V and VI. That the intact vesicles were oriented mainly so that their extracellular, α-BuTx-binding sides faced outward was verified at the electron microscopic level by the binding of α-BuTx and anti-BuTx Ig, visualized with the Hcy/A marker. As can be seen in Figure 7a, the outsides of several vesicles treated with α-BuTx and anti-BuTx Ig, visualized with Hcy/A, were coated with the Hcy/A markers. Upon sonication, the inside surfaces of the vesicles were exposed but no BuTx binding could be discerned (Figure 7b). Control experiments in which vesicles were treated with NRG and Hcy/A showed no labeling on either the intact or the sonicated vesicles (Figure 8). With all six anti-AcChR Ig prepara-

tions, the extracellular faces of the membranes were labeled (Figure 9a). With both anti-BuTx and anti-AcChR Ig's, a high proportion (70-80%) of the larger intact vesicles (approximately 0.5 μ in diameter and larger) were heavily labeled while most of the smaller vesicles and some of the larger ones are not labeled at all with either antibody.

When the sonicated vesicles were treated with anti-AcChR Ig there was a dramatic difference in labeling from one antibody preparation to the next. With anti-AcChR Ig's from rabbits #1, 2, 3, 4, and 6 there was little if any labeling on the insides of the vesicles (Figure 9b). Occasionally, inside labeling of the vesicles was observed (Figure 9c), but it was usually sparse and could be correlated with an increase in the background labeling of the Alcian blue, suggesting that insufficient washing may have caused non-specific disposition of Hcy/A on the vesicle surface. In other cases (Figure 9d) label was observed only around free edges of the vesicle which had curled over and faced upward, exposing a narrow rim of external AcChR sites to the anti-AcChR Ig solution.

With anti-AcChR Ig from rabbit #5, sonicated vesicles were heavily labeled (Figure 9e). The density

of Hcy/A markers on the vesicles was up to 15 times higher than that on the Alcian blue background, an increase comparable to that observed in the labeling of intact vesicles with anti-AcChR Ig (compare Figures 9a and 9e). As in the labeling of intact vesicles it was mainly the larger sonicated vesicles that were labeled with anti-AcChR Ig from rabbit #5; most of the smaller vesicles and some of the larger ones did not label at all (Figure 9f).

The labeling results are summarized in Tables V and VI. All vesicles in large fields (each containing $\sim$ 200 vesicles) of intact or sheared vesicles labeled with each of the anti-AcChR preparations, α-BuTx + anti-BuTx, or NRG were examined and the percentage of vesicles labeled with Hcy/A markers for each of four arbitrary size ranges (diameter of $\geq$ 5000 Å, 3000-5000 Å, 1000-3000 Å and $\leq$ 1000 Å) was determined. As is clear from Table V, the AcChR-containing vesicles are essentially all larger than 3000 Å in diameter, with 70-80% of the intact vesicles larger than 5000 Å and 20-30% of those in the 3000-5000 Å range containing AcChR as determined by Hcy/A visualization of both anti-AcChR and α-BuTx and anti-BuTx binding. To provide a better statistical sample from which to determine whether anti-AcChR from rabbit #5 did indeed consistently label the

insides of AcChR-containing vesicles, 40 or more vesicles with diameters of 3000-5000 Å and 40 or more with diameters $\geq$ 5000 Å were examined for Hcy/A labeling after treatment with anti-AcChR, α-BuTx + anti-BuTx, or pre-immune Ig, with the results given in Table VI. Anti-AcChR from rabbits #1-4 and #6 and anti-BuTx behaved the same way, again labeling 70-80% of the largest vesicles and 20-30% of the 3000-5000 Å vesicles on the extracellular side of the membrane and essentially none on the cytoplasmic side. This larger sampling also confirmed the result indicated in the smaller sampling in Table II: anti-AcChR from rabbit #5 labeled the cytoplasmic side of the membranes on 78% of the $\geq$ 5000 Å vesicles and 25% of the 3000-5000 Å vesicles, the same amount of labeling observed on the extracellular sides of these vesicles with α-BuTx + anti-BuTx and with all six anti-AcChR preparations (including that from rabbit #5). Pre-immune Ig labeled on neither the insides nor the outsides of vesicles in any size range.

Ouchterlony immunodiffusion assays of each anti-AcChR Ig preparation against purified AcChR, Triton-solubilized membrane fragments (MFTS), and MFTS-R showed a strong reaction of all anti-AcChR Ig against AcChR and against MFTS (see Figure 10). Only one

preparation (from rabbit #2) reacted moderately with the MFTS-R (see Table I). With one other anti-AcChR Ig preparation, a barely discernible reaction was recorded. With the other four anti-AcChR Ig preparations (including that from rabbit #5), no reaction with the MFTS-R was observed.

DISCUSSION

The experimental evidence presented here demonstrates that the AcChR, composed of four subunits with molecular weights of 40,000, 50,000, 60,000, and 65,000 daltons (Raftery, et al, 1974b; Weill, et al, 1974; Raftery, et al, 1975; Chang and Bock, 1977; Lindstrom, et al, 1978; Vandlen, et al, 1979; Froehner and Rafto, 1979; Elliott, et al, 1980; Raftery, et al, 1980), is a transmembrane protein. The method of immunospecific labeling used permits complete exposure of both the extracellular and cytoplasmic faces of the membrane to the antibody solution. These faces could clearly be identified as the extracellular face of the synapse corresponding to the outside face of the membrane vesicle and the cytoplasmic face ~~to~~ the inside of the vesicle by Hcy/A visualization of the binding of α-BuTx and anti-α-BuTx.

Hemocyanin has proven to be a highly visible marker in both transmission and scanning electron microscopic studies of smooth cellular or membrane surfaces (Smith and Revel, 1972; Brown and Revel, 1978), and the specificity of Hcy/A for IgG, thoroughly established by Miller, et al (1980), is here shown again by the lack of Hcy/A labeling of membrane vesicles treated with NRG. The use of the electron-dense markers colloidal gold and ferritin in preliminary experiments proved disappointing due to a lack of specificity and an intolerably high background level of ferritin. Moreover, both of these markers are best used in solution with samples to be examined by thin-section; their small size and high electron density makes them most useful under these circumstances. Because of the considerable tendency of the intact vesicles used in these studies to reseal after osmotic shock and then collapse upon resuspension in higher osmotic strength buffer, it was not possible to label their inside surfaces in solution. Such attempts generated the same accessibility problems noted by others using this method (Karlin, et al, 1978; Tarrab-Hazdai, et al, 1978).

Because of its large size and distinctive shape, hemocyanin can readily be seen using the replica technique for both transmission and scanning electron micro-

scopy. Linkage to Protein A, which binds tightly to the F_c region of immunoglobulin from several species (Goding, 1978), produces a useful and versatile labeling reagent for immuno-electron microsocpy. Hcy/A is also visible in thin-section electron micrographs; the distinctive shape of hemocyanin makes it easily distinguishable despite its lack of any electron dense core. However, the large size of the molecule (350 x 500 Å) would make it less useful for labeling the insides of vesicles, even in the absence of the resealing problem.

The technique used here, therefore, is ideally suited for the reagents used. The intact vesicles are sheared by mechanical forces while immobilized on coverslips and thus physically prevented from resealing. In this way, the cytoplasmic face of the membrane is completely exposed to the immunolabeling reagents. With accessibility problems removed, the large Hcy/A complex then provides a clearly visible marker for electron microscopic visualization. The use of osmotically intact vesicles permitted the use of a [^{125}I] α-BuTx binding assay to confirm that most of the vesicles were indeed "right-side out"; i.e., most of the α-BuTx sites were exposed on the outside surface of the vesicles, in agreement with the observations of Hartig and Raftery (1979).

In the labeling of intact vesicles with anti-AcChR Ig, the results obtained with antisera from different rabbits were in excellent agreement. Antisera from all rabbits showed equal labeling of the outside (extracellular) faces of intact vesicles. The observation that the same proportion of vesicles of each size range were labeled with both anti-BuTx and anti-AcChR Ig is indicative of the specificity of the antibodies for the AcChR since it suggests that the same vesicles were labeled with both reagents. The differences in labeling from one antibody preparation to the next became apparent only in the labeling of the cytoplasmic faces of sonicated vesicles, where anti-BuTx and five of the six preparations of anti-AcChR showed little or no labeling and one of the anti-AcChR preparations showed labeling comparable to that seen on the extracellular face of the membranes.

That the labeling seen with anti-AcChR from rabbit #5 was specific labeling of the AcChR on the cytoplasmic face of the membrane was the conclusion drawn from several lines of evidence. (i) The AcChR injected into the rabbit producing these antibodies was highly purified as judged by its SDS gel electrophoresis profile, having a gel pattern consistent with that of pure T.

californica AcChR (Raftery, et al, 1974b; Weill, et al, 1974; Raftery, et al, 1975; Chang and Bock, 1977; Lindstrom, et al, 1978; Vandlen, et al, 1979; Froehner and Rafto, 1979; Elliott, et al, 1980; Raftery, et al, 1980). Furthermore, the same AcChR preparation was injected at the same time into another rabbit (#6) whose antisera were also used in these studies. The antibodies from rabbit #6 did not react with the cytoplasmic sides of the vesicles, ruling out the possibility that there was simply a variation in the AcChR preparation used to immunize the rabbit which caused this labeling of the cytoplasmic face. (ii) The immunodiffusion results indicate that it is in all likelihood not a contaminant in the AcChR injected into the rabbit that causes labeling of the cytoplasmic face with the antibodies from rabbit #5. In the interaction between the various anti-AcChR Ig preparations and MFTS-R, there was no discernible reaction with immunoglobulins from rabbit #5. There was, however, a very faint reaction with the antisera from rabbit #2 and a barely discernible reaction with antisera from rabbit #4, neither of which labeled the cytoplasmic face. (iii) All labeling experiments were repeated three times with three different intact vesicle preparations, yielding the same results. Therefore,

the labeling of the cytoplasmic face of the membrane by this anti-AcChR preparation was a real phenomenon and not an artifact caused by insufficient washing of one sample. (iv) Finally, the statistics of labeling given in Tables V and VI indicate that the AcChR-containing vesicles can be identified by their large size. It is these large vesicles that label with both anti-AcChR (77%) and anti-BuTx (71%) on the extracellular face and which also label with this anti-AcChR preparation (78%) on the cytoplasmic face. This statistical agreement also holds for vesicles in the 3000-5000 Å range, 20-30% of which contain AcChR. That none of the vesicles labeled with preimmune Ig on either side nor with α-BuTx + anti-BuTx on the cytoplasmic side also indicates that the labeling observed is specific for the given antibody-antigen interaction.

It is not unusual that different rabbits injected with the same antigen would produce antibodies of differing specificities; these experimental rabbits are an outbred population and their immune responses are not homogeneous. Such differences in the specificities of rabbit antibodies have been found in studies of the functional effects of anti-AcChR antibodies (Sanders, et al, 1976; Karlin, et al, 1978) and of anti-Na^+-K^+ ATPase antibodies (Kyte, 1974). That only one out of

six rabbits displayed an immune response to antigens on the inside face of the membrane indicates that either the portion of the receptor exposed on this side is not very antigenic and/or only a small portion of the molecule is exposed on the cytoplasmic face. This concurs with the findings of Ross, et al (1977), who determined that the protein present protruded only 15 ± 5 Å on one side of the membrane of AcChR-enriched preparations while extending 55 ± 5 Å from the other side.

In thin-section electron micrographs of the labeling of membrane fragments in solution with ferritin-conjugated anti-AcChR antibodies, Tarrab-Hazdai et al (1978) saw some labeling of both sides of open vesicles, while Karlin et al (1978) saw some inside labeling, but were unable to distinguish it from non-specific sticking of ferritin. This difference may also have resulted from differing specificities of the anti-AcChR antibody preparations used. In the labeling of whole vesicles with antibody molecules, the insides are often not accessible to the labeling reagents and there can be non-specific trapping of labels inside the vesicles. The observations mentioned above which indicate that Torpedo membrane vesicles reseal after osmotic shock also complicate this experimental approach. The labeling method described here avoids any ambiguities caused by inaccessi-

bility or by trapping of label inside the vesicles, since only the extracellular side, or only the cytoplasmic side, of the membrane is completely exposed to the labeling solution.

The AcChR consists of four subunits of molecular weights 40,000, 50,000, 60,000 and 65,000 daltons (Raftery, et al, 1974; Weill, et al, 1974; Raftery, et al, 1975; Chang and Bock, 1977; Lindstrom, et al, 1978; Vandlen, et al, 1979; Froehner and Rafto, 1979; Elliott, et al, 1980; Raftery, et al, 1980). By lactoperoxidase catalyzed iodination of the outsides of intact vesicles, Hartig and Raftery (unpublished) have shown that portions of all four of the AcChR subunits are exposed on the extracellular face of the synaptic membrane. Lindstrom, et al (1978) have shown that antibodies to each of the AcChR subunits cross-react with rat muscle receptors in vivo, causing a decrease in muscle AcChR content. Hence, each of these four polypeptide chains must contain at least some antigenic determinants exposed on the extracellular face of the membrane.

Attempts to label the membrane vesicles with antibodies to individual AcChR subunits have been unsuccessful. This is probably due to the failure of such antibodies (prepared against the denatured individual subunits

isolated from preparative SDS-polyacrylamide gels) to react with native, membrane-bound AcChR as observed with electron microscopy. Therefore, it was not possible to determine by this method which of the subunits are exposed on the extracellular side and which on the cytoplasmic side of the membrane. Further studies regarding this question are reported in Chapter III of this thesis.

In conclusion, we have developed an unambiguous method for immunospecific labeling of the extracellular face or of the cytoplasmic face of membrane vesicles enriched in AcChR. The AcChR is a complex molecule consisting of four polypeptide subunits which remain together as a complex in the presence of Triton (Schmidt and Raftery, 1973a) and cholate (Elliott, et al, 1980) and which dissociate only when denatured in SDS. Antibodies to the AcChR containing only these four subunits were prepared and used to label a preparation of vesicles (Hartig and Raftery, 1979), in which 94-100% of the α-BuTx binding sites were on the outside surface of the membrane. Thus, in these vesicles, almost all of the AcChR molecules were at least partially exposed to the outside surface of the vesicles (corresponding to the extracellular face of the post-synaptic membrane).

All preparations of anti-AcChR antibodies were found to bind to the outer surface of the vesicles and one preparation also bound to the inner surface of the vesicles. Therefore, one or more of the four polypeptide subunits of the AcChR used as the antigen for production of these antibodies is exposed on the extracellular face of the membrane and one or more is exposed on the cytoplasmic face of the membrane. Thus, it may be concluded from this evidence that the AcChR is a transmembrane protein, with many antigenic determinants on the extracellular face of the synaptic membrane and with few of the antigenic determinants protruding on the cytoplasmic face of the membrane.

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Table I

Characterization of Anti-AcChR Antisera

		Titers*		Ouchterlony Reactions†			E.M. Studies with Hcy/A	
rabbit	Contracted EAMG	nmol α-BuTx sites	pmol AcChR	AcChR	MFTS	MFTS-R	extra cellular labeling	cyto-plasmic labeling
#1	yes	0.32	2.00	++++	++++	-	yes	no
#2	no	0.51	3.00	++++	++++	++	yes	no
#3	yes	0.65	4.06	++++	++++	-	yes	no
#4	yes	0.54	3.37	++++	++++	+	yes	no
#5§	yes	1.95	12.17	++++	++++	-	yes	yes
#6§	yes	2.35	14.16	++++	++++	-	yes	no

* Titers are given per ml of serum

† Ouchterlony results are as judged visually, with ++++ indicating a very strong reaction, + a very weak reaction, and - no reaction.

§ These two rabbits were injected with the same preparation of AcChR.

Table II

Effects of Osmotic Shock on Intact Vesicles

membrane / preparation	intact vesicles	osmotically shocked
single bilayer	89%	28%
double bilayer	11%	72%

Table III

Molar Ratio of Protein A and Hemocyanin in Hcy/A

	Initially Added	After Hcy/A Purification
Protein A	1.55 mg	2.86 µg/ml
Hemocyanin	35 mg	7.50 µg/ml
Molar Ratio (Protein A:Hcy)	10:1	0.8:1

Table IV

Determination of Hemocyanin-Hemocyanin Crosslinking in Hcy/A

Preparation	Particles Counted	% Dimers	% Oligomers
Hcy	2054	4	0.4
Hcy/A	1715	5	0.2
Hcy/IgG	1614	9	2.0

TABLE V

ANTIBODY LABELING OF VESICLES IN A SINGLE FIELD, ACCORDING TO SIZE

Vesicle Diameter		≥ 5000 Å		3000-5000 Å		1000-3000 Å		≤ 1000 Å	
Antibody Preparation	Vesicle Preparation	# Labeled / Total	% Labeled	# Labeled / Total	% Labeled	# Labeled / Total	% Labeled	# Labeled / Total	% Labeled
Anti-AcChR #1	Intact	5/7	71%	11/36	31%	3/152	2%	0/72	0%
	Sheared	0/3	0%	1/6	17%	0/30	0%	0/22	0%
Anti-AcChR #2	Intact	7/13	54%	14/40	35%	3/106	3%	0/66	0%
	Sheared	0/3	0%	0/10	0%	0/51	0%	0/120	0%
Anti-AcChR #3	Intact	7/8	88%	10/42	24%	6/147	4%	0/73	0%
	Sheared	0/2	0%	0/10	0%	0/22	0%	0/92	0%
Anti-AcChR #4	Intact	8/11	73%	9/31	29%	6/116	3%	0/89	0%
	Sheared	0/4	0%	1/12	8%	1/11	9%	0/43	0%
Anti-AcChR #5	Intact	5/7	71%	7/24	29%	6/163	2%	0/70	0%
	Sheared	5/6	83%	10/38	26%	3/45	7%	0/78	0%
Anti-AcChR #6	Intact	4/5	80%	10/40	25%	5/152	3%	0/87	0%
	Sheared	0/3	0%	1/34	3%	3/47	6%	0/49	0%
Anti-BuTx	Intact	4/5	80%	8/36	22%	2/108	2%	0/95	0%
	Sheared	0/2	0%	0/21	0%	0/26	0%	0/92	0%
NRG	Intact	0/10	0%	0/52	0%	0/104	0%	0/83	0%
	Sheared	0/8	0%	0/12	0%	0/27	0%	0/17	0%

SUMMARY, TABLE V

(A) EXTRACELLULAR SURFACE LABELING

Vesicle Diam. / Ab	≥ 5000 Å	3000-5000 Å	1000-3000 Å	≤ 1000 Å
Anti-AcChR #1-6	73 ± 9%	29 ± 4%	3 ± 0.8%	0%
Anti-BuTx	80 ± 10%	22 ± 3%	2 ± 0.5%	0%
NRG	0%	0%	0%	0%

(B) CYTOPLASMIC SURFACE LABELING

Vesicle Diam / Ab	≥ 5000 Å	3000-5000 Å	1000-3000 Å	≤ 1000 Å
Anti-AcChR #1-4,6	0%	6 ± 7%	3 ± 4%	0%
Anti-AcChR #5	83 ± 10%	26 ± 4%	7 ± 7%	0%
Anti-BuTx	0%	0%	0%	0%
NRG	0%	0%	0%	0%

All vesicles in a single field were counted for each antibody category and the percentage of labeled vesicles for each size range determined. The results are summarized in the lower half of the Table.

TABLE VI

ANTIBODY LABELING OF INTACT AND SHEARED VESICLES

(A) EXTRACELLULAR SURFACE LABELING

Vesicle Diam. / Ab	≥ 5000 Å		3000-5000 Å	
	# Labeled / Total	% Labeled	# Labeled / Total	% Labeled
Anti-AcChR #1-6	56/73	77 ± 9%	54/189	29 ± 4%
Anti-BuTx	35/49	71 ± 9%	11/49	22 ± 3%
NRG	0/52	0%	0/52	0%

(B) CYTOPLASMIC SURFACE LABELING

Vesicle Diam. / Ab	≥ 5000 Å		3000-5000 Å	
	# Labeled / Total	% Labeled	# Labeled / Total	% Labeled
Anti-AcChR #1-4,6	2/58	3 ± 3%	3/78	4 ± 3%
Anti-AcChR #5	39/50	78 ± 10%	13/51	25 ± 3%
Anti-BuTx	2/52	4 ± 3%	1/57	2 ± 2%
NRG	0/51	0%	0/42	0%

Figure 1

Titration curve showing the amount of anti-rabbit IgG needed to stabilize 1 ml of colloidal gold against flocculation. The uptake saturates at ∿ 0.8 µg of IgG per ml of gold.

