Interactions of Perhydrohistrionicotoxin with Acetylcholine Receptors from *Torpedo californica* Electroplax

Thesis by

Janet Ruth Elliott

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ABSTRACT

Electrophysiological evidence has shown that the histrionicotoxins reversibly block neuromuscular transmission, acting only in the presence of agonists (Albuquerque, E. X., Barnard, E. A., Chiu, T. H., Lapa, A. J., Dolly, J. O., Jansson, S.-E., Daly, J., and Witkop, B. (1973) Proc. Natl. Acad. Sci. USA 70, 949-953). The use of tritiated perhydrohistrionicotoxin (H_{12}-HTX) and the fluorescent probe ethidium bromide allowed biochemical characterization of the H_{12}-HTX binding site in postsynaptic membranes and after detergent extraction.

[^{3}H]H_{12}-HTX bound with high affinity ($K_D \sim 0.5\ \mu M$) to acetylcholine receptor (AcChR) enriched membranes isolated from Torpedo californica electroplax. The ratio of α-bungarotoxin sites to H_{12}-HTX sites was 4:1. This stoichiometry implied that one H_{12}-HTX bound per AcChR dimer. H_{12}-HTX was not competitive with α-bungarotoxin or agonists, but the agonist recognition site and the H_{12}-HTX site were conformationally linked. The K_D for [^{3}H]H_{12}-HTX was decreased twofold in the presence of carbamylcholine in concentrations sufficient to convert the receptor to the form having high affinity for agonists. Decahydrohistrionicotoxin increased the binding of [^{3}H]acetylcholine. H_{12}-HTX had no effect on [^{3}H]carbamylcholine binding. In this system the twofold higher affinity of H_{12}-HTX for the membrane in the high affinity state was not sufficient to strongly affect the process of agonist induced conversion from the low affinity state to the high affinity state for agonists (in vitro "desensitization"). $3\ \mu M$ H_{12}-HTX did not

directly desensitize nor did it accelerate the rate of agonist induced desensitization or decelerate the rate of recovery from agonist induced desensitization. 30 μM H_{12}-HTX did not directly desensitize nor did it accelerate the rate of agonist induced desensitization or decelerate the rate of recovery from agonist induced desensitization. 30 μM

H_{12}-HTX increased the rate of in vitro desensitization only slightly.

Local anesthetics displaced [^{3}H]H_{12}-HTX bound to membranes in an apparently competitive fashion. The presence of carbamylcholine sufficient to convert the receptor to the high affinity state for agonists modulated the apparent K_Is for some local anesthetics. The differences in their apparent affinities for the low and high affinity states of the receptor roughly paralleled the effects of these local anesthetics on the rate of carbamylcholine induced "desensitization" (Blanchard, S. G., Elliott, J., and Raftery, M. A. (1979) Biochemistry, in press). For example dibucaine, which greatly accelerated the rate of conversion to the high affinity state, bound 17-fold more tightly to the high affinity state, and tetracaine, which slowed the rate of conversion, bound 20-fold more tightly to the low affinity state. This phenomenon may not have been due to a specific binding, since the detergent Triton X-100 also blocked [^{3}H]H_{12}-HTX binding and accelerated in vitro "desensitization" at micromolar concentrations.

Specific ethidium bromide fluorescence was quenched by H_{12}-HTX with an apparent K_I very similar to the K_D determined by direct binding studies. This quenching was probably due to a change in the environment of the bound ethidium rather than an actual displacement.

[^{3}H]H_{12}-HTX binding could be extracted from AcChR enriched membranes by the detergent sodium cholate. Binding activity was sensitive to detergent concentration and was only recovered at low cholate concentrations where some aggregation occurred. The ratio of α-bungarotoxin sites to H_{12}-HTX sites remained 4:1, though the K_D (1.6 μM) was higher than with membranes. The cholate extracts were fractionated by several techniques, yielding an enrichment of AcChR. Treatment of cholate extracts with an α-bungarotoxin-Sepharose resin removed H_{12}-HTX and α-bungarotoxin binding activities in parallel.

Treatment of AcChR enriched membranes with base selectively removed most non-AcChR polypeptides without affecting [^{3}H]H_{12}-HTX binding. This provided positive identification of the H_{12}-HTX binding component with the AcChR, eliminating the possibility that the binding site was located on the 43,000 M_r polypeptide found in native membranes. The local anesthetics dibucaine and tetracaine displaced [^{3}H]H_{12}-HTX bound to base treated membranes with unaltered K_Is Carbamylcholine modulated the affinity of the membranes for H_{12}-HTX and the local anesthetics. [^{3}H]H_{12}-HTX binding could be reconstituted from cholate extracts of treated membranes. Thus the high affinity H_{12}-HTX site appears to be located on one or more of the AcChR polypeptides, and reconstitution of that binding site from detergent extracts does not require the presence of the 43,000 M_r polypeptide.

[^{3}H]H_{12}-HTX binding was largely unaffected by reduction and alkylation of AcChR enriched membranes, a treatment which reduced α-bungarotoxin binding activity. It was likewise unaffected by treatment of the membranes with the carboxyl-modifying reagent trimethyl-

oxonium tetrafluoroborate. Divalent cations (Ca^{2+}, Mg^{2+}) increased the affinity of membranes for [^{3}H]H_{12}-HTX about twofold. [^{3}H]H_{12}-HTX binding was little affected by changes in NaCl concentration of the buffer.

ABBREVIATIONS USED:

AcCh	acetylcholine
AcChE	acetylcholinesterase
AcChR	acetylcholine receptor
BrAcCh	bromoacetylcholine
α-BuTx	α-bungarotoxin
Carb	carbamylcholine
CBB	Coomassie Brilliant Blue
DNPP	Diethylnitrophenyl phosphate
EDTA	ethylenediaminetetracetic acid
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid
HTX	histrionicotoxin
H_2-HTX	dihydroisohistrionicotoxin
H_8-HTX	octahydrohistrionicotoxin
H_{10}-HTX	decahydrohistrionicotoxin
H_{12}-HTX	perhydrohistrionicotoxin
PMSF	phenylmethanesulfonyl fluoride
SDS	sodium dodecyl sulfate
Torpedo Ringers	250 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 4 mM $CaCl_2$, 20 mM Hepes (pH 7.4)
Tris	tris-(hydroxymethyl) amino methane

I. INTRODUCTION

1. General Overview

Transmission of impulses at the motor endplate is initiated by the combination of acetylcholine (AcCh) with specific receptors in the postsynaptic membrane. This leads to a transient increase in the permeability of this membrane to Na^+ and K^+, generating an endplate potential and thus triggering an action potential. These acetylcholine receptors (AcChR) are classed pharmacologically as nicotinic; they largely lack specificity (Barlow, 1965) and nicotine and tetramethylammonium ions block AcCh effects.

The AcChR is an integral membrane glycoprotein consisting of at least two functionalities: (1) an AcCh recognition site which binds agonists such as AcCh or carbamylcholine and antagonists such as curare and α-bungarotoxin, and (2) a site responsible for ion translocation. The ion translocation mechanism is usually thought to be a channel rather than a carrier because of its high conductance ($\sim 10^7$ ions/channel/sec (Neher and Sakmann, 1976)).

The AcChR is found in high densities on the crests of the synaptic folds ($\sim 25,000/\mu M^2$ (Barnard et al., 1975)), where it is advantageously oriented with respect to sites where quanta of AcCh are released from the nerve ending. The receptor density decreases along the postsynaptic membrane towards the depths of the folds, and is estimated to decrease as much as tenfold at the bottom of the folds. By contrast, the density of acetylcholinesterase (AcChE) appears to remain fairly constant from the crests to the base of the folds (Salpeter et al., 1972).

For the purposes of the biochemical characterization of the AcChR, tissues from various sources such as denervated muscles or diaphragms have not been the most suitable because of their complexity and low density of motor endplates. By far the most biochemical work has been carried out using electric organs obtained from fish such as the fresh-water electric eel, Electrophorus electricus, or the elasmobranch electric rays of genus Torpedo and Narcine. These electric organs consist of modified neuromuscular junctions and are relatively simple systems. The large, multinucleate cells have no muscle-like contractile proteins. Their surface is heavily innervated with of the order of 10^5 synapses per cell (Bourgeois et al., 1972). The cells are arrayed in stacks and electrical discharges are achieved by synchronous discharge of the membrane potential of these cells. Electric organs can be obtained in kilogram quantity and from them large amounts of AcChR may be isolated. Torpedo has a higher concentration of AcChR than Electrophorus because all current passes through AcChRs, whereas Electrophorus cells are also electrically excitable, and so current flows through both AcChRs and action potential channels (Bennett, 1970).