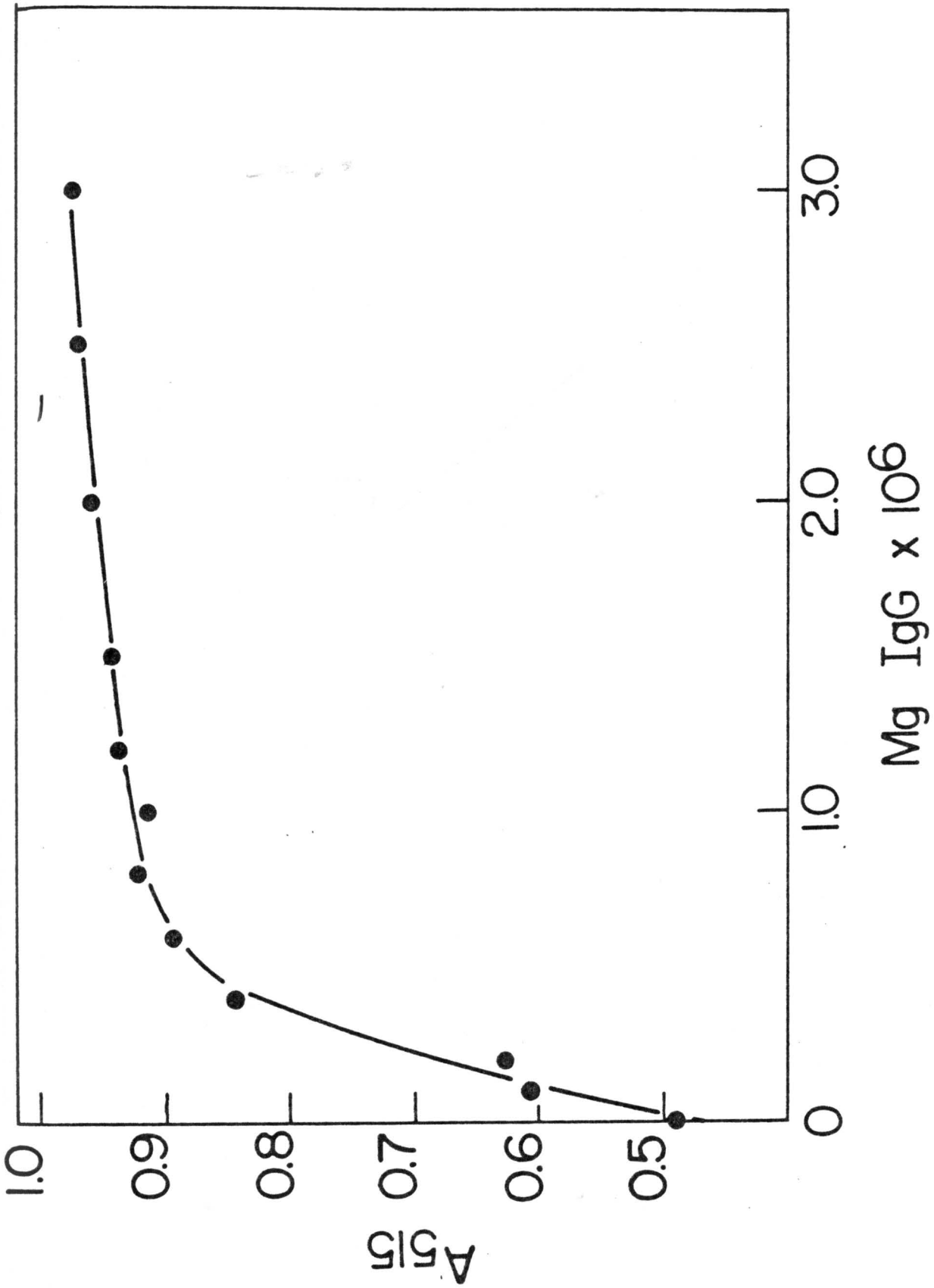

1.0
0.9
0.8
0.7
0.6
0.5
A515
0
1.0
2.0
3.0
Mg IgG x 10⁶

Figure 2

Anti-AcChR-gold labeling of AcChR-enriched membrane fragments. In thin-section, the vesicles appear as hollow circles, while the extremely electron-dense gold particles (G) are clearly visible. Occasionally, the gold spheres appear aligned along a membrane (arrow), but they are generally present in clusters among the vesicles. X90,000.

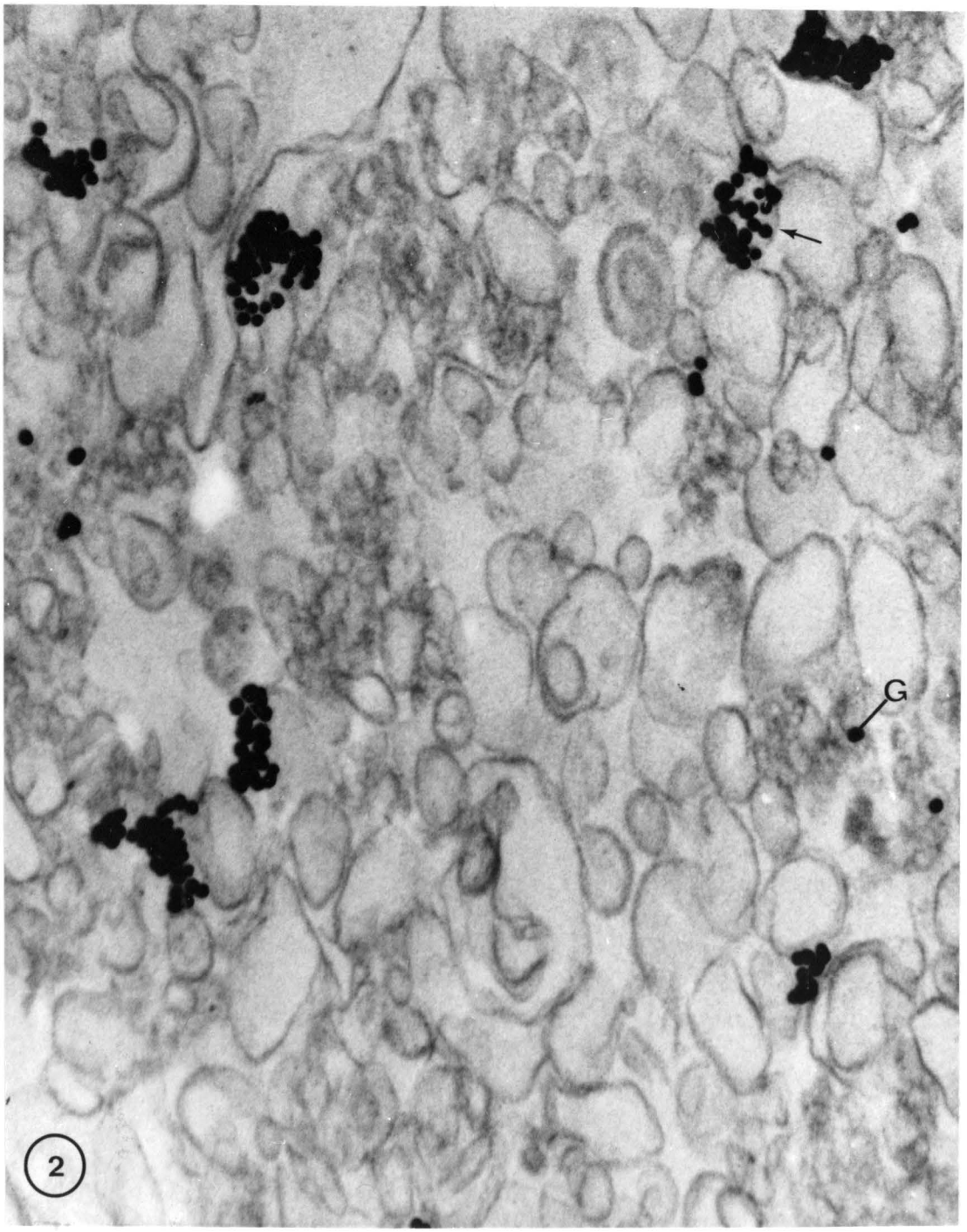
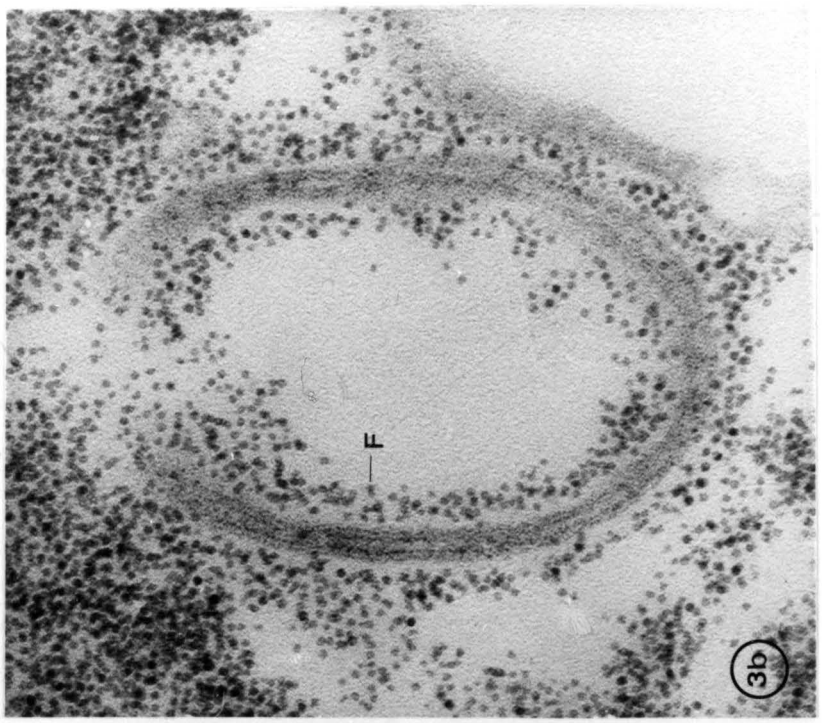

G
2

Figure 3

Anti-AcChR labeling of membrane vesicles visualized in thin-sections by ferritin coupled to anti-rabbit IgG. (a) Labeling of intact vesicles. The ferritin (F) is dispersed around the outsides of most, but not all of the membrane vesicles. X78,000. (b) Labeling of vesicles after osmotic shock and resuspension in NPE buffer. The double bilayer of the collapsed vesicle can clearly be seen, labeled on the extracellular side with ferritin (F). X180,000.


F
3b

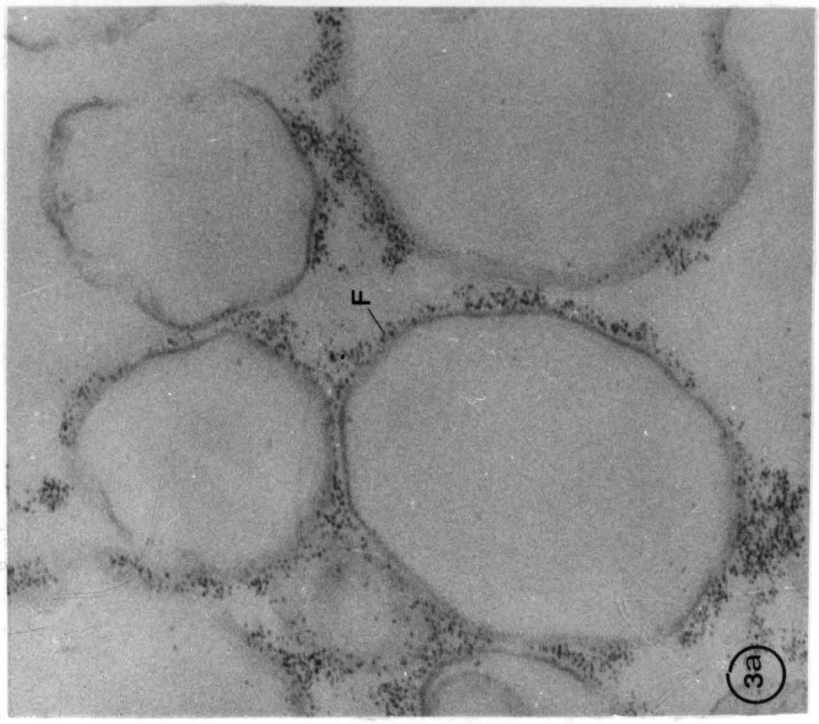
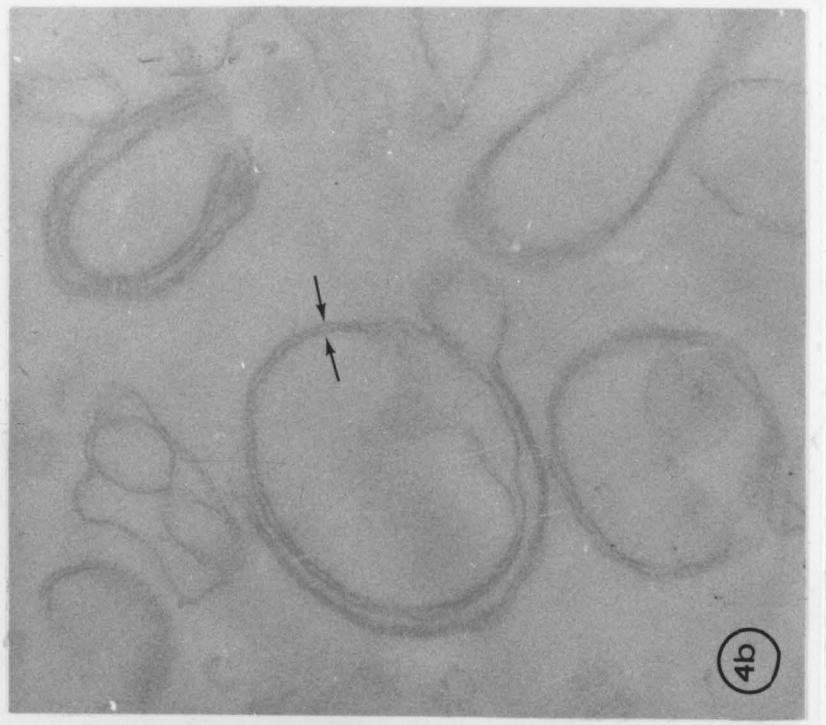
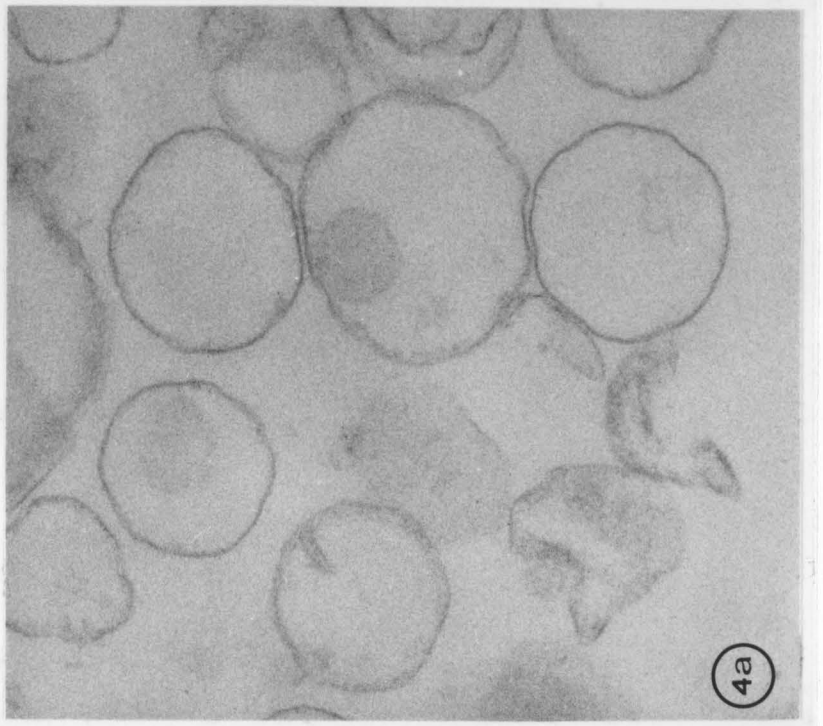

F
3a

Figure 4

Comparison of intact vesicles before and after osmotic shock. (a) Unshocked vesicles appear in thin section as hollow spheres, outlined by a single membrane bilayer. x 55,000. (b) After osmotic shock and resuspension in NPE buffer, vesicles assume a variety of shapes, usually with a double membrane bilayer (arrows). x 55,000. Compare the thickness of the single bilayer in (a) ($\sim$ 80 Å) with that of the double bilayer in (b) ($\sim$ 250 Å).



4b



4a

Figure 5

Elution profile of Sepharose 2B chromatography on Hcy/A. The Hcy/A conjugate is in fractions 16-23, with the uncoupled Protein A following in fractions 27-32. (●) indicates absorbance at 280 nm and (■) the [^{125}I]-Protein A.

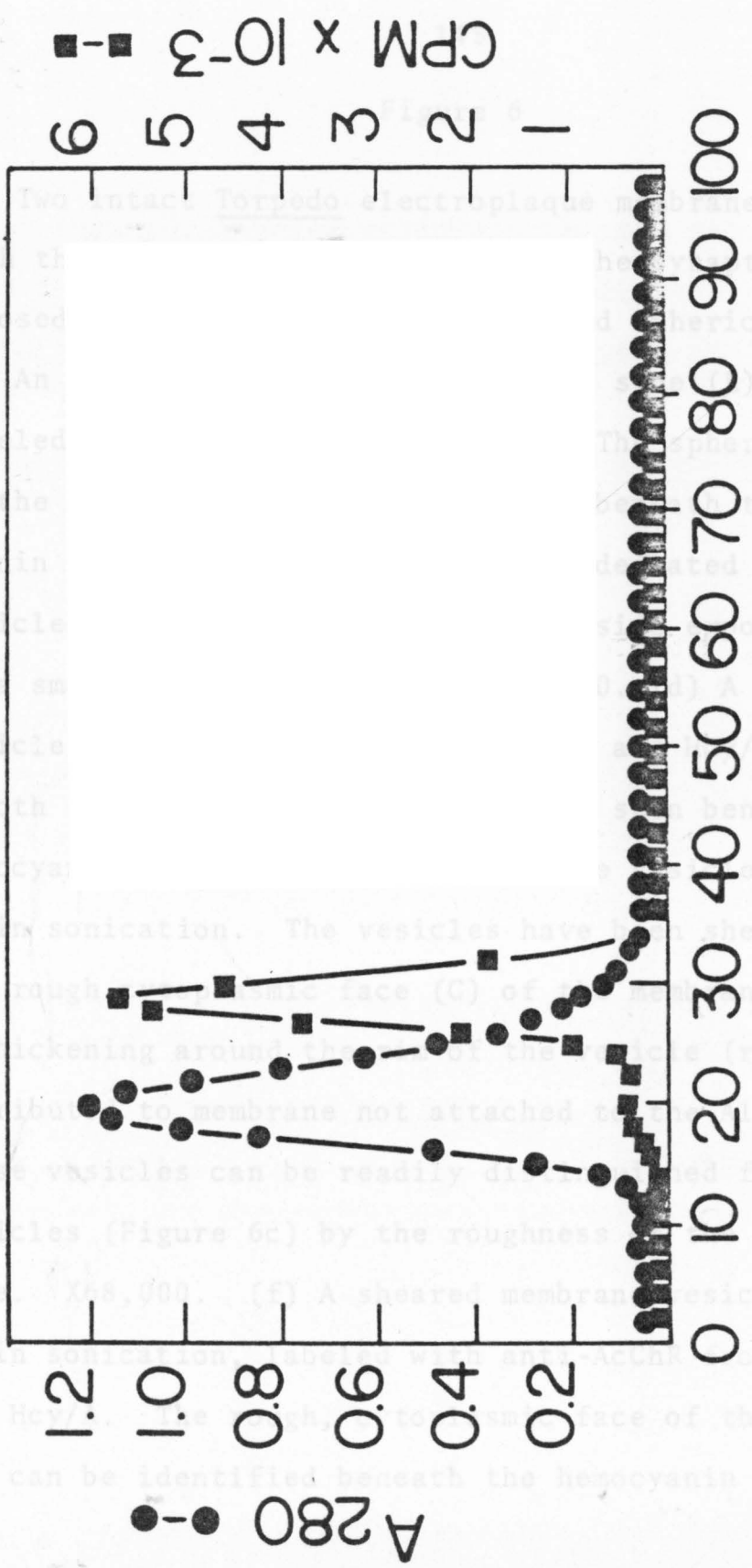

A280 ●-●
1.2
1.0
0.8
0.6
0.4
0.2
0 10 20 30 40 50 60 70 80 90 100
FRACTION NUMBER
CPM x 10⁻³ ■-■
6 5 4 3 2 1

Figure 6

(a) Two intact Torpedo electroplaque membrane vesicles, with the extracellular side (E) of the synaptic membrane exposed. The vesicles are smooth and spherical. X60,000 (b) An intact vesicle, extracellular side (E) exposed, labeled with anti-AcChR and Hcy/A. The spherical shape of the vesicle can be distinguished beneath the hemocyanin markers (h). X50,000. (c) A deflated membrane vesicle (D), with its extracellular side exposed, appears as a smooth but flat circle. X43,000. (d) A deflated vesicle (D), labeled with anti-AcChR and Hcy/A. The smooth surface of the vesicle can be seen beneath the hemocyanin markers (h). (e) Membrane vesicles after 5 min sonication. The vesicles have been sheared, leaving the rough cytoplasmic face (C) of the membrane exposed. A thickening around the rim of the vesicle (r) can be attributed to membrane not attached to the Alcian blue. These vesicles can be readily distinguished from deflated vesicles (Figure 6c) by the roughness of the cytoplasmic face. X68,000. (f) A sheared membrane vesicle after 5 min sonication, labeled with anti-AcChR from rabbit #5 and Hcy/A. The rough, cytoplasmic face of the membrane (C) can be identified beneath the hemocyanin markers (h).

These sheared, labeled vesicles can be easily distinguished from intact, labeled vesicles and deflated, labeled vesicles, as can be seen by a comparison of (b), (d) and (f). X50,000.

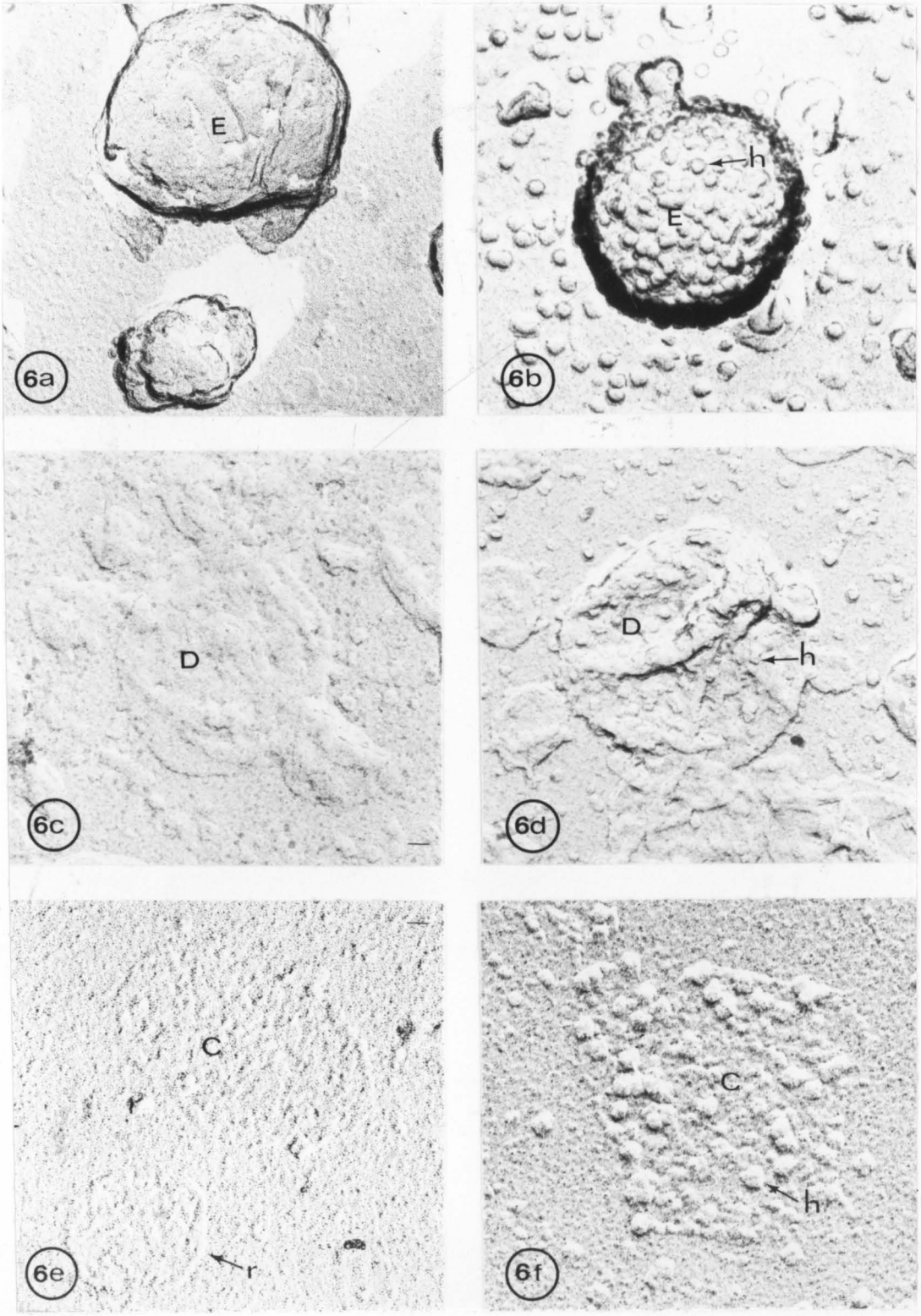

E
6a
h
E
6b
D
6c
D
h
6d
C
r
6e
C
h
6f

Figure 7

Vesicles treated with α-BuTx followed by anti-BuTx and Hcy/A. (a) Hemocyanin molecules (h) can be seen as round or square particles on the external surface (E) of the larger intact vesicles. The small vesicles are not labeled and are probably lipid vesicles. A deflated vesicle (D), can be identified by its distinctive shape and smoothness. X31,000. (b) After sonication, the vesicles are sheared, exposing their cytoplasmic sides (C). Hemocyanin molecules (h) appear in the background but only one can be detected at the edge of a vesicle. X49,500.

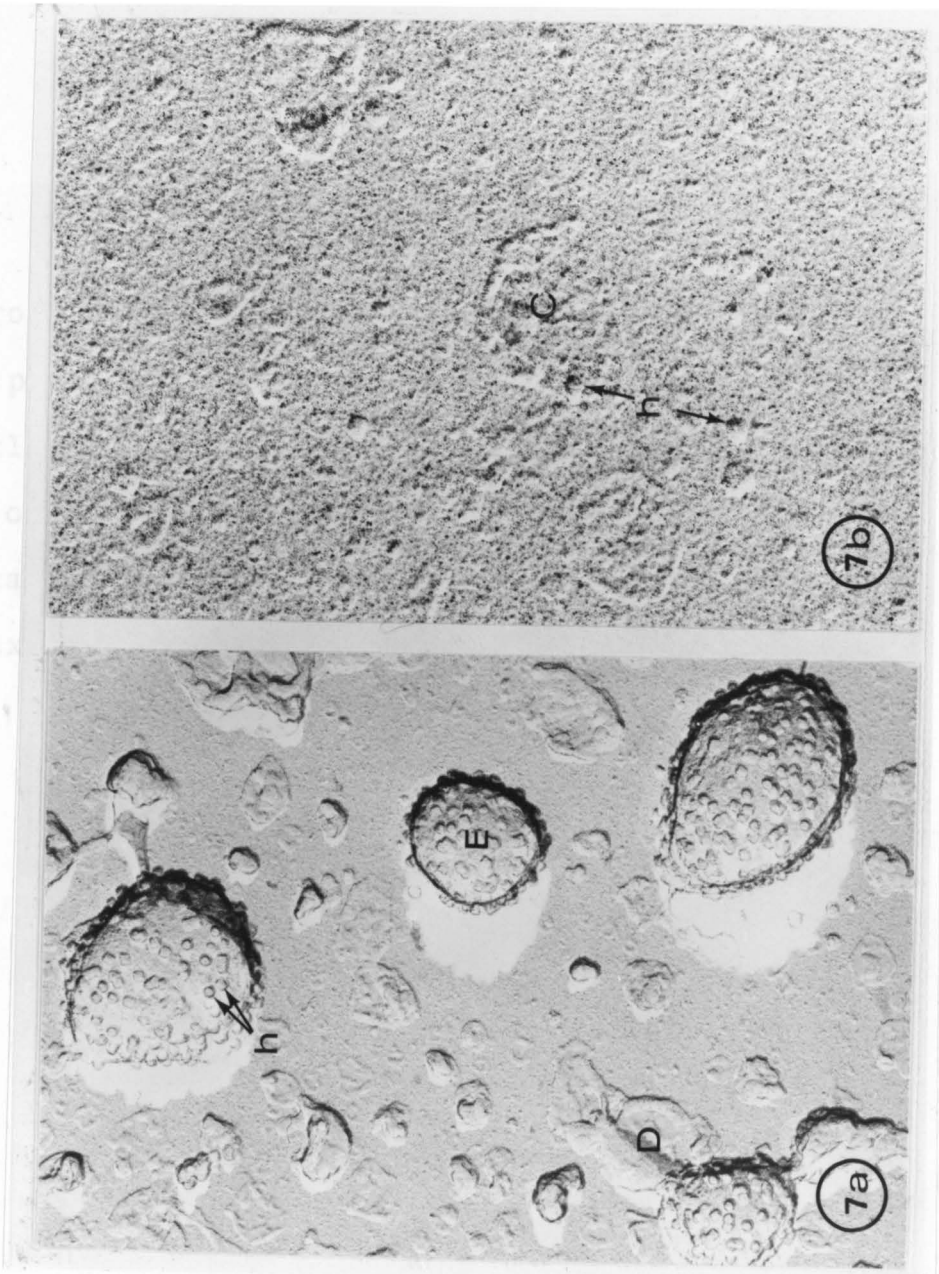

C
f
7b
E
h
D
7a

Figure 8

Control experiment in which vesicles were treated with pre-immune rabbit Ig and Hcy/A. (a) With intact vesicles there was no labeling of the extracellular side of the membrane (E). X50,000. (b) After sonication, the cytoplasmic side of the membrane (C) was exposed and did not label. X81,000.

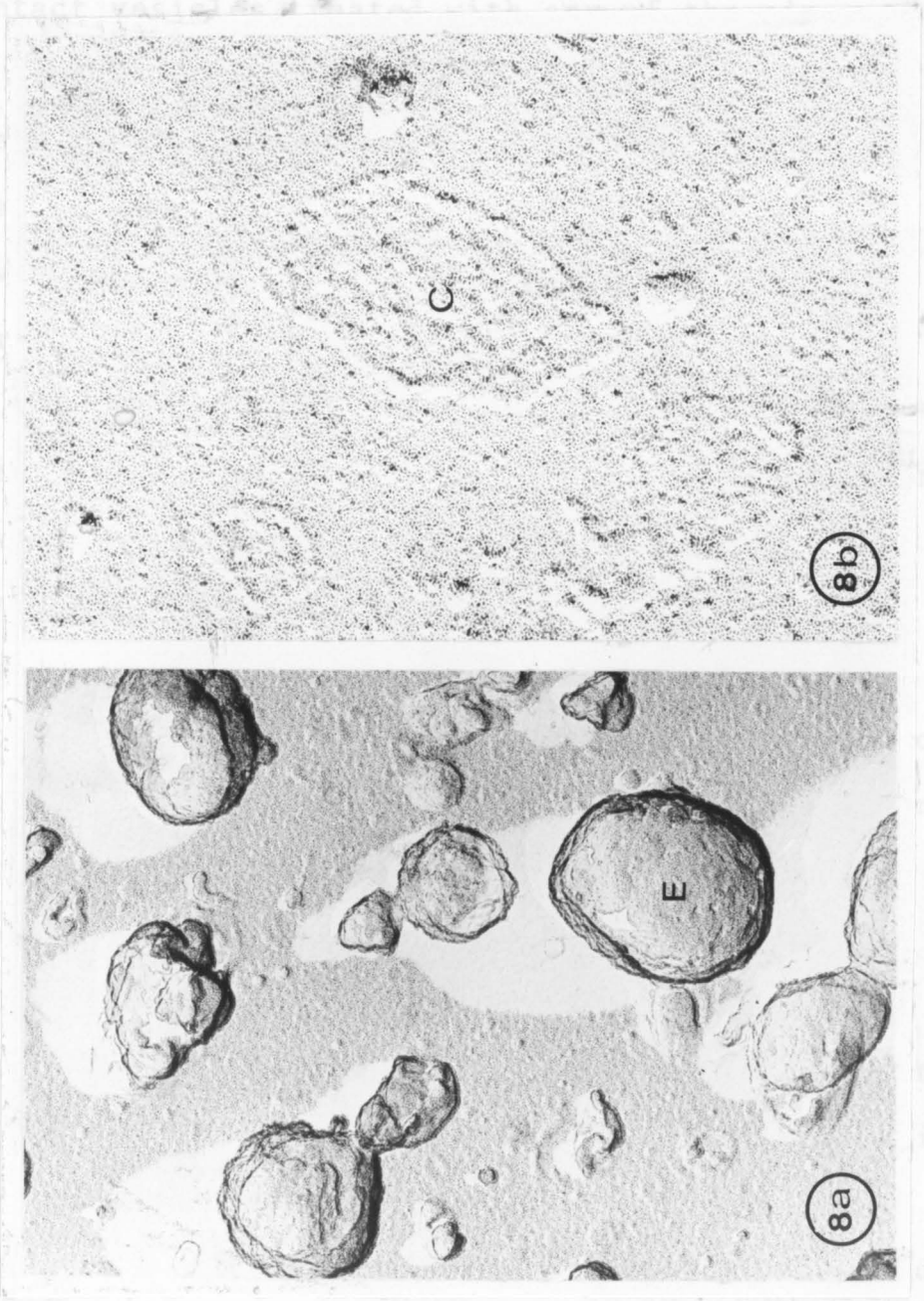
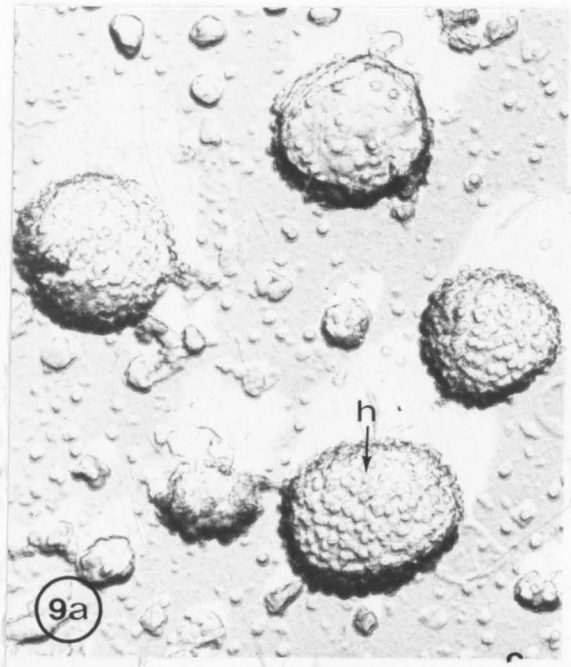
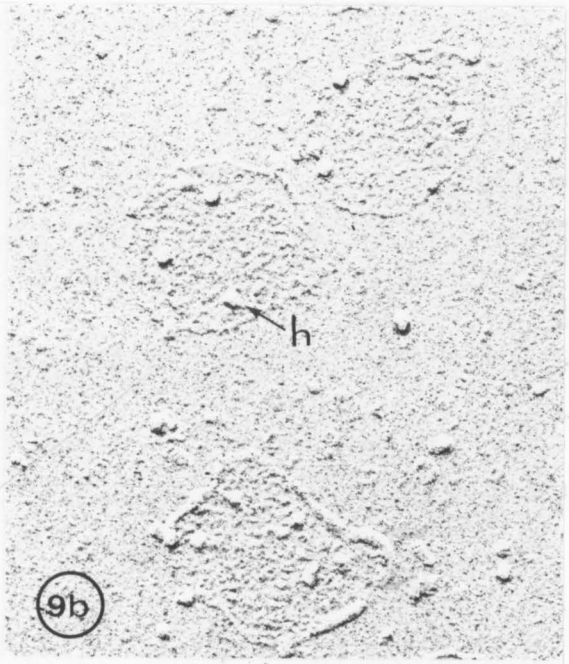

C
8b
E
8a

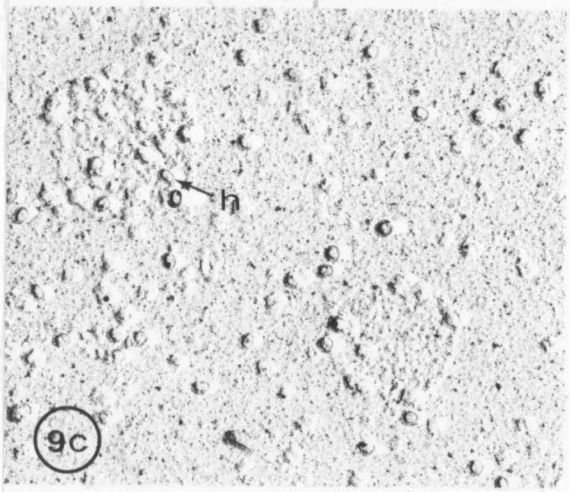
Figure 9

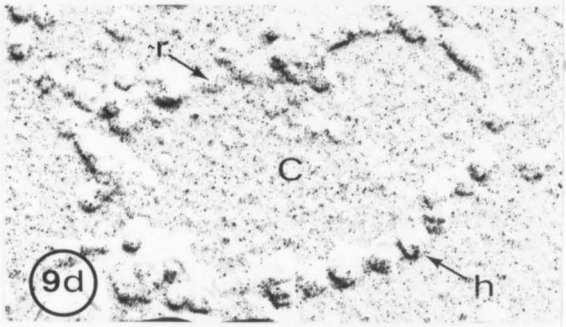
Vesicles treated with anti-AcChR followed by Hcy/A. (a) Intact vesicles treated with any of the six anti-AcChR preparations were labeled with hemocyanin (h) on their external surfaces. As in Figure 7 most of the larger vesicles were labeled, while the smaller ones were not. X31,000. (b, c, d) Vesicles after sonication, treated with anti-AcChR from rabbits #1, 2, 3, 4, or 6. (b) Few hemocyanin molecules (h) can be seen on the cytoplasmic side of the membrane or on the background. X37,000. (c) An increase in the hemocyanin (h) labeling of the cytoplasmic membranes corresponds to an increase in the hemocyanin in the background. X43,000. (d) The cytoplasmic face (C) of the sheared vesicle can be seen with thickening around the free edges (r). The entire rim is labeled with hemocyanin molecules (h), with none in the center. X66,000. (e, f) Vesicles after sonication, treated with anti-AcChR from rabbit #5. (e) A close-up of one vesicle shows the characteristic rough cytoplasmic side of the membrane heavily labeled with hemocyanin (h). X47,000. (f) A view of a larger field shows sheared vesicles with their cytoplasmic sides exposed (L).

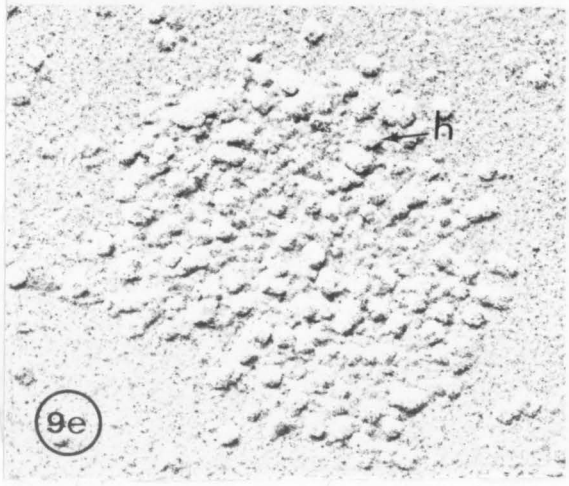
A large vesicle is labeled with hemocyanin (h), while several smaller vesicles are unlabeled (U). X22,000


h
9a


h
9b


h
9c


r
C
h
9d


h
9e

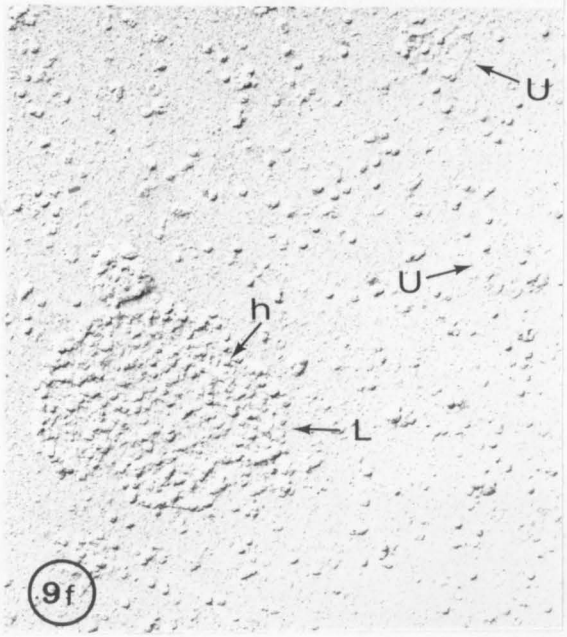
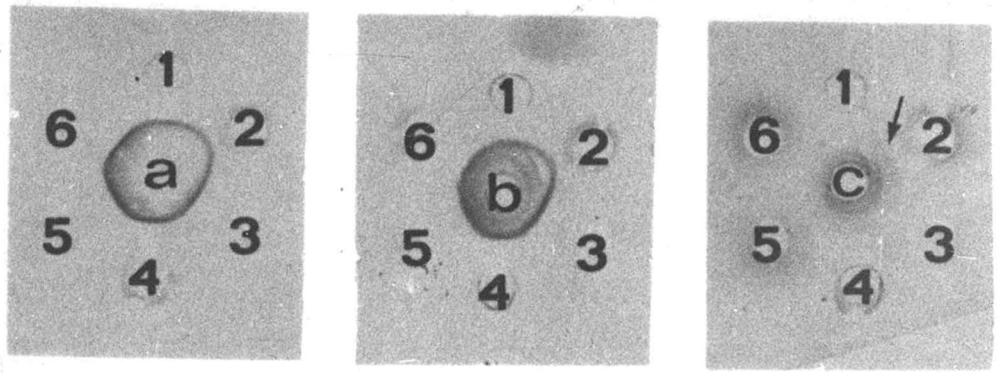

U
U
h
L
9f

Figure 10

Ouchterlony immunodiffusion assays. The numbers 1-6 indicate the wells which were filled with anti-AcChR Ig from rabbits 1-6. The antigens in the center wells were (a) AcChR, (b) MFTS, and (c) MFTS-R. The arrow points out the faint cross-reaction observed between MFTS-R and Ig from rabbit #2.