The snake venom α-toxins have been particularly useful tools in the isolation and characterization of AcChRs. These include α-bungarotoxin (α-BuTx) from Bungarus multicinctus, cobrotoxin from Naja nigricollis, and erabutoxin from Laticaudata fasciata (for a review see Lee, 1972). These toxins are compact basic polypeptides with 61-74 amino acids which bind noncovalently to the AcChR with K_D as low as 10^{-11} M and extremely slow reversibility. They are specific blockers

of postsynaptic depolarization, and there is little evidence that they have any other effects in vivo (Lee and Chang, 1966). Since they bind to the AcChR with high affinity and specificity, they can be used in radiolabeled form to measure binding in equilibrium or kinetic experiments (Raftery et al., 1975). They can also be coupled to solid supports for affinity chromatography (Changeux, 1975).

2. Isolation and Characterization of the Structure of the AcChR

Several methods have been published for the preparation of membranes enriched in AcChR from Torpedo species (Duguid and Raftery, 1973; Reed et al., 1975; Cohen et al., 1972; Sobel et al., 1977) employing sucrose gradients in swinging bucket and zonal rotors. The separation results from the unusually high density of the AcChR containing membranes (specific gravity ~ 1.18 compared with 1.14 for the average density of membranes from Torpedo electroplax (Hartig and Raftery, 1979)). The separation has recently been made faster and more reproducible by the use of reorienting sucrose gradients (Elliott et al., 1979).

Electron microscopy shows that these preparations consist primarily of spherical vesicles ranging in diameter from 1000 to 10,000 Å, many of which are covered by dense arrays of rosettes 60-70 Å in diameter which have an electron-dense pit in the center (Nickel and Potter, 1973; Cartaud et al., 1973; Raftery et al., 1974). These rosette structures have been positively identified with the AcChR (Klymkowsky and Stroud, 1979). Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis shows that the major polypeptides

found in these membranes have apparent molecular weights of 40,000, 43,000, 50,000, 60,000, 65,000, and 90,000 daltons.

The AcChR has been solubilized and purified from the electric organs of a variety of fish (reviewed by Heidmann and Changeux, 1978). In general, non-ionic detergents such as Triton X-100 are used to solubilize the receptor which is then purified on an affinity column consisting of a cholinergic ligand coupled to a solid support. The ligands used are a variety of quaternary ammonium derivatives or snake α-toxins. The specific activity of the best preparations varies from 8-12 nmole/mg protein in α-toxin binding sites. Electron microscopy of these purified preparations also reveals the rosettes described above (Cartaud et al., 1973).

The purified AcChR is reported to have four types of polypeptide subunits of molecular weights 40,000, 50,000, 60,000, and 65,000 daltons as determined by SDS polyacrylamide gel electrophoresis (Raftery et al., 1974; Weill et al., 1974; Raftery et al., 1975; Karlin et al., 1975; Vandlen et al., 1976; Lindstrom et al., 1978). The stoichiometry of these subunits is unknown. Conflicting reports assert that the receptor consists only of 40,000 subunits, the higher molecular weight polypeptides being artifacts of purification (Sobel et al., 1977). The four subunits have different amino acid compositions (Raftery et al., 1975; Vandlen et al., 1979). Antibodies raised against each subunit precipitate the whole AcChR (Claudio and Raftery, 1977). The agonist binding site has been shown by affinity labeling to be located on the 40,000 subunit (Weill et al., 1974; Moore and Raftery, 1979a).