1
6
2
a
5
3
4
1
6
2
b
5
3
4
1
6
2
c
5
3
4

Chapter III: Topographic Studies of *Torpedo* Acetylcholine Receptor Subunits as a Transmembrane Complex

INTRODUCTION

Excitable membrane vesicles highly enriched in the acetylcholine receptor (AcChR) have been purified from the electric organs of several species of electric fish. These membrane preparations possess the properties of nicotinic postsynaptic membranes: they bind α-neurotoxins (Cohen, et al, 1972; Duguid and Raftery, 1973) and cholinergic ligands (Raftery, et al, 1974a; Weber and Changeux, 1974), and they possess distinct binding sites for local anesthetics (Weber and Changeux, 1974; Cohen, et al, 1974; Schimerlik and Raftery, 1976; Blanchard, et al, 1979a) and the alkaloid histrionicotoxin (Elliott and Raftery, 1977; Eldefrawi, et al, 1977). Binding of cholinergic agonists results in the flux of inorganic cations through the vesicular membrane (Kasai and Changeux, 1971; Clark, et al, 1972; Hess and Andrews, 1977; Miller, et al, 1978) and the demonstration of such flux through membrane preparations containing only the AcChR protein (Moore, et al, 1979) suggests that the AcChR functions as a cation translocating protein complex.

Detergent extracted, chromatographically purified Torpedo californica AcChR sediments both as a monomer of

9S and as a 13.7S dimer (Raftery, et al, 1972). Except for one account (Sobel, et al, 1977), both species have been found to consist of four subunits with molecular weights of 40,000, 50,000, 60,000, and 65,000 daltons (Raftery, et al, 1974b; Weill, et al, 1974; Raftery, et al, 1974; Chang and Bock, 1977; Lindstrom, et al, 1978; Vandlen, et al, 1979; Froehner and Rafto, 1979; Elliott, et al, 1980; Raftery, et al, 1980), with the binding sites for α-neurotoxins, agonists, and some antagonists located on the 40,000 dalton subunit (Chang, et al, 1977; Witzemann and Raftery, 1977; Damle, et al, 1978; Moore and Raftery, 1979; Witzemann, et al, 1979). These four subunits were found to possess a high incidence of amino acid sequence homology (Raftery, et al, 1980) and to exist in both membrane-bound (Raftery, et al, 1980) and detergent-solubilized (Lindstrom, et al, 1979; Raftery, et al, 1980) receptor as a pentameric complex with a stoichiometry of 2:1:1:1. This stoichiometry dictates a molecular weight of 225,000 daltons (Martinez-Carrion, et al, 1975; Hucho , et al, 1978; Reynolds and Karlin, 1978). In light of this molecular definition of the AcChR and its defined function alluded to above, it is of interest to determine the topography of its subunits in the membrane and eventual assignment

of a specific function to each polypeptide.

Using antibodies to purified AcChR, it has been found at the electron microscopic level by both thin-sectioning (Tarrab-Hazdai, et al, 1978) and by examination of replicas of intact and sheared membrane vesicles (Strader, et al, 1979; Chapter II of this Thesis) that the receptor is a transmembrane protein, being exposed on both the cytoplasmic and the extracellular surfaces of electroplax membrane vesicles. The evidence that the antigenic determinants exposed on the extracellular face of the synaptic membrane were more prominent than those exposed on the cytoplasmic face of the membrane (Strader, et al, 1979; Chapter II of this Thesis) concurs with the findings of Ross, et al (1977), who reported that the protein present in Torpedo membrane preparations extended 55 Å on one side of the bilayer, while protruding only 15 Å on the other side. Which of the four AcChR subunits are exposed on either face of the membrane could not be determined by immuno-electron microscopy due to the failure of antibodies to individual subunits, isolated from preparative SDS gels, to cross-react with membrane-bound receptor.

Evidence for the exposure of all four AcChR subunits on the external surface of the membrane has been obtained. With lactoperoxidase catalysed iodination of AcChR-enriched membrane surfaces, the 40,000, 50,000, and 60,000 dalton subunits were labeled and, thus, exposed on the external synaptic surface of the membrane; the 65,000 dalton subunit was labeled very little under these conditions (Hartig and Raftery, 1977). Both the 40,000 and 65,000 dalton subunits were labeled with a photo-labile derivative of α-bungarotoxin (α-BuTx) however, indicating their exposure on the exterior membrane surface (Witzemann and Raftery, 1978). Lindstrom, et al (1978) have shown that antibodies to each of the AcChR subunits cross-react with rat muscle receptors in vivo, causing a decrease in muscle AcChR content, demonstrating that each of these four polypeptide chains must contain at least some antigenic determinants exposed on the extracellular face of the membrane.

In studies described here, an investigation of the topography of the AcChR molecular complex in the post-synaptic membrane is presented. Using tryptic degradation of the extracellular and cytoplasmic membrane surfaces separately, it has been determined that all four of the AcChR subunits span the membrane bilayer and

are exposed on both faces of the postsynaptic membrane. These results are discussed in terms of a possible model for the disposition of the AcChR complex in its membrane environment.

MATERIALS AND METHODS

Membrane fragments enriched in AcChR were prepared from Torpedo californica electric organs by the method of Elliott, et al (1980). For further enrichment in AcChR, the membranes were extracted with base (Steck and Yu, 1973; Neubig, et al, 1979; Elliott, et al, 1980) and resuspended in 10 mM Tris, pH 7.4, giving a preparation containing approximately 4 nmol α-BuTx sites per mg protein. Intact, right-side out vesicles enriched in AcChR (0.5-1 nmol α-BuTx binding sites per mg total protein) were prepared in NTC buffer (50 mM Tris, 0.35M NaCl, 5 mM $CaCl_2$, pH 7.4) by the method of Hartig and Raftery (1979). The "sidedness" of the vesicles was determined by the α-BuTx sites assay described by Hartig and Raftery (1979), using NTC buffer for determinations on the intact vesicles and 10 mM Tris, pH 7.4 for the base extracted membrane vesicles.

The concentration of α-BuTx sites was determined according to Schmidt and Raftery (1973) using DEAE-cellulose filter discs and [^{125}I] α-BuTx (Clark, et al, 1972; Blanchard, et al, 1979b). Protein concentration was determined by the method of Lowry, et al (1951), using bovine serum albumin as the standard. SDS polyacrylamide gel electrophoresis was done according to Laemmli (1970) with a 10% acrylamide, 0.27% bisacrylamide separating gel and a 3% acrylamide, 0.08% bisacrylamide stacking gel. After staining with Coomassie brilliant blue (CBB), gels were scanned with a Gilford linear transport unit at 550 nm.

For negative staining, membrane vesicles were diluted to 1 mg/ml. One drop of this membrane suspension was incubated on a carbon-coated copper grid for 1 min, and the excess removed with filter paper. Grids were then stained with 1 drop of 2% phosphotungstic acid, pH 7.4, for 5 min, dried, and examined with a Phillips 201 electron microscope.

Transglutaminase (TGase) from guinea pig liver was the generous gift of David Gard, prepared by the method of Connellan, et al (1971). TGase activity was determined by the method of Folk and Cole (1966), using CBZ-L-glutaminyl glycine and hydroxylamine HCl.

Exposure of Membrane Surfaces on Positively Charged Beads:

Polylysine-coated polyacrylamide beads were prepared by the method of Cohen, et al (1977). 200 mg polylysine was coupled to 5g Biogel P-2 polyacrylamide beads, which had previously been extensively washed, in 2.6 M pyridine with carbodiimide for 60 hrs at room temperature. After thorough washing by multiple centrifugations, the beads were resuspended in buffer and stored at 4°C for up to several months. Alternatively, Affi-gel 731, a positively charged derivative of polyacrylamide beads, was purchased from Bio-Rad.

For attachment of intact vesicles to beads, the beads were first washed in NTC buffer, then membrane vesicles were added in a volume approximately equal to that of the packed beads, and the suspension mixed by gentle inversion for 30 mins at room temperature. Too vigorous mixing caused the breakage of membrane vesicles or their removal from the beads. After washing to remove unattached membranes, the interior surfaces of the vesicles were exposed by shearing away the portions of the membranes not attached to the surface of the beads as follows. The beads, in a plastic test tube, were suspended in a volume of buffer approximately five times that of the packed beads, the test tube suspended in a bath sonicator, and sonicated at 80 kHz for 5 mins, with stirring every 1 min

to prevent settling of the beads. After washing to remove the free membranes, the beads, coated with the exposed interior surfaces of membrane vesicles, were labeled as described below. To remove the attached membrane fragments from the beads, the beads were vortexed vigorously for 2 mins with 1 M K_2HPO_4; this step was repeated twice more, before these membrane fragments were pelleted by centrifugation and resuspended in a small volume of buffer. For quantitation of the binding to and removal from the positively charged beads of the membrane vesicles, membrane-bound AcChR was incubated with a small amount ($\sim$ 10% of its total binding capacity) of [^{125}I] α-BuTx for several hours before mixing with the beads.

Transglutaminase-Catalyzed Labeling of Membrane Proteins:

(a) Dansyl cadaverine labeling of the exterior surface of membrane vesicles - Intact vesicles (0.15 mg of membrane protein) and TGase (0.012 mg) were mixed in 0.3 ml NTC buffer, containing 5 mM DTT and 5 mM dansyl cadaverine. After incubation for 2 hrs at 37°C, the reaction was quenched with the addition of 10 mM EGTA, and the membrane fragments washed by centrifugation and prepared for gel electrophoresis.

(b) Dansyl cadaverine labeling of the interior surface of membrane vesicles - Intact vesicles (1-2 mg) were attached to polylysine coated beads (∿ 1 ml packed beads) and sheared by sonication, as described above, for exposure of the interior face of the vesicles to the labeling reagents. After washing, ∿ 0.4 mg of protein remained on the beads. The beads were incubated with TGase (28 μg) and dansyl cadaverine (8 mM) in 1 ml NTC, containing 10 mM DTT, for 2 hrs at 37°C. During the incubation period, the beads were suspended by gentle inversion every 10 mins. After 2 hrs, the reaction was quenched by the addition of 10 mM EGTA, the beads washed, and the membrane fragments removed from the beads with 1 M K_2HPO_4, as described above. After centrifugation, the membranes were dissolved in gel sample buffer and electrophoresed on SDS gels.

Control samples were prepared with the omission of TGase from the mixture, or consisted of intact vesicles which had been labeled as above with non-dansylated cadaverine before attachment to the beads. As an alternative to attached of the membrane vesicles to the beads, interior labeling was performed by the addition of TGase, dansyl cadaverine, and DTT to intact vesicles in a Ca^{++}-Tris buffer withonly 65% the osmolarity of NTC. After

loading the reagents into the vesicles by osmotic shock, the membrane vesicles were resuspended in a buffer of the same osmolarity, containing 10 mM EGTA, to prevent labeling of the exterior surface of the vesicles.

After electrophoresis, gels were photographed on an ultraviolet light box with a Polaroid camera equipped with an orange filter, with exposure times ranging from 2-5 mins. After the most prominent bands were marked with India ink, the gels were stained with CBB and the fluorescent bands identified.

(c) [^{3}H]-Putrescine labeling of membrane surfaces - Labeling of the exterior and interior surfaces of intact vesicles was performed as described above for dansyl cadaverine labeling, except that 1.5 µmol of [^{3}H]-putrescine (0.1 mCi) was substituted for the dansyl cadaverine for 0.15 mg of membrane protein. After electrophoresis and staining, tritium-labeled protein bands were identified by fluorography (Bonner and Laskey, 1974; Laskey and Mills, 1975). Briefly, the gel was rocked in two changes of DMSO for 1 hr, then in 2,5-diphenyloxazole (22.2g) in 100 ml DMSO for 3 hrs, then washed in two changes of H_2O for 1 hr before being dried on a sheet of filter paper. After drying, the gel was exposed to X-ray film, which had been "pre-flashed"

with a brief pulse of light to heighten its sensitivity, at -80°C for 2-7 days. The labeled proteins appeared as sharp dark bands on the developed film.

For opening and resealing of membrane vesicles, base-treated membrane fragments (1 mg/ml) were subjected to two cycles of freezing and thawing by the method of Moore and Raftery (1980), with modification, as follows. 40 µl of membrane fragments in a small pyrex tube were frozen in liquid nitrogen for 5 mins, then thawed at 4°C for 1 min. The second cycle was performed immediately after the first thawing was complete. The sidedness of the vesicles was determined using the α-BuTx sites assay described by Hartig and Raftery (1979) for preparations of intact vesicles, except that the buffer used here was 10 mM Tris, pH 7.4.

Tryptic Degradation of the Exterior Surface of Membrane Vesicles:

After two cycles of freezing and thawing, membrane vesicles (1 mg/ml) were treated with trypsin (100 nM) for two hrs at 37°C. Following this soybean trypsin inhibitor was added to a final concentration of 50 µM

and the sample heated in gel sample buffer, containing SDS and β-mercaptoethanol for electrophoresis. A control sample, in which both the trypsin and the inhibitor were added before the freezing and thawing cycles, was included in each experiment. To measure the time course of the digestion, samples were incubated at 37°C for times ranging from one min to two hrs; at the appropriate time, inhibitor and gel sample buffer were added and the sample heated (100°C for three mins) and frozen at -20°C until electrophoresis was performed.

Tryptic Degradation of the Interior Surface of Membrane Vesicles:

Membrane vesicles (1 mg/ml) were added to trypsin (100 nM) at 4°C and immediately frozen in liquid nitrogen for five mins. After thawing at 4° for $\leq$ 1 min the freezing was repeated. Soybean trypsin inhibitor was added (50 μM) immediately upon completion of the second thawing, and the sample incubated at 37°C for two hrs. As a control each time, a sample was treated as above with trypsin, frozen and thawed twice, treated with inhibitor, and then immediately mixed with gel sample buffer and frozen until electrophoresis was begun. The time course of the tryptic degradation of the interior surface

was measured by quenching with gel sample buffer at the appropriate time, as described above for degradation of the exterior surface.

RESULTS

Intact vesicles, prepared in NTC buffer by the method of Hartig and Raftery (1979), were determined to be oriented 94-100% "outside-out"; i.e., with 94-100% of the α-BuTx binding sites located on the vesicle exterior. The TGase used in these studies had a specific activity of 0.13 U/mg, where 1 U was defined by Folk and Cole (1966) as the amount of enzyme needed to catalyze the formation of 0.5 μmol of hydroxamate per minute. This specific activity is low when compared to freshly prepared enzyme (preparations of which commonly have specific activites of ∿ 2 U/mg) but was sufficient when larger amounts of the enzyme were used for labeling.

Use of [^{125}I] α-BuTx-labeled membrane preparations permitted quantitation of the attachment of membrane vesicles to the positively-charged beads, as summarized in Table I. When an excess of membrane-bound AcChR was incubated with 0.2 ml of packed beads for 30 mins in NTC buffer, 52 μg of protein was attached to the beads.

Shearing the vesicles by sonication for 5 mins caused the removal of 20 µg (39%) of this protein, leaving 32 µg (61%) attached to the beads. Of this, 55% (17 µg) could be removed by vortexing in 1M K_2HPO_4, leaving 45% (15 µg) permanently attached to the beads. These results agree in general with those of Jacobson and Branton (1977), who first used this technique for the exposure of the cytoplasmic surface of erythrocyte membranes and their subsequent isolation.

This technique, then, represents the in-solution extension of the method used in Chapter II for the immuno-labeling of the extracellular and cytoplasmic faces of the postsynaptic membranes. After intact vesicles, oriented 94-100% extracellular-side-out, are immobilized on the beads and sheared, their cytoplasmic membrane faces are exposed to the labeling reagents. Labeling the interior and exterior faces of the membrane vesicles prepared by this method with TGase-catalyzed covalent attachment of dansyl cadaverine gave the results shown in Figure 1. When the labeling reagents were in contact with only the exterior surface of intact vesicles, the labeling pattern was that seen in track B. After staining, the fluorescent bands were

shown to correspond to the CBB stained band at 90,000 daltons, a faintly staining one at ∿ 64,000 daltons, and sharp bands at ∿ 55,000, 50,000, 47,000, 45,000, 40,000, 35,000 daltons. The 35,000 dalton band was identified as a degradation product of TGase, the 47,000 dalton band as actin (Strader, et al, 1980; Chapter IV of this thesis), and the 40,000 and 50,000 dalton bands as subunits of the AcChR. In addition, there was a less distinct fluorescent band in the range of ∿ 60,000 daltons, which probably corresponded to the AcChR subunit of that molecular weight. The band at ∿ 64,000 daltons did not correspond to the 65,000 dalton subunit of the AcChR; during the several times this experiment was repeated, the 65,000 dalton subunit was never labeled from the vesicle exterior.

When the TGase-catalyzed dansyl cadaverine labeling was performed on membrane fragments attached to beads with their interior (cytoplasmic) faces exposed, a different labeling pattern was observed (track C on Figure 1). The major fluorescent bands were those at 90,000, 65,000, 60,000, and 40,000 daltons, with a less distinct band centered around 50,000 daltons. These bands corresponded to the subunits of the AcChR, with the additional band at 90,000 daltons, which has frequently

been observed in Torpedo membrane preparations, but the identity of which is unknown.

A comparison of tracks B and C shows that, of the four AcChR subunits, the 40,000 dalton chain was labeled in both preparations, the 50,000 labeled from the exterior and less distinctly from the interior, the 60,000 labeled from the interior and less distinctly from the exterior, and the 65,000 dalton chain labeled only from the membrane interior.

Examination of track A, in which TGase was omitted from the labeling reaction on intact vesicles reveals a labeling pattern slightly fainter, but essentially identical to that observed in track B, indicating that the labeling was not specifically TGase-catalyzed. This result was not reproducible, however; in many experiments, little or no labeling was observed upon omission of TGase, as is shown in Figure 2, track A. In Figure 1, track D, the intact vesicles were labeled with cadaverine before attachment to the beads. A comparison of tracks C and D shows that this prelabeling of the exterior surface of the vesicles had no effect on the interior labeling observed. In Figure 2, tracks B and C, are shown intact vesicles labeled with dansyl cadaverine both with (B) and without (C) prelabeling with cadaverine. It

can be seen that the prelabeling accounts for such a small degree of the total labeling that fluorescent labeling is unaffected by this treatment. Thus no conclusion as to the specificity of the labeling reaction can be drawn from these data.

In addition to the specific labeling of protein bands noted on the gels, fluorescence was observed at the top and bottom of all the gels. These bands were attributed to high molecular weight aggregates, possibly resulting from TGase-induced crosslinking, and to unbound dansyl cadaverine, respectively.

When TGase and dansyl cadaverine were loaded into intact vesicles by osmotic shock, very little labeling of protein bands was observed; this technique thus proved less useful than the mechanical shearing method for exposure of cytoplasmic membrane surfaces to the labeling reagents.

With the [^{3}H]-putrescine in the amounts used in these experiments, only very faint labeling was observed by fluorography. Much higher concentrations appear to be needed to take advantage of this potentially quantitative labeling method.

In experiments on base-extracted membranes, the membrane vesicle preparations were found, after two cycles of freezing and thawing, to be oriented such that 95% ± 5% of their α-BuTx binding sites were exposed to the external solution; i.e. only ∿ 5% of these vesicles were oriented with the extracellular (synaptic) surface of the postsynaptic membrane oriented inward. Neither the number nor the orientation of α-BuTx binding sites changed after two hrs at 37°C.

Preliminary experiments of degradation of membrane fragments with various concentrations of trypsin, quenching with various concentrations of trypsin inhibitor, and incubation at various temperatures led to the final experimental conditions described above. It was found that incubation with concentrations of greater than 100 nM trypsin resulted in an increased rate of degradation to the point where one min at 4°C was sufficient for significant degradation to occur. A slower rate was desired to permit thawing of the samples to which trypsin had been added to the interior before the addition of inhibitor to the outside with no significant degradation of the outer surface of the membrane occurring. Likewise, thawing at 4°C was used to avoid the noticeable degradation which occurred at room temperature or at 37°C in a period of one min. The large excess of inhibitor (50 μM

inhibitor for 100 nM trypsin) used to quench the hydrolytic reaction was necessary to obtain immediate quenching; with even 10 fold less inhibitor, a significant amount of hydrolysis occurred after inhibitor addition. Two cycles of freeze-thawing were found to be sufficient to load the trypsin into the vesicles; one cycle resulted in less complete degradation of AcChR subunits, but no further improvement was observed with three cycles.

When vesicles were treated with trypsin on the outside or inside surfaces and the protein electrophoresed on SDS gels, the results shown in Figure 3 were obtained. Neither the freeze-thawing process nor the incubation with trypsin plus inhibitor for two hrs at 37°C had any effect on the gel pattern (compare tracks A and E). When trypsin was added after the freeze-thawing, so that only the extracellular surface of the vesicles was exposed to the enzyme, all four AcChR subunits were degraded, as can be seen from the decrease in their staining intensity in track B. At the same time new bands appeared on the gel; a broad band at $\sim$ 45,000 daltons and sharper ones at $\sim$ 34,000 and $\sim$ 27,000 daltons. In a parallel experiment, trypsin was loaded into the vesicles by freeze-thawing and degradation of the exterior surface prevented by the addition of trypsin in-

hibitor to the thawed solution of resealed vesicles; in this way, only the cytoplasmic face of the postsynaptic membrane was exposed to trypsin. These results are shown in tracks C and D of Figure 3. In track C, gel sample buffer was added to the sample immediately after the addition of inhibitor. A comparison with track E shows that little degradation occurred during the thawing period, when trypsin but not inhibitor was present on both the inside and the outside the vesicles: only a small band at ∿ 34,000 daltons indicates any degradation at all, and none of the subunit bands decreased measureably in staining intensity. In track D, the results of a two hr incubation at 37°C with trypsin sealed inside the vesicles and trypsin-inhibitor complex present outside are shown. The staining intensities of the 65,000, 60,000, and 50,000 daltons bands decreased considerably, while that of the 40,000 dalton band decreased a little. New bands also appeared on the gel: a broad band at ∿ 48,000 daltons and sharp bands at ∿ 38,000, ∿ 37,000, ∿ 34,000, ∿ 33,000, and ∿ 31,000 daltons.

The time course of a typical experiment is shown in Figure 4. Tryptic hydrolysis of the protein on the extracellular surface of the vesicles (Figure 4A) proceeded at approximately the same rate for all four

subunits, with the most rapid proteolysis occurring during the first 15 mins. After this time, degradation of the 60,000 and 65,000 dalton subunits had almost reached its limit, while the 40,000 and 50,000 dalton subunits continued to disappear more slowly. Degradation of the protein on the cytoplasmic electroplaque membrane surface (Figure 4B) proceeded at a rapid initial rate, as well, with degradation of the 50,000, 60,000 and 65,000 dalton subunits being almost complete after five mins. The 40,000 dalton subunit showed little degradation during this period, however, and continued to disappear at a slow rate during the two hr incubation period. Although the quantitative data varied from one experiment to the next, with maximum digestion of the 60,000 and 65,000 dalton subunits on either face of the membrane being reached at 5-30 mins after the incubation was begun, the qualitative results remained consistent; a fast initial hydrolysis was observed for all four subunits, followed by the slower disappearance of the 40,000 and 50,000 dalton subunits, which was especially noticable for the interior surface of the membrane.

The time courses of tryptic hydrolyses on both sides of the membrane were determined on three separate preparations and averaged (each normalized to its control sample) in terms of the percentage of each subunit remaining at

a given time, as plotted in Figure 5. On the extracellular side of the membrane, all four subunits disappeared at roughly the same rate, with all reaching approximately the same level (27-33% of original) after two hours. On the cytoplasmic face of the membrane, however, there were marked differences in the rates and degrees of degradation of the four subunits. The 65,000 dalton subunit was the most thoroughly degraded; only 12% of the original CBB-staining material remained after two hrs. Hydrolysis of the 60,000 dalton subunit was almost as complete, with 20% remaining after two hrs. The 50,000 dalton subunit disappeared more slowly, with 48% of the original remaining after digestion for two hrs, and the 40,000 dalton subunit was removed even more slowly, with 70% remaining after two hrs.

To investigate possible ultrastructural disruption during the tryptic treatment, samples of untreated membranes, controls with trypsin and inhibitor added simultaneously to membrane vesicles, and membranes degraded by trypsin from either the interior or the exterior were examined bynegative-stain electron microscopy. Membranes in all four samples appeared mostly in the form of

spherical vesicles covered with the rosette-shaped structures previously identified as AcChR molecules. In the untreated membranes and in the control sample, the rosettes were regularly spaced over the entire vesicle surface (Figure 6A and D). On vesicles treated with trypsin on either the exterior (Figure 6B) or the interior (Figure 6C), however, the rosettes were present on the membrane surface in clusters, separated by patches of bare membrane. This phenomenon, similar to that observed by Klymkowsky, et al (1980) was more pronounced for samples degraded from the outside than for those degraded from the inside; in neither case was any erosion of the rosette structure itself observed.

DISCUSSION

Transglutaminase-catalyzed dansyl cadaverine labeling of the extracellular and the cytoplasmic membrane surfaces, separately, of AcChR-enriched membrane vesicles showed differential labeling of the four subunits of the AcChR on the two membrane faces. As demonstrated in Figure 1, the 40,000 dalton subunit was labeled from both sides of the membrane and the 65,000 dalton subunit only from the interior surface of the vesicles, with the

labeling of the 50,000 and 60,000 dalton chains being less well-defined, but apparently from both membrane faces.

Evidence from photo-affinity labeling (Witzemann and Raftery, 1977) and from immunological studies (Lindstrom, et al, 1978) has previously indicated that all four AcChR subunits are exposed on the membrane exterior, despite reported difficulties in labeling of the 65,000 dalton subunit from that surface (Hartig and Raftery, 1977). The evidence found here with TGase-catalyzed dansyl cadaverine labeling of the exterior surface of intact vesicles concurs with these findings: the 40,000, 50,000, and 60,000 dalton subunits appear to be labeled to varying degrees, with no labeling of the 65,000 dalton subunit observed. These results were consistent from one experiment to the next, with a band at ∿ 63,000-64,000 daltons being labeled, but not the 65,000 dalton AcChR subunit. This lack of labeling of the 65,000 dalton subunit may be due to steric factors, since both dansyl cadaverine and the 85,000 dalton TGase molecule must be in simultaneous contact with a glutamine residue for labeling to occur.

From the data shown in Figure 1, all four subunits appear to be exposed on the cytoplasmic membrane surface.

This labeling, however, was not reproducible from one experiment to the next. In many experiments only very faint labeling or no labeling at all was observed when reagents were in contact with the interior surface of the membrane. A large part of this irreproducibility stemmed from fluctuations in the amount of protein removed from the beads with 1 M K_2HPO_4. The range of protein concentrations adequate for CBB staining on SDS gels is relatively narrow; less than 100 µg of the proteins present in these membrane preparations gave only faint staining, while, with more than 200 µg, staining was too dark, lowering the resolution of individual bands. In attempts to extract ∿ 150 µg of protein from the beads, it was not unusual to obtain less than 100 µg, resulting in little staining or fluorescence on the gel. Thus, the irreproducibility of this labeling technique precluded the drawing of firm conclusions as to topography of the AcChR subunits from these data.

A more serious drawback lies in the nature of the method used for these preliminary studies. In theory, the attachment of intact, extracellular-side-out AcChR containing vesicles to positively charged beads and exposure of their cytoplasmic membrane faces by mechanical shear represents the extension in solution of the method

used in Chapter II for the immuno-labeling of the extracellular and cytoplasmic faces of postsynaptic membranes. The large increase in surface area of the beads over that of the coverslips permits the use of this technique on a scale such that enough material can be recovered from a small volume of beads for analysis on SDS gels. The drawbacks of the technique, which can be avoided when individual vesicles are examined under the electron microscope, however, introduce a great deal of uncertainty into the biochemical labeling studies reported here. As was discussed in Chapter II, a certain percentage of the vesicles do not shear during sonication, but, rather, collapse into double membrane sheets with their extracellular faces exposed. By electron microscopy, these deflated vesicles can be readily identified and distinguished from sheared vesicles by their smoother and more three-dimensional appearance; thus they can be disregarded in analysis of the data. In solution, however, labeling of these vesicles cannot be distinguished from that of sheared vesicles, leaving the distinction between extracellular and cytoplasmic labeling less clear. In the same way, labeling of the edges of sheared vesicles, which may represent the extracellular face of the membrane detached from the bead and curled over the cytoplasmic

face, can be identified and disregarded at the electron microscopic level, but not in solution. The percentage of deflated vesicles in a preparation, which would aid in data analysis, could not be determined by electron microscopy; the surfaces of the spherical beads would have to be examined by scanning electron microscopy, the resolution of which is too low to permit distinctions between deflated and sheared vesicles to be made.

The limitations of this method for exposure of the two membrane faces were avoided and unambiguous identification of the subunits exposed on each side of the membrane achieved by the use of the freeze-thaw method of Moore and Raftery (1980) for this purpose. The experimental evidence presented here from tryptic hydrolysis indicates that all four of the AcChR subunits span the postsynaptic membrane, being exposed on both the extracellular and the cytoplasmic faces of the membrane bilayer. The freeze-thaw technique for introduction of agents within sealed vesicles results in preparations which we show here are oriented with 95% of the α-BuTx binding sites outside, i.e. 95% extracellular (synaptic) side facing out. The tryptic hydrolysis of subunits observed from inside the vesicles did not result from residual inside-out vesicles since much more than 5% of the CBB staining

material for each subunit disappeared from the gel in all cases, while only 5% of the vesicles were inside-out. Unsealed vesicles, whether oriented inside-out or outside-out, would not be detected by the method used. α-BuTx would have access to bind to either side of such vesicles but these membranes would not be degraded under the experimental conditions used, since trypsin inhibitor added to the solution would also have access to both sides of these open vesicles. Thus, only the sealed membrane vesicles, 95% of which were oriented with their extracellular sides facing outward, constituted the population of vesicles studied in these experiments.

Under the conditions of the experiments, all four subunits were hydrolyzed to some extent from both the extracellular and the cytoplasmic sides of the vesicles, indicating their exposure on both faces of the membrane, thus implying that all four subunits span the membrane bilayer and are exposed to the hydrophobic core of the membrane. In experiments designed to determine which of the AcChR subunits penetrate into the hydrophobic membrane bilayer, Sator, et al (1979) used the lipophilic photolabel [^{3}H] pyrenesulfonyl azide to label membrane bound AcChR and determined that the 50,000 and 60,000 dalton subunits were exposed to the hydrophobic membrane core.

In similar experiments using 5-[^{125}I] iodonapthyl-1-azide as the lipophilic label, Tarrab-Hazdai, et al (1980) found that only the 40,000 dalton subunit gave evidence of exposure to the hydrocarbon interior of the membrane bilayer.

Evidence that different domains of the subunits are exposed on the two faces of the membrane is obtained from an inspection of the gels of the tryptic degradation patterns observed (Figure 3). The CBB staining patterns of the two samples were quite different; only the new band at ∿ 34,000 daltons was common to both while many distinct products were obtained in each case. This diversity in the new peptides produced upon tryptic hydrolysis of the AcChR from the extracellular vs. the cytoplasmic face of the membrane demonstrates clearly that the distribution of the subunits in the postsynaptic membrane is assymetric, with different domains of the subunits being exposed on each side of the membrane.

At the electron microscopic level, vesicles degraded with trypsin from either the inside or the outside had a similar appearance, with the AcChR rosette structures themselves unaffected, but clustered in the membrane, as opposed to the evenly spacedrosettes seen in untreated controls. Thus, it appears that digestion by trypsin

hydrolysis affects some anchoring mechanism holding the molecule in the membrane. This is consistent with the results of Bartfield and Fuchs (1979) who observed that after hydrolysis of detergent-solubilized AcChR with 10^3 times as much trypsin as was used here, the receptor retained its pharmacological and pathological myasthenic specificities. Similarly, Klymkowsky, et al (1980) reported morphological changes similar to those found here for trypsin-digested AcChR-enriched membrane vesicles, while α-BuTx binding was unaffected. While we cannot completely rule out the possibility that morphological changes were responsible for the exposure of some of the subunits to tryptic hydrolysis, this does not seem likely. If tryptic degradation led to the opening of holes or tears in the membrane to allow hydrolysis of other subunits, then the trypsin inhibitor on the outside would reach the trypsin inside the vesicle and quench the reaction. It is more likely that the clustering of receptor rosettes seen under these conditions reflects only a translation in the membrane, especially since the rosette structure itself appeared to be unaffected.

From a comparison of the time course of the degradative processes on the two sides of the membrane, qualitative estimates of the relative degrees of exposure of trypsin-sensitive residues for each subunit may be made. On the extracellular membrane face, all four subunits disappeared at roughly the same rate, indicating, that, with respect to hydrolysis by trypsin, they are all exposed to approximately the same degree. On the cytoplasmic face, however, after the initial rapid hydrolysis, the 65,000 dalton subunit was degraded the most readily, followed closely by the 60,000 dalton subunit, with the 50,000 dalton protein disappearing more slowly and the 40,000 dalton subunit much more slowly still. This indicates that, with respect to trypsin sensitive residues, the 65,000 and 60,000 dalton subunits are the most exposed on the cytoplasmic face of the membrane, followed by the 50,000 dalton subunit, with the 40,000 dalton subunit exposed very little. More evidence is needed with probes for other amino acids before it will be possible to state whether this differential degree of exposure on the cytoplasmic face is in fact the case for the four subunits or is merely a reflection of differing sensitivity to trypsin of the exposed regions.

At the ultrastructural level, high resolution electron micrographs of the AcChR rosettes show from four to six similar "subunits" arranged about an electron dense core (Raftery, et al, 1974b; Cartaud, et al, 1973; Nickel and Potter, 1973; Klymkowsky and Stroud, 1979). This is consistent with recent reports that the receptor molecule consists of four homologous subunits present in the ratio of 2:1:1:1 (Raftery, et al, 1980), a total of five similar subunits, all exposed on the extracellular surface of the membrane. The four subunits show a considerable amount of sequence homology at their amino termini (Raftery, et al, 1980); however, it is not known whether the homology continues through the carboxyl-termini of all four polypeptides. The similar rates of tryptic degradation of the four subunits on the exterior of the membrane, when compared to the vastly different rates of degradation observed for the four on the vesicle interior, suggests that the four subunits are, by this criterion, equally exposed on the extracellular face of the membrane, with the longer chains more exposed and the shortest (40,000 dalton) least exposed on the cytoplasmic membrane face. This evidence is consistent with a model of the AcChR (Figure 7) as a

pentameric structure of two identical and three pseudo-identical polypeptide chains on the exterior face of the membrane, and continuing to an unknown extent within the bilayer. This complex extends through the membrane, with the additional carboxyl-terminal portions of the longer chains exposed at least partially on the cytoplasmic face and the carboxyl-terminus of the shortest chain barely exposed on the interior surface of the membrane. Studies are currently under way to further investigate this model and its functional significance.

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Table I

Attachment of Intact Vesicles to Positively Charged Beads (0.2 ml packed beads)

Procedure	Amount Attached	Amount Removed
initial attachment	52 µg	----
sonication	32 µg (61%)	20 µg (39%)
1 M K_2HPO_4	15 µg (45%)	17 µg (55%)

Figure 1

SDS polyacrylamide gel of TGase-catalyzed dansyl cadaverine labeled membrane vesicles. (A) Control samples of labeling of intact vesicle exterior in which TGase was omitted; (B) TGase-catalyzed labeling of intact vesicle exterior; (C) TGase-catalyzed labeling of interior face of vesicles (immobilized on polylysine coated beads and sheared by sonication); (D) TGase-catalyzed labeling of interior surface of membrane vesicles on which the exterior surface was previously labeled with cadaverine. The positions of the four AcChR subunits are marked.

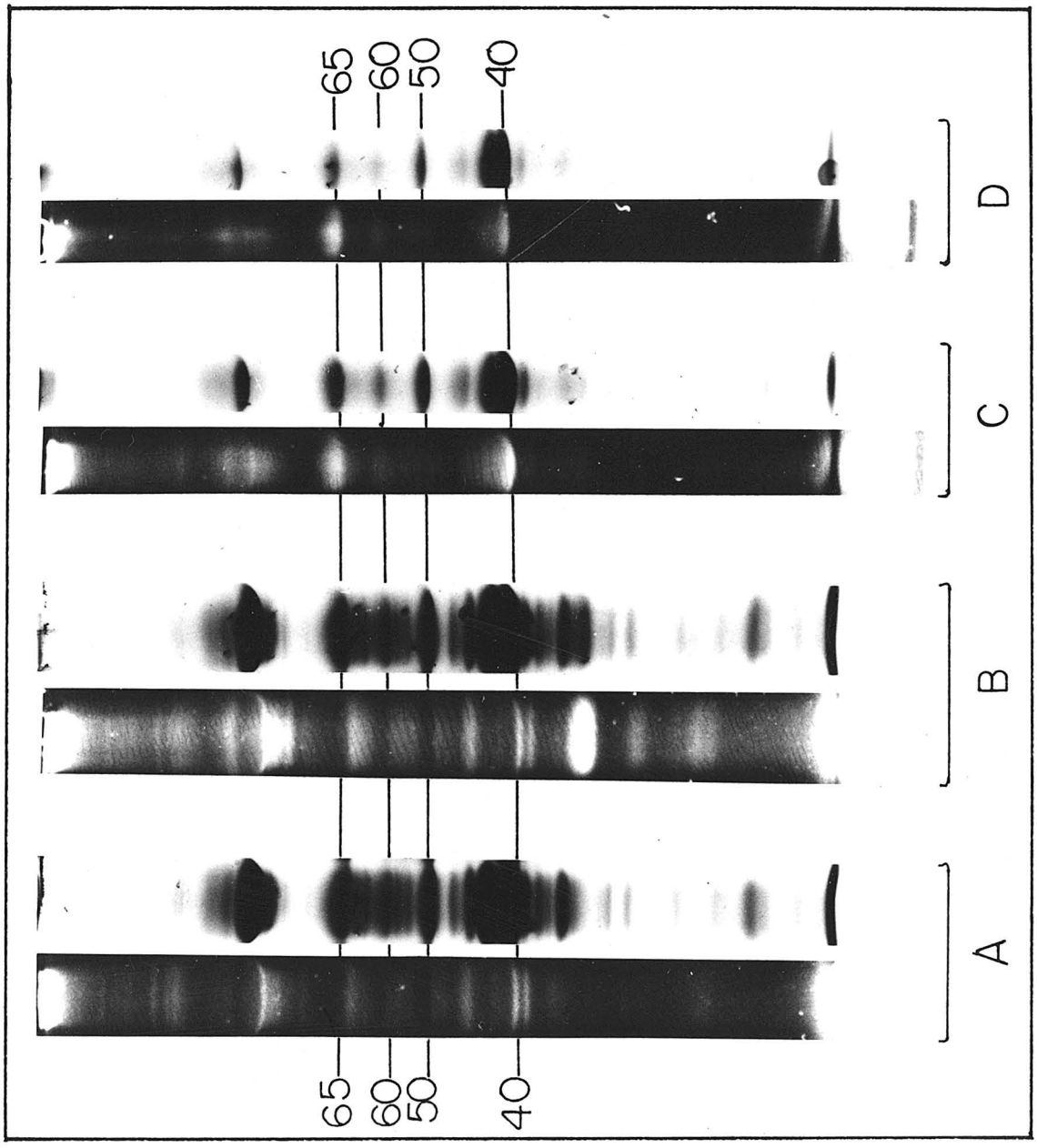

65
60
50
40
D
C
B
A
65
60
50
40

Figure 2

SDS polyacrylamide gel of TGase-catalyzed dansyl cadaverine labeled exterior surfaces of intact vesicles. (A) Control sample in which TGase was omitted; (B) TGase-catalyzed labeling of exterior face of intact vesicles previously labeled with cadaverine; (C) TGase-catalyzed labeling of exterior face of intact vesicles not previously labeled.