Upon detergent extraction the AcChR exists primarily in two forms, one having sedimentation coefficient ~9 S and the other ~13 S (Raftery et al., 1972). The binding properties of these forms for cholinergic ligands and α-toxins appear to be essentially the same (Raftery et al., 1972; Sugiyama and Changeux, 1975; Gibson et al., 1976). The two forms represent monomers and dimers formed by an intermolecular disulfide linkage between 65,000 subunits (Chang and Bock, 1977; Hamilton et al., 1977; Witzemann and Raftery, 1978; Hucho et al., 1978). The molecular weight of the monomer has been determined by several methods: SDS polyacrylamide gel electrophoresis after crosslinking gives 260,000 (Biesecker, 1973) or 275,000 (Hucho and Changeux, 1973), osmometry gives 270,000 (Martinez-Carrion et al., 1975), sedimentation coefficients determined in sucrose gradients give about 250,000 (Raftery et al., 1972, 1974; Meunier et al., 1972), and the most recent sedimentation equilibrium experiments give 250,000 (Reynolds and Karlin, 1978). The most recent estimates of specific activity for α-BuTx binding (Vandlen et al., 1979) indicate that two toxin molecules bind per AcChR monomer.

The amino acid compositions of the AcChR oligomer and its subunits reveal average hydrophobicity intermediate between typical membrane and non-membrane proteins (Raftery et al., 1975; Vandlen et al., 1979), indicating that a substantial part of the receptor molecule may extend beyond the domain of the membrane or that the increased polarity might be due to the requirement for a hydrophilic channel through the receptor protein. Indeed, X-ray difraction studies

of AcChR enriched T. californica membrane fragments have shown that the protein present spanned the membrane, extending 55 Å on one side of the bilayer and 15 Å on the other (Ross et al., 1977).

The AcChR contains 20-34 nmole/mg protein free sulfhydryl groups, comprising 18-30% of the total cysteic acid residues (Eldefrawi et al., 1975; Chang and Bock, 1977). A Cys residue in or near the agonist binding site can be selectively alkylated with bromoacetylcholine (BrAcCh) after treatment of the receptor by dithiothreitol (Moore and Raftery, 1979a). Moore and Raftery (1979b) have demonstrated that transformations between the different conformational states of the membrane-bound AcChR are regulated by disulfide-sulfhydryl interchange reactions.

Purified AcChR contains approximately seven phosphoserine residues per molecule, and these residues occur in all four subunits (Vandlen et al., 1979). No phosphothreonine was detected. It has recently been proposed that the level of phosphorylation of the membrane bound receptor may regulate some of its functional properties, such as conformational changes or its localization in the neuromuscular junction (Gordon et al., 1977; Teichberg et al., 1977).

All four subunits of purified AcChR contain carbohydrate. The neutral sugars mannose, glucose, and galactose in the approximate molar ratio of 10:1:2 are present in the whole receptor at the level of 2.7-3.5% by weight and glucosamine is also detected (Vandlen et al., 1979).

3. Ligand Binding to the AcChR

Binding of agonists such as AcCh, carbamylcholine (Carb), and decamethonium and antagonists such as d-tubocurarine to the AcChR in membrane bound and in detergent solubilized form has been measured directly using radiolabeled ligands (Moody et al., 1973; Raftery et al., 1974; Meunier et al., 1974) and indirectly by the inhibition of radiolabeled α-toxin binding (Raftery et al., 1974; Weber and Changeux, 1974). Solubilization of the membranes in many cases greatly alters the observed K_Ds; extrapolations from solubilized preparations to membrane bound AcChR are dangerous. Rang (1975) has tabulated a great many of the K_Ds reported in the literature for agonists and antagonists to both membrane bound and solubilized AcChR.

Values from 1 to 2 are reported for the ratio of α-toxin binding sites to agonist binding sites (see Heidmann and Changeux, 1978). Most recently Raftery et al. (1979) have shown that the agonists AcCh, Carb, and BrAcCh all bind to AcChR enriched membranes with K_D in the range of $1\text{-}5 \times 10^{-8}$ M and stoichiometry of 2:1 toxin sites: agonist sites. One possible explanation for the inconsistency in reported stoichiometries is the recent observation that reduction with dithiothreitol followed by alkylation with iodoacetamide could greatly reduce the number of α-BuTx sites while not significantly affecting the number of [^{3}H] AcCh sites (Dunn and Raftery, unpublished). Thus it is conceivable that the amount of oxidation undergone by membranes during purification affects the number of α-toxin sites detectable.