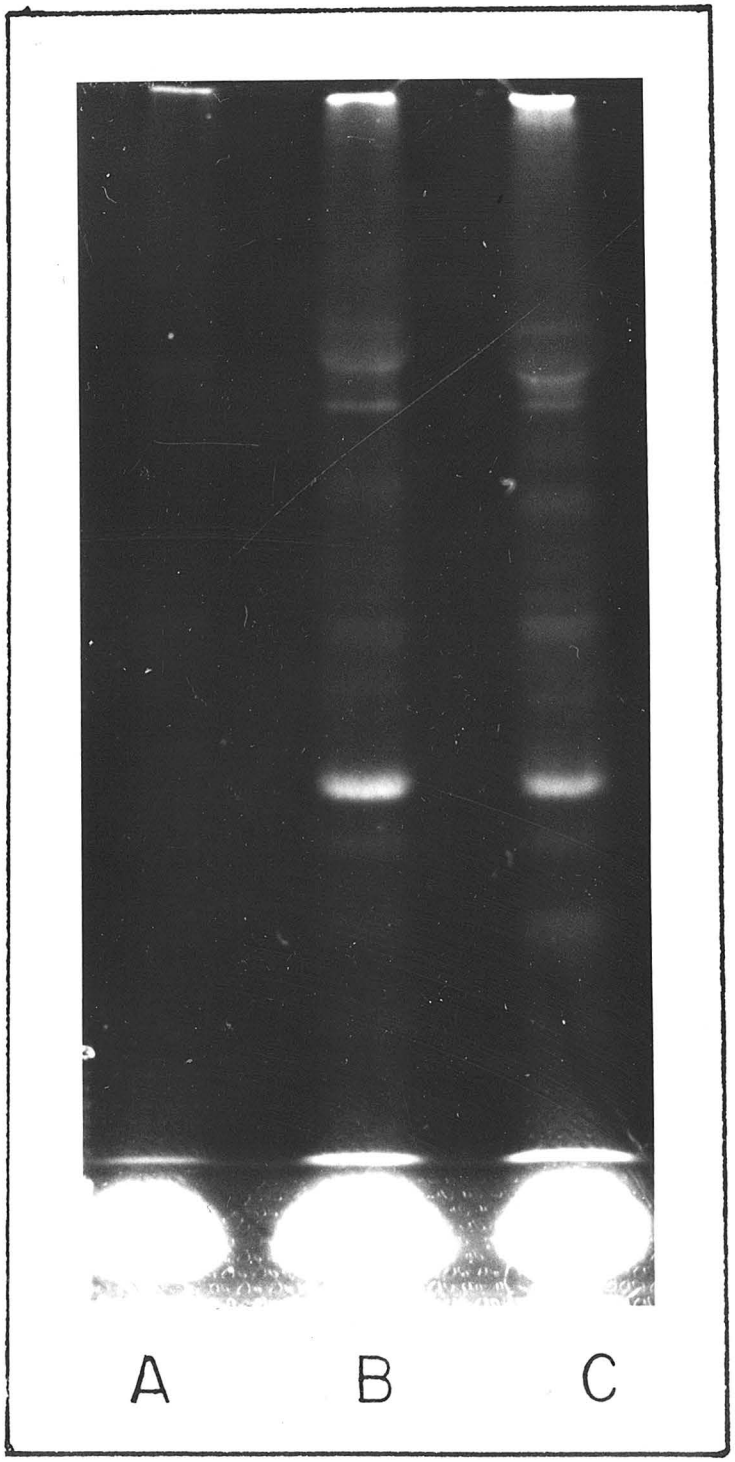

A
B
C

Figure 3

SDS gel electrophoresis of (A) membrane fragments treated with trypsin and inhibitor, subjected to two cycles of freezing and thawing, and incubated at 37°C for two hrs; (B) membrane fragments treated with trypsin after freeze-thawing, incubated at 37°C for two hrs (outside tryptic hydrolysis; (c) membrane fragments treated with trypsin, then frozen and thawed, treated with inhibitor immediately after thawing, then heated in gel sample buffer; (D) same as (C), but incubated at 37°C for two hrs instead of heating in gel sample buffer (inside tryptic hydrolysis); (E) untreated membrane fragments. The four AcChR subunits are marked.

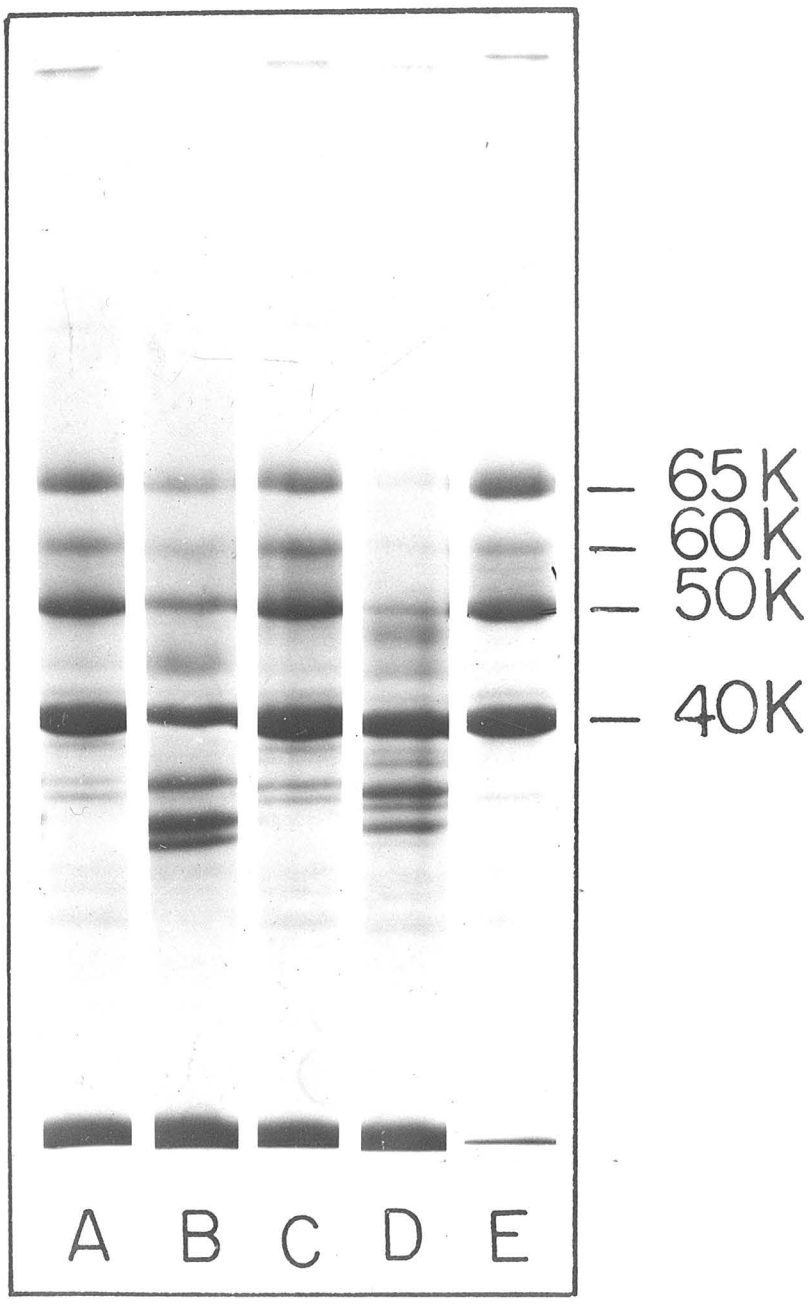

65K
60K
50K
40K
A
B
C
D
E

Figure 4

Time course of tryptic degradation (A) from the outside and (b) from the inside of the membrane vesicles. Peak heights from scans of CBB stained gels are given for the 40,000 (●), 50,000 (○), 60,000 (■), and 65,000 (□) dalton subunits.

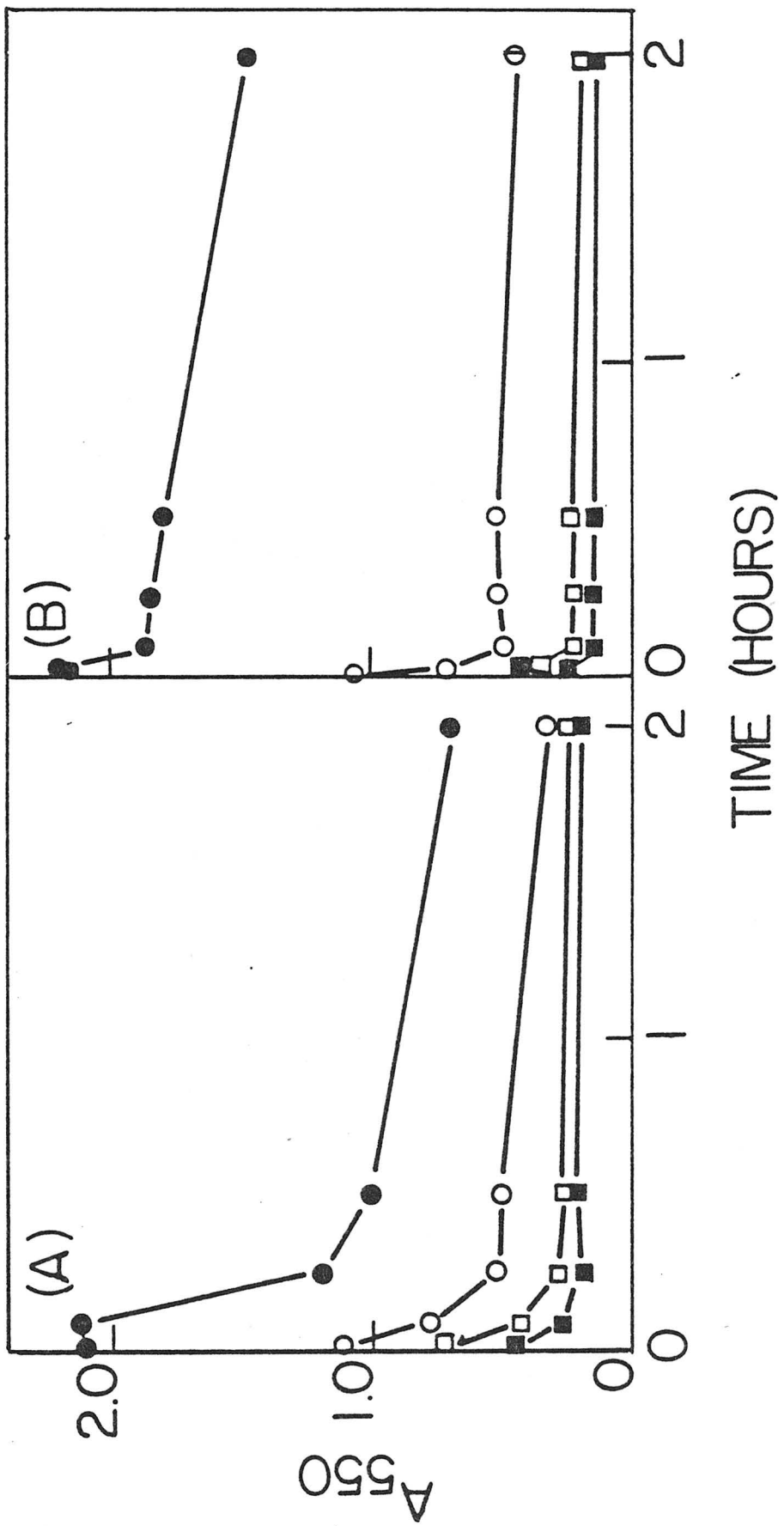

(A)
(B)
2.0
1.0
0
A_{550}
0
1
2
TIME (HOURS)

Figure 5

Time course of tryptic degradation (A) from the outside, and (B) from the inside of the membrane vesicles. Peak heights from scans of CBB stained gels are given as the average of three experiments, each normalized to its own controls. The 40,000 (●), 50,000 (○), 60,000 (■), and 65,000 (□) dalton subunits are shown.

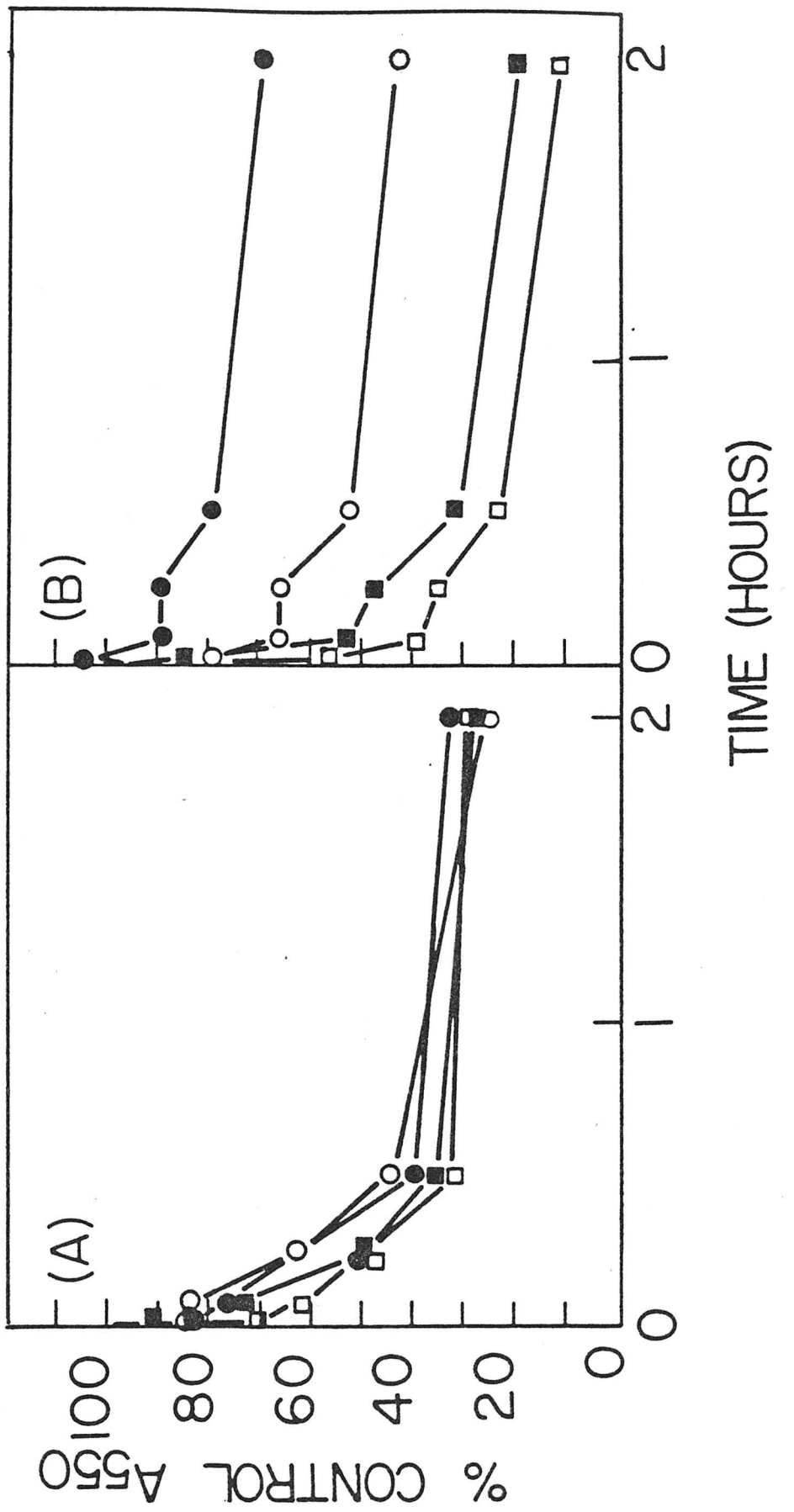

% CONTROL A550
100
80
60
40
20
0
(A)
(B)
0
1
2
0
1
2
TIME (HOURS)

Figure 6

Negative stain electron micrographs of (A) control membrane vesicle treated with trypsin and inhibitor (X425,000); (B) membrane vesicle degraded with trypsin from the outside (X210,000); (C) membrane vesicle degraded with trypsin from the inside (X210,000); and (D) an untreated membrane vesicle (X210,000).

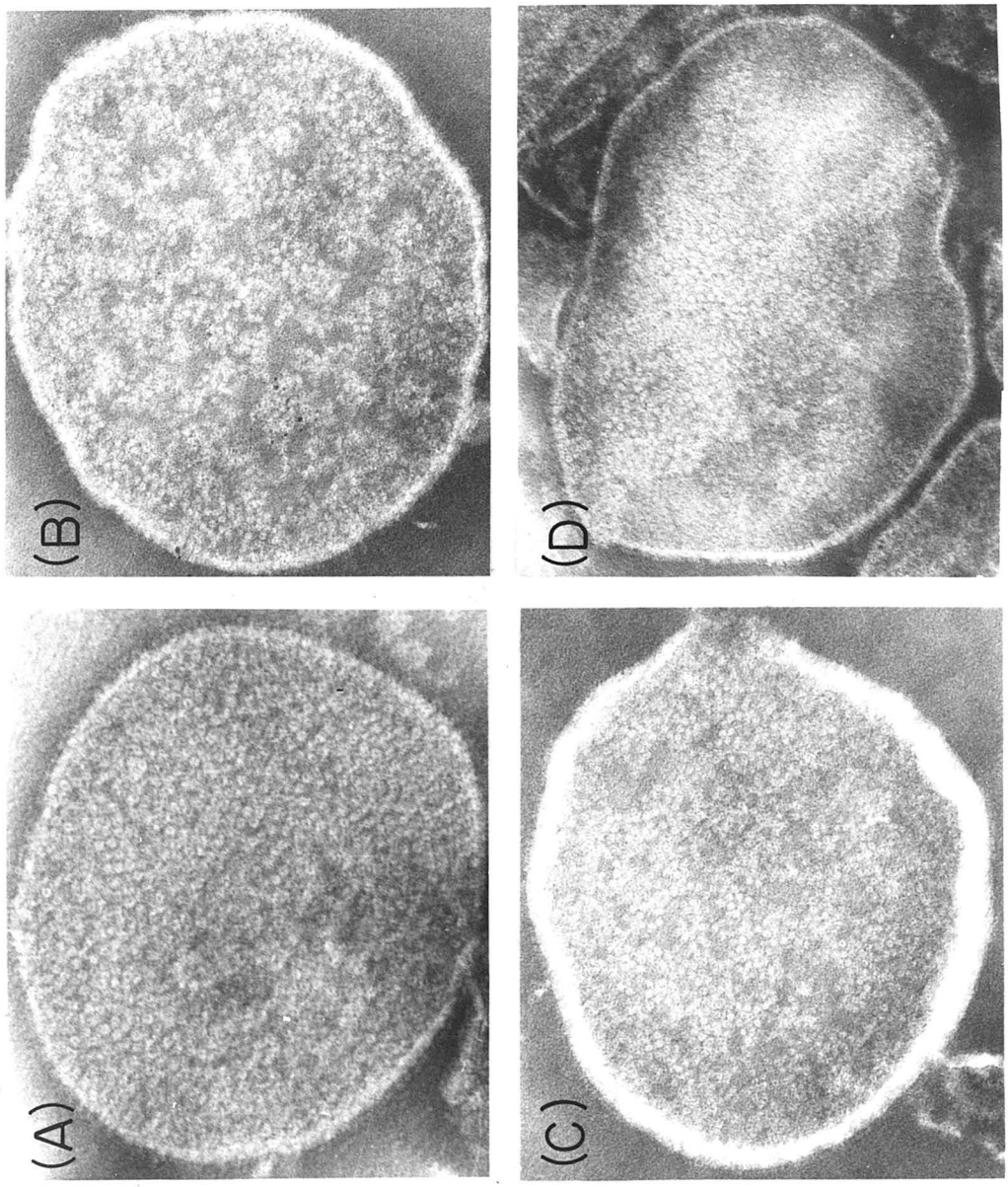
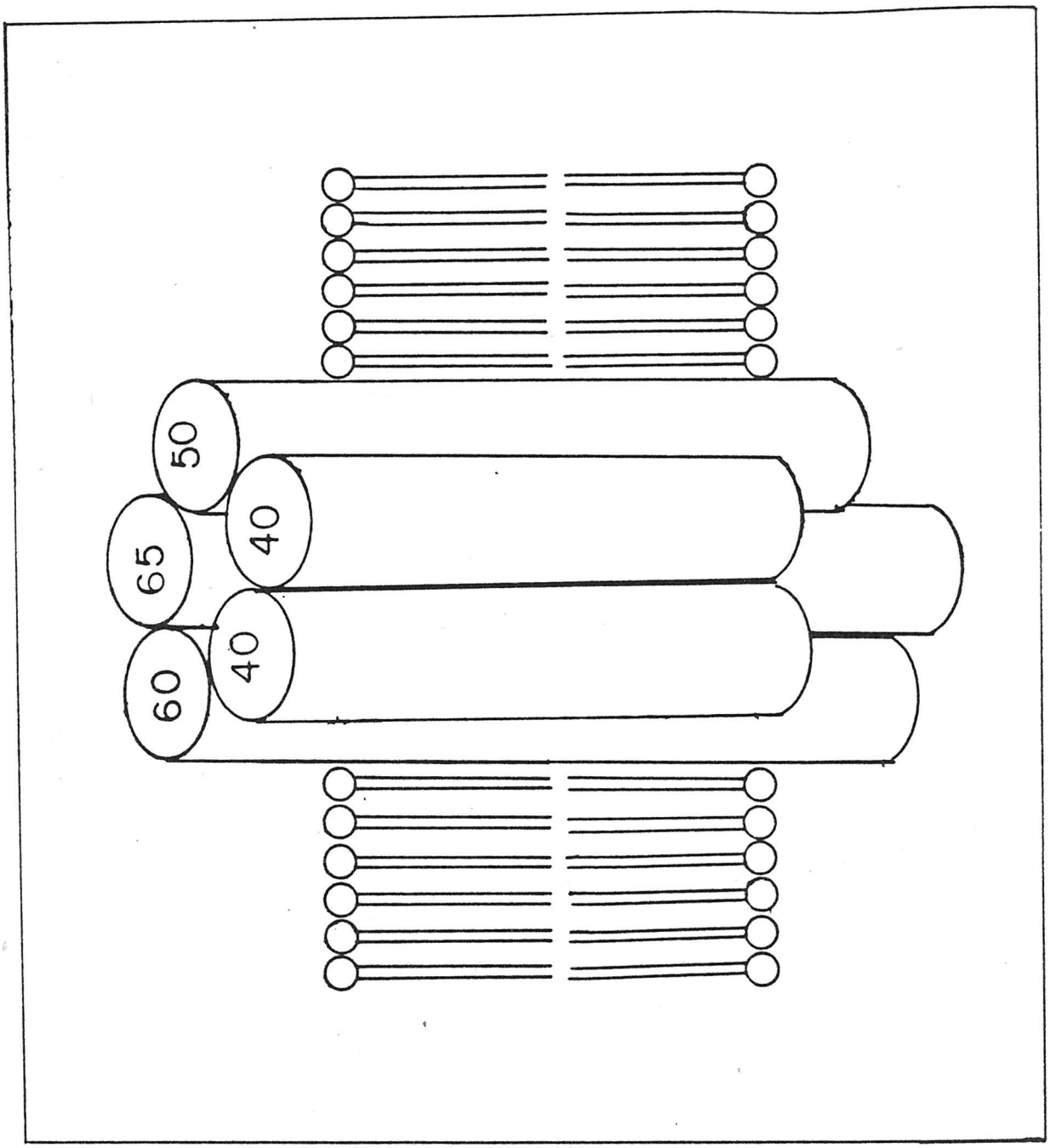

(B)
(D)
(A)
(C)

Figure 7

A model of the position of the acetylcholine receptor in the postsynaptic membrane that is consistent with the data presented in Chapters I, II, and III of this thesis. The receptor is a pentameric transmembrane complex of two identical (40,000 dalton) and three pseudoidentical (50,000, 60,000, and 65,000 dalton) subunits, all exposed approximately equally on the exterior membrane surface, and extending to varying degrees on the interior surface, with the 65,000 dalton exposed the most, and the 40,000 dalton chain barely exposed on this side of the membrane.


50
40
65
40
60

Chapter IV: The Characterization of Actin Associated with Postsynaptic Membranes from *Torpedo californica* Electroplax

INTRODUCTION

In addition to its well-documented function in muscle contraction, actin has been shown to be a component of many types of non-muscle cells, where there is evidence for its involvement in motility and other cellular processes, such as blood clot retraction, cytokinesis, cytoplasmic streaming, mitosis, and phagocytosis; it is also implicated in cytoskeletal formation (Goldman, et al, 1976; Hitchcock, 1977; Clarke and Spudich, 1977). Actins have been studied from a wide variety of non-muscle sources, such as platelets (Adelstein, et al, 1971), erythrocytes (Tilney and Detmers, 1975), macrophages (Hartwig and Stossel, 1975), sea urchin eggs (Miki-Noumura and Oosawa, 1969), and several species of unicellular organisms, such as Acanthamoeba (Pollard, 1976) and Nitella (Palevitz, 1976). These non-muscle actins are similar in structure and physical properties to each other and to muscle actin, having similar, although non-identical, amino acid compositions, mobilities on SDS gels, peptide maps, and polymerization properties (Clarke and Spudich, 1977). Like muscle actin, actin from non-muscle sources appears to have a Ca^{++} requirement for its role in cell motility (Hoffman-Berling, 1964; Hitchcock, 1977); the

mechanism for Ca^{++} involvement is not yet understood.

In many cases, actin has been found associated with cell membranes, where it is thought to function in both cell motility and anchoring (Goldman, et al, 1976; Hitchcock, 1977). In association with the plasma membrane, actin is capable of polymerization, but is often found in the monomer state, rather than in the filaments common to muscle cells (Tilney, 1976). When examined under the electron microscope, this monomeric actin tends to appear as an amorphous material directly under the plasma membrane. The method of association of both monomeric and filamentous actin with the membrane is not known, although it is thought to be by attachment to either a peripheral protein or to an integral membrane protein of less than 100,000 daltons, from freeze-fracture electron microscopic studies (Tilney, 1976).

Actin and myosin have been observed in the electric organ of the Torpedo (Kaminer and Szonyi, 1973) and at the synapse in cerebral tissue from chicken and a variety of small mammals (Toh, et al, 1976). Studies of the function of actin and myosin at the synapse using synaptosomes prepared from brain tissue indicate that actin is associated with the presynaptic membrane and myosin with the presynaptic vesicles. Berl, et al (1973)

postulate a transmitter release mechanism by which interactions between the actin and myosin would cause conformational changes in the membranes leading to the expulsion of transmitter from the vesicles into the synaptic space. In dissected rabbit Deiters' neurons, actin was found localized at the synapses on both the pre- and postsynaptic sides of the junction (Bray, 1977). Although the presence of actin and related muscle and tubular proteins at the synapse is well documented, their structural and functional roles there remain to be determined.

Recently, the question of the association of actin with the acetylcholine receptor (AcChR) has been raised. The observation under the electron microscope of a dense, amorphous coating on the cytoplasmic face of the postsynaptic membrane (Rosenbluth, 1975) brings to mind parallels with theappearance of monomeric, membrane-associated actin, and leaves room for speculation on a role for actin in the anchoring of the AcChR molecules in the postsynaptic region of the membrane.

Purified Torpedo AcChR has been found to be composed of four subunits with molecular weights of 40,000, 50,000, 60,000 and 65,000 daltons (Raftery, et al, 1974; Weill, et al, 1974; Chang and Bock, 1977; Lindstrom, et al, 1978;

Vandlen, et al, 1979; Froehner and Rafto, 1979; Elliott, et al, 1980; Raftery, et al, 1980; Chapter I of this thesis). In addition to these subunits, SDS gels of all but the purest preparations of AcChR-enriched membrane fragments contain a strong band migrating at 43,000 daltons; the function of this protein and its relationship to the AcChR have recently been the subject of much investigation. It has been reported that this protein contains the binding site for histrionicotoxin (HTX), thus implicating it in the function of the ion channel (Sobel, et al, 1978). However, more recent studies on membrane fragments which have been extracted with base to remove the 43,000 dalton protein have shown the HTX binding site to be associated with the AcChR rather than with the 43,000 dalton chain (Neubig, et al, 1979; Elliott, et al, 1979). Affinity labeling of AcChR-enriched membrane fragments with a local anesthetic analog showed labeling of this 43,000 dalton component, as well as the 40,000 dalton AcChR subunit (Blanchard and Raftery, 1979). Because of the wide range of activities of local anesthetics, the significance of the labeling of the 43,000 dalton protein with a local anesthetic analog is unclear (Blanchard, 1980), and the nature of this protein is still not defined.

There has been speculation that this 43,000 dalton protein may be actin due to their similar molecular weights and to the growing knowledge that actin is a common protein in non-muscle systems. Karlin, et al (1979) have stated that all or part of the 43,000 dalton species is composed of actin, based on anti-actin antibody precipitation and on gel electrophoresis patterns. Sobel, et al (1979), however, have observed that actin does not co-migrate with the 43,000 dalton component on SDS gels of AcChR-enriched membrane preparations from the related species Torpedo marmorata.

One of the unusual properties of actin is its ability to act as a specific inhibitor of deoxyribonuclease I (Lazarides and Lindberg, 1974); DNase I causes the depolymerization of F-actin by formation of a complex with a stoichiometry of 1:1 with G-actin (Hitchcock, et al, 1976). In the studies described in this Chapter, the DNase I inhibition properties have been utilized, along with electrophoresis in several SDS gel systems, to determine the relationship between actin and the 43,000 dalton protein. Evidence is presented that actin is a component of most preparations of AcChR-enriched membranes, but one that is distinct from the 43,000 dalton protein.

MATERIALS AND METHODS

Crude membrane fragments were prepared from Torpedo californica electroplax by the method of Reed, et al (1975) involving sequential centrifugation and homogenization. Membrane fragments enriched in AcChR were prepared either by the method of Reed, et al (1975), using sucrose gradient centrifugation in a zonal rotor, or by the faster method of Elliott, et al (1980), using a vertical rotor for the sucrose gradient centrifugation, giving preparations binding 1.3-2.0 nmol α-bungarotoxin (α-BuTx) per mg of protein. The concentration of α-BuTx sites was determined according to Schmidt and Raftery (1973) using DEAE-cellulose filter discs and ^{125}I-α-BuTx (Clark, et al, 1972; Blanchard, et al, 1979). Protein concentrations were determined by the method of Lowry, et al (1951) using bovine serum albumin as the standard. SDS gel electrophoresis was done according to Laemmli (1970) with the acrylamide concentrations varied as presented in the text. After electrophoresis, gels were stained with Coomassie brilliant blue (CBB) in 25% methanol, 10% acetic acid. Analysis for 3-methylhistidine was done on a Beckman model 120C amino acid analyzer using System A from Keuhl and Adelstein (1969) and Dowex-50 resin.

Preparation of Actin:

Chicken smooth muscle actomyosin was purified from a gizzard muscle homogenate by the method of Driska and Hartshorne (1975). Pure actin was isolated from the actomyosin fraction by preparative SDS gel electrophoresis on gels with dimensions of 11 x 13.5 x 0.6 cm, using a 12.5% acrylamide, 0.1% methyline-bis-acrylamide separating gel and a 3% acrylamide, 0.08% bis-acrylamide stacking gel. After staining with CBB, the actin band was cut out and the protein eluted from the gel by the method of Lazarides (1975).

Pure 43,000 dalton protein was isolated from Torpedo membrane fragments by this same technique of preparative gel electrophoresis.

DNase Inhibition Assay:

DNase inhibition activity of membrane fragments was determined by a modification of the method of Lindberg (1964). Membrane fragments were solubilized in 1% Triton X-100 for 30 mins and the insoluble material pelleted by centrifugation at 15,000 xg for 10 mins. Aliquots of the supernatant were incubated for five mins with 1 µg of bovine pancreatic DNase I (Sigma), then mixed by vortexing with 3 ml of DNA substrate solu-

tion (0.04 mg/ml DNA (calf thymus-Sigma), 4 mM Mg^{++}, 2 mM Ca^{++}, pH 7.5) for 10 sec and the hyperchromicity upon DNA depolymerization monitored immediately as an increase in absorbance at 260 nm. This result was compared to the absorbance increase when 1 µg DNase I alone was added to the DNA substrate solution. One inhibition unit was defined by Lindberg (1964) as the amount of inhibitor causing a decrease of 0.001 OD/min in 1 µg of DNase.

DNase I Affinity Chromatography:

DNase I-agarose was prepared by the method of Lazarides and Lindberg (1974), using cyanogen bromide-activated Sepharose 2B (Axen, et al, 1967). Membrane fragments were solubilized in 1% Triton and clarified by centrifugation before affinity chromatography, which was performed according to Lindberg and Eriksson (1971). The actin was eluted from the column with 3 M guanidine HCl, then dialyzed extensively against water and lyophilized.

RESULTS

The ability of actin to inhibit DNase I activity provided a means for determining the presence of actin in fractions from Torpedo californica electric organ. Using this assay, actin was found in the supernatant after the first centrifugation of electric organ homogenates in isolated crude membrane extracts, and in most, but not all, membrane preparations enriched in AcChR (see Figure 1). The amount of actin in these AcChR-enriched membrane fragments varied from zero to 90 DNase I inhibition units per mg of protein, or up to 90 inhibition units per nmole of α-BuTx binding sites. Approximately 1-3 μg of purified chicken smooth muscle actin are required to completely inhibit I μg of DNase I (Lazarides and Lindberg, 1974); on the basis of this information, it can be calculated that AcChR-enriched membrane preparations contain approximately 6 μg of active actin per mg of protein. The membrane fragments were solubilized in 1% Triton for the DNase I inhibition assays; control experiments showed that intact membrane preparations did not inhibit the enzyme and that all the DNase I inhibitor extractable from the membranes was contained in the solubilized supernatant after extraction with Triton. Further controls showed

no effect on DNase I activity by Triton alone or by any of the other components of the buffers used.

Membrane-bound AcChR, over a period of time, becomes desensitized, or locked in a high-affinity state for ligand binding (Lee, et al, 1977; Quast, et al, 1978). Testing the hypothesis that the level of detectable actin monomer in these membrane preparations is related to the ligand affinity state of the AcChR, DNase I inhibition and the affinity state of the AcChR in membranes were measured periodically for several days after the isolation of the membrane fragments. No relationship was observed between levels of DNase I inhibition and the ligand affinity state of the AcChR molecule.

DNase I affinity chromatography was used to isolate actin from Triton-solubilized membrane preparations enriched in AcChR. A typical elution profile is given in Figure 2. As shown in Figure 3, all DNase I inhibitory activity can be removed from the membrane fragments by this method. After elution from the affinity column with 3 M guanidine HCl, the actin no longer acted as a DNase I inhibitor, presumably as a result of the destruction of its DNase binding site. SDS gel electrophoresis on the material showed it to co-migrate with both

purified chicken smooth muscle actin and with a minor component of the membrane preparations with a molecular weight of ∿ 47,000 daltons (see Figure 4). This band was distinct from the major 43,000 dalton band observed on the gels.

Confirmation that the 43,000 dalton polypeptide was not actin was provided by analysis of this band (cut from a preparative SDS gel of the AcChR-enriched membrane fragments and determined to be pure by re-electrophoresis) for the amino acid 3-methylhistidine. 3-methylhistidine is a relatively rare amino acid, found as a component of actin from several species (Weihing and Korn, 1971; Booyse, et al, 1973; Adelstein and Keuhl, 1979; Pardee and Bamburg, 1979). Under conditions where less than one residue per molecule would be readily detected, no 3-methylhistidine was found in this protein.

When AcChR-enriched membrane fragments were electrophoresed on SDS gels of varying acrylamide concentrations, the CBB staining pattern of the proteins changed slightly from one gel to the next, as shown in Figure 5. As the concentration of acrylamide was increased from 7.5% to 12.5%, the separation between the 43,000 dalton protein and actin improved and became optimal using the 12.5% acrylamide, 0.1% bis-acrylamide gels. The separation

worsened in gels run in half-strength Laemmli running buffer (Figure 5E) and in cases where electrophoresis was carried out at currents of $\geq$ 15 mA/cm^2 (data not shown).

DISCUSSION

Actin has been identified in the supernatant after the first centrifugation of homogenized Torpedo electric organ, in crude electroplax membrane fragment preparations, and in membrane fragments enriched in AcChR. This protein, which has been identified both by its ability to inhibit DNase I and by co-migration with purified actin on SDS gels, is present at higher concentrations in the original supernatant than in either of the membrane preparations, leading to the conclusion that the majority of actin in electroplax is a component of the cytoplasm. A portion, however, is associated with the membranes sufficiently tightly to remain with them through sucrose density gradient centrifugation. This membrane-associated actin is freed to inhibit DNase I only upon solubilization of the membrane with Triton.

Thus, there appear to be at least two classes of actin present in these preparations: a large fraction of the total actin capable of inhibiting DNase I appears to be located in the cytoplasm of the electroplaques, while a

smaller fraction is membrane-associated. The fact that the membrane-associated actin, upon solubilization with detergent, inhibits DNase I indicates that the actin bound to the membrane is in the monomeric form (G-actin), since only G-actin is capable of binding to DNase I (Hitchcock, 1976). If the membrane-associated actin were in the form of filaments (F-actin), the addition of DNase I to the unsolubilized membrane suspension would cause the depolymerization of F-actin to G-actin with the formation of G-actin-DNase I complexes (Hitchcock, et al, 1976), resulting in the inhibition of DNase I activity without prior solubilization. This is not the case; DNase I inhibition by membrane preparations is only observed upon solubilization with Triton, which is not capable of depolymerizing the actin filaments. This observation of the association of monomeric actin with Torpedo membranes concurs with the findings of Tilney (1976), who reported that actin associated with plasma membranes is often found in the monomeric state.

SDS gel electrophoresis of the material from solubilized Torpedo membranes bound to the DNase I affinity column shows it to co-migrate with actin purified from chicken smooth muscle and to correspond to a band in the membrane fragments migrating at $\sim$ 47,000 daltons.

The separation of this actin band from the 43,000 dalton component of membrane fragments was poor in all of the gels tried, except for the Laemmli gel using 12.5% acrylamide and 0.1% bis-acrylamide. This may account for the lack of agreement of these results with those from another laboratory (Hamilton, et al, 1979; Karlin, et al, 1979), who have stated that the 43,000 dalton protein co-migrates with actin on SDS gels. Since the four AcChR subunits are glycoproteins (Vandlen, et al, 1979) and actin is not, their relative mobilities may be expected to vary in different gel systems. That actin co-migrates with the 47,000 dalton component of the AcChR-enriched membranes and with the DNase inhibitor purified from these membranes in all gel systems used here indicates that the threeproteins are identical.

The 43,000 dalton protein is a major component of AcChR-enriched Torpedo membrane preparations from several laboratories (Elliott, et al, 1980; Sobel, et al, 1979; Neubig, et al, 1979; Karlin, et al, 1979). It has been tentatively implicated in the binding of histrionicotoxin (Sobel, et al, 1979) and local anesthetics (Blanchard and Raftery, 1979), although the evidence for HTX binding has been contradicted and its

implications for receptor function debated (Elliott, et al, 1979; Blanchard, 1980). Furthermore, the removal of this protein from the membrane preparations by treatment with base has been shown to have no effect on the function of the AcChR (Moore, et al, 1979). That this protein is not actin has been shown here by its lack of the amino acid 3-methylhistidine and by its failure to co-migrate with purified actin on SDS-polyacrylamide gels. Actin has, instead, been identified as a separate component of AcChR-enriched membrane preparations migrating on SDS gels between the 43,000 dalton protein and the 50,000 dalton subunit of the AcChR. The role of actin in these preparations and whether it has a structural or functional relationship to the AcChR molecule remains to be investigated.

It is clear that the function of actin in these membrane preparations is not related to any known function of the receptor; after treatment with base to remove peripheral proteins, the membrane-bound AcChR remains fully functional (Moore, et al, 1979) in the absence of any other protein components of the membranes, including actin (Elliott, et al, 1980). It is possible, however, that actin plays a structural role relative to the AcChR. Again, results with base-treated AcChR-enriched membrane fragments can be used to demonstrate that actin is not

directly involved in the anchoring of the receptor in the membrane; the AcChR remains fixed in the membrane in the absence of actin (Elliott, et al, 1980). However, actin may be involved in the process by which the receptor molecules are concentrated in the regions of membrane directly opposite the nerve terminals. Ultrastructural studies on electroplax membranes in vivo have shown the concentration of receptors to be two orders of magnitude higher in the postsynaptic membrane than extrasynaptically (Bourgeois, et al, 1972). Other studies have identified a distinctive amorphous coating on the cytoplasmic side of the postsynaptic membrane, frequently associated with filaments (Rosenbluth, 1975). The composition of this coating is unknown, but its description bears an interesting resemblance to the observations of Tilney (1976) that monomeric, membrane-associated actin appears at the electron microscopic level as an amorphous coating on the cytoplasmic side of the plasma membrane. This correlation and the observations from ultrastructural studies that actin is present at the synapse in several species (Toh, et al, 1976), are in agreement with a role for actin in the anchoring of AcChR molecules in the postsynaptic region of the membrane.

At the same time, it is important to keep in mind the heterogeneous nature of these membrane populations. In these preparations, the AcChR constitutes no more than 50% of the total protein present, and electron microscopic investigations show that the receptor in the AcChR-containing vesicles is closely packed, occupying most of the membrane surface (Klymkowsky and Stroud, 1979). From this, it is clear that many of the membrane vesicles must contain little or no AcChR; this, too, is confirmed by electron microscopic observations. Thus, it is possible that the actin present in these AcChR-enriched membrane preparations may be associated with the non-receptor containing vesicles and have no relationship to the AcChR molecule.

The presence of actin in AcChR-enriched membrane fragments may have implications for the in vivo mechanism of the concentration of receptors in the postsynaptic membrane. However, the function of actin in these preparations is, at the present time, unknown and its relationship to the AcChR remains to be determined.

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Figure 1

DNase I inhibition assay on increasing amounts of Triton-solubilized AcChR-enriched membrane fragments: 0.12 mg of protein (●), 0.24 mg (△), 0.30 mg (x) and 0.45 mg of protein (□) show increasing inhibition of DNase I activity over the control with no membrane protein (○). 0.45 mg of membrane protein causes 62% inhibition of 1 μg of DNase I.

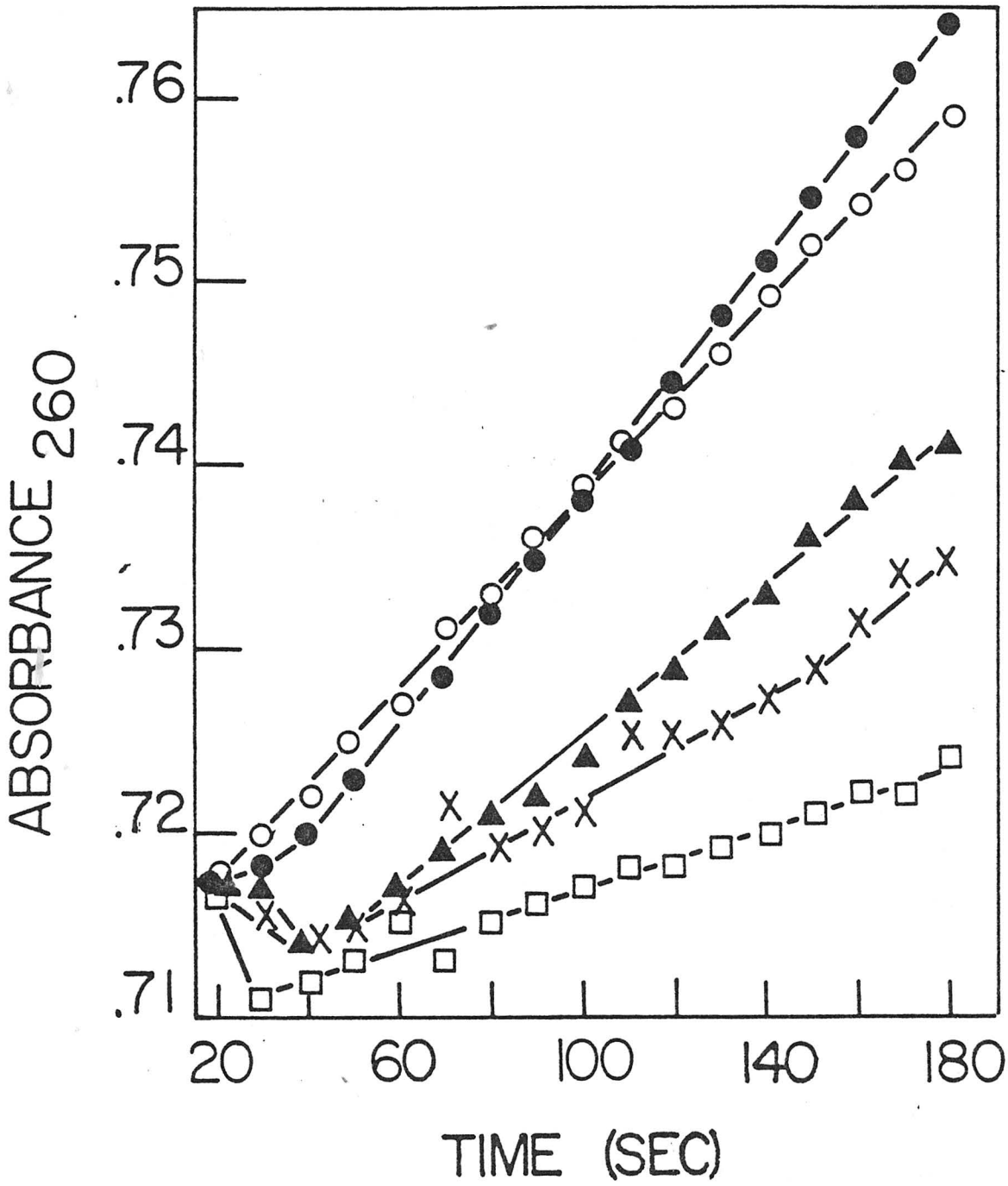

.76
.75
.74
.73
.72
.71
ABSORBANCE 260
20
60
100
140
180
TIME (SEC)

Figure 2

Elution profile of DNase I affinity chromatography on Triton-solubilized AcChR-enriched membrane fragments. Pool C contains the DNase I inhibitor.

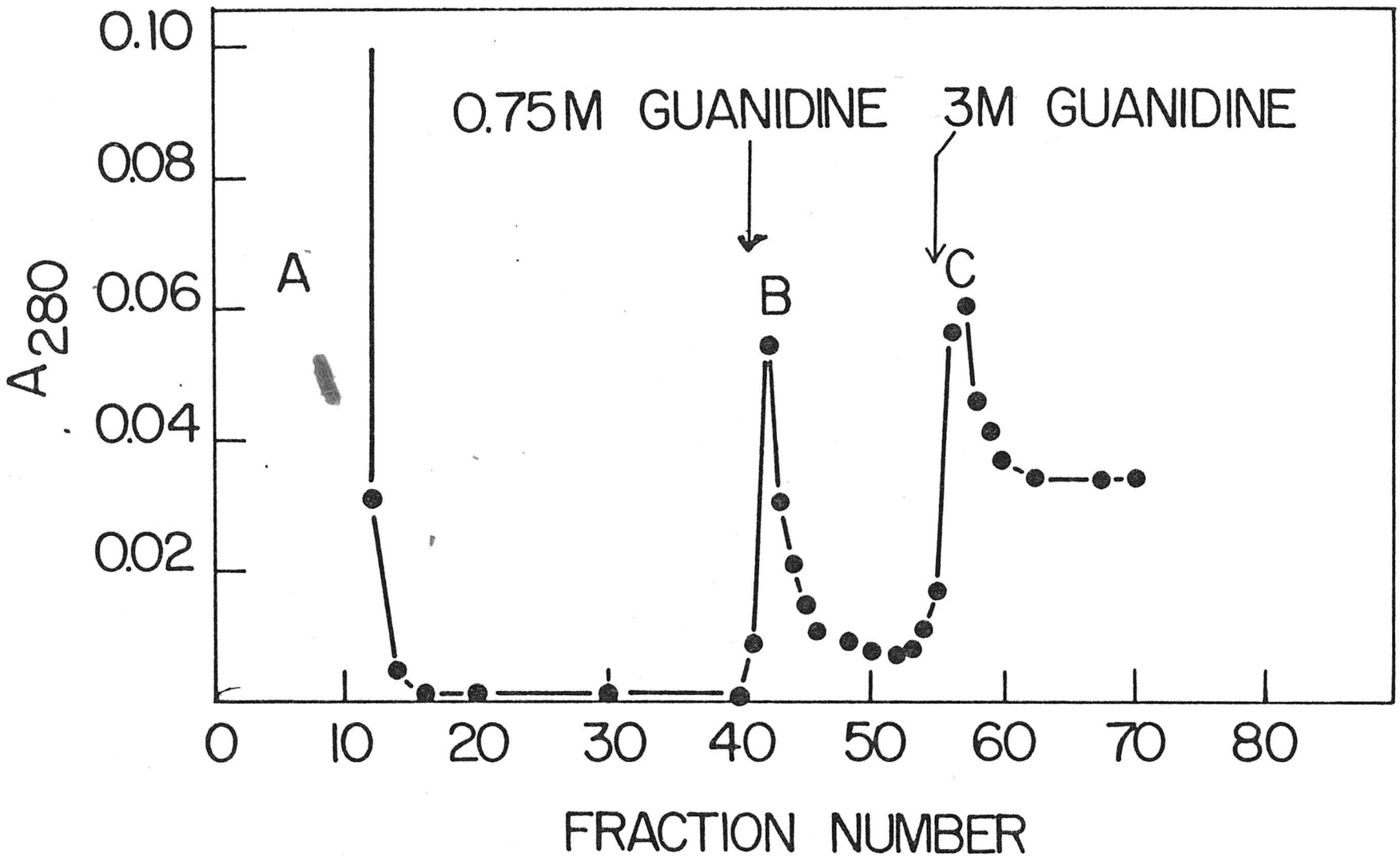

0.10
0.08
0.06
0.04
0.02
A_{280}
A
0.75M GUANIDINE
B
3M GUANIDINE
C
0
10
20
30
40
50
60
70
80
FRACTION NUMBER

Figure 3

DNase I inhibition assay on Triton-solubilized AcChR-enriched membrane fragments before (○) and after (△) DNase I affinity chromatography. Before treatment, the membrane preparation caused 60% inhibition of 1 μg of DNase I; afterwards, the DNase I activity was the same as that of the control (●).

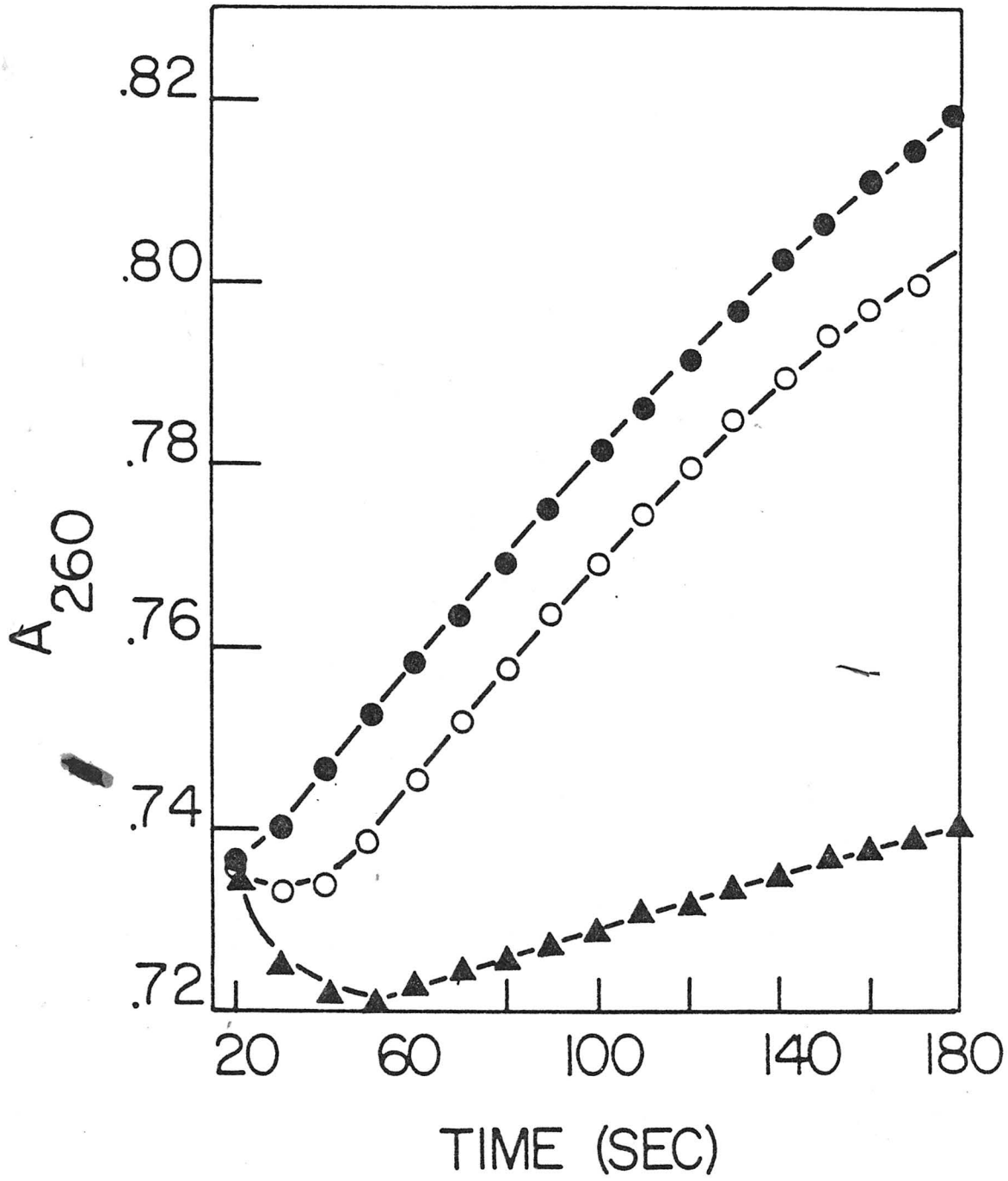

.82
.80
.78
.76
.74
.72
A260
20
60
100
140
180
TIME (SEC)

Figure 4

SDS-polyacrylamide gel (12.5% acrylamide, 0.1% bis-acrylamide) on (a) Triton-solubilized, purified AcChR, (b) AcChR-enriched membrane fragments, (c) DNase I affinity column purified actin from AcChR enriched membrane fragments, (d) chicken smooth muscle actin. The four AcChR subunits (1 = 40,000 daltons, 2 = 50,000, 3 = 60,000, and 4 = 65,000 daltons) are marked as well as the 43,000 dalton band and actin. It can be seen that actin and the 43,000 dalton band are distinct from each other.

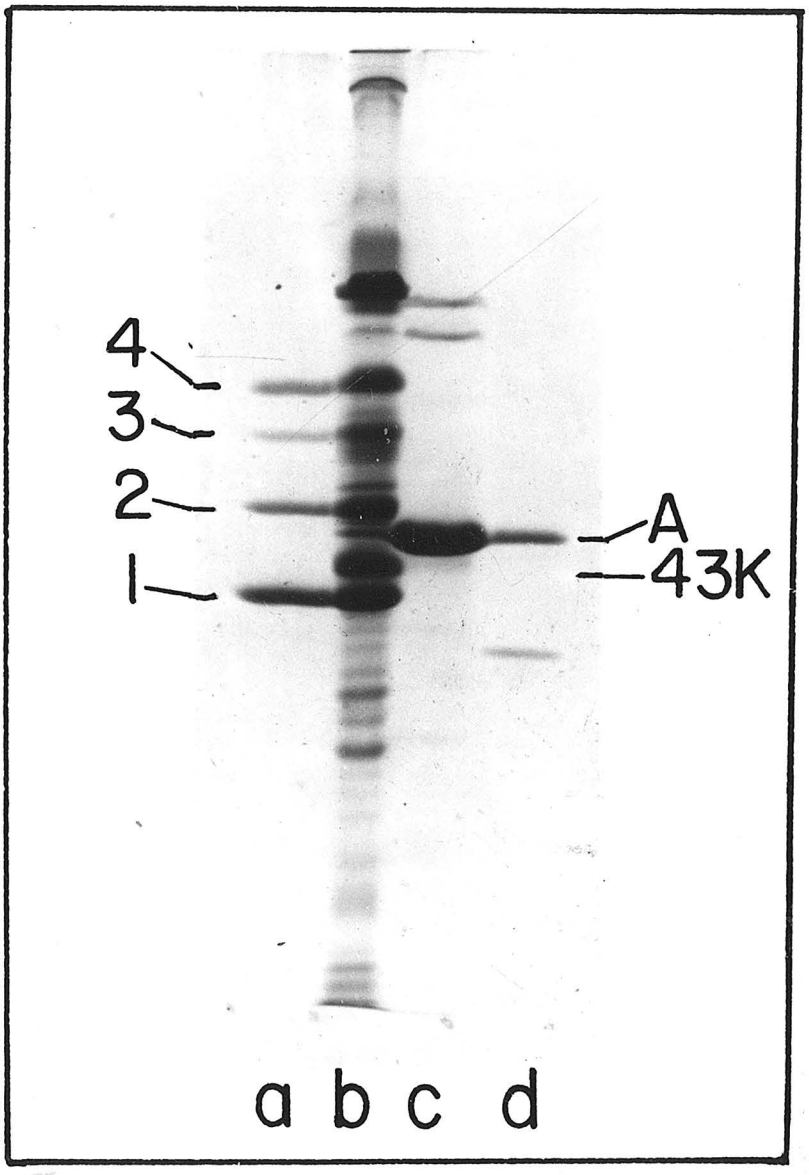
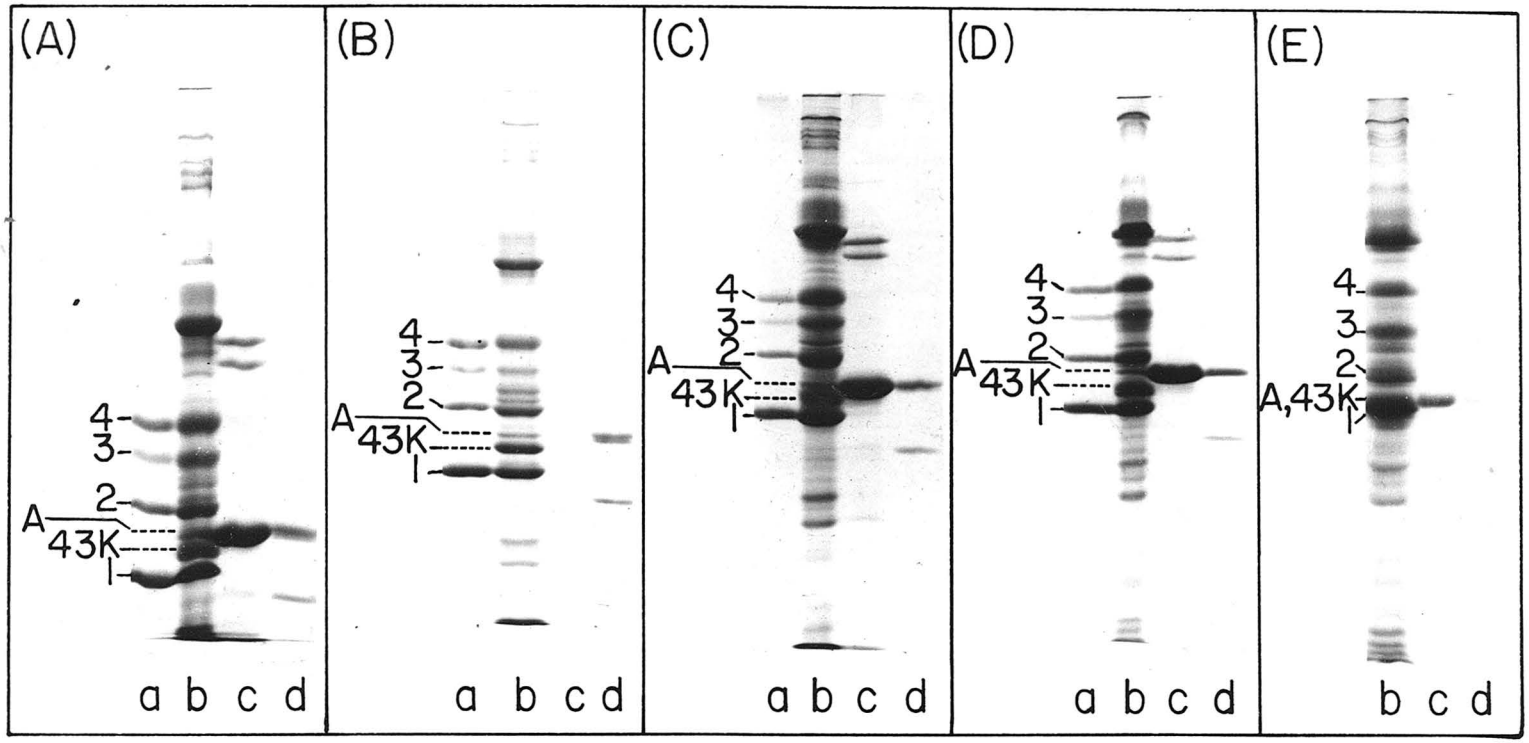

4
3
2
1
A
43K
a b c d

Figure 5

SDS-polyacrylamide gels on (a) Triton-solubilized, purified AcChR, (b) AcChR-enriched membrane fragments, (c) DNase I affinity column purified actin from AcChR enriched membranes, and (d) chicken smooth muscle actin. Laemmli SDS gels are: (A) 7.5% acrylamide, 0.2% bis; (B) 8.7% acrylamide, 0.23% bis; (C) 10% acrylamide, 0.26% bis; (D) 12.5% acrylamide, 0.1% bis; and (E) 12.5% acrylamide, 0.1% bis in half strength Laemmli buffer. The four AcChR subunits are marked, as are the 43,000 dalton protein and actin. The separation of actin from the 43,000 dalton protein is clearest in gel D.


(A)
4
3
2
A
43K
1
a b c d
(B)
4
3
2
A
43K
1
a b c d
(C)
4
3
2
A
43K
1
a b c d
(D)
4
3
2
A
43K
1
a b c d
(E)
4
3
2
A,43K
1
b c d