A number of fluorescent ligands have been used as probes of AcChR function including dansylcholine (Cohen and Changeux, 1973), quinacrine (Grünhagen and Changeux, 1976), and ethidium bromide (Schimerlik and Raftery, 1976; Schimerlik et al., 1979a). Stopped flow kinetic studies with quinacrine (Grünhagen et al., 1977) and ethidium (Quast et al., 1978a; Quast et al., 1979) have led to the proposal of kinetic schemes for agonist binding to AcChR enriched membranes. However, it is difficult at this time to correlate the spectroscopic events in vitro with the events of receptor function in vivo.

4. Transitions Between Ligand Affinity States

It has been reported that preincubation with agonists and certain antagonists induces a conformational change in the membrane bound AcChR from a pre-existing state having low affinity for agonists to one(s) having high affinity. This has been described based on the effects of preincubation with agonists on the initial rate of α-toxin binding to membrane preparations (Weber et al., 1975; Weiland et al., 1976; Bonner et al., 1976; Barrantes, 1976; Colquhoun and Rang, 1976; Lee et al., 1977; Quast et al., 1978 b). Conversion of the receptor by agonists from a state of low affinity to one(s) of high affinity has been suggested to be an in vitro correlate of in vivo desensitization as originally described by Katz and Thesleff (1957). This is a decrease in membrane conductance to normal levels in the presence of continued high agonist concentrations. The state of low affinity presumably represents the receptor in its resting state in the

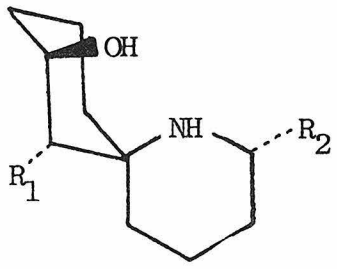
membrane prior to interaction with an agonist, and low affinity binding is associated with activation of the system leading to membrane depolarization. The significance of high affinity binding in the normal physiological situation is as yet unclear.

The rate of in vivo desensitization increases with increasing agonist concentration, and the rate of recovery is constant and independent of the rate of desensitization or which agonist was used to induce desensitization (Katz and Thesleff, 1957). In addition, there are certain agents which, though they are not agonists and do not directly desensitize, can affect the rate of desensitization in the presence of agonist. These are termed "metaphilic" agents. These include some local anesthetics and multivalent cations, which accelerate desensitization in the order:
$Mg^{2+} \ll Ca^{2+} \leq Ba^{2+} < Sr^{2+} \ll Al^{3+} < La^{3+}$ (Magazanik and Vyskocil, 1973; Magazanik, 1976). In the above respects the change in ligand affinity state in vitro parallels desensitization.

An approach which has been used in the study of desensitization and other functional properties of the AcChR in vitro is the measurement of rapid $^{22}Na^{+}$ efflux from preloaded membrane vesicles in response to agonists. Recent improvements in this technique have greatly increased its usefulness (Hess and Andrews, 1977; Moore et al., 1979).

5. The Histrionicotoxins

The histrionicotoxins were first isolated and characterized in 1971 (Daly et al., 1971). The two major basic alkaloids, histrionicotoxin (HTX) and dihydroisohistrionicotoxin (H_2-HTX) were isolated from methanol extracts of the skins of 400 poison arrow frogs, Dendrobates histrionicus, collected in the tropical rainforest of western Columbia. Later several other minor constituents were isolated differing only in the degree of unsaturation of the side chains (Tokuyama et al., 1974). The X-ray structures of HTX (Daly et al., 1971) and H_2-HTX (Karle, 1973) were reported. The fully saturated derivative perhydrohistrionicotoxin (H_{12}-HTX), which was not isolated from the extracts, was prepared by catalytic reduction and shown to have biological activity similar to HTX (Albuquerque et al., 1973a). The structures of histrionicotoxins are shown below:



HTX: R_1 = -CH=CH-C≡CH, R_2 = $-CH_2CH=CH-C\equiv CH$

H_2-HTX: R_1 = -CH=CH-C≡CH, R_2 = $-CH_2CH_2CH=C=CH_2$

H_8-HTX: R_1 = $-CH_2CH_2CH=CH_2$, R_2 = $-CH_2CH_2CH_2CH=CH_2$

H_{12}-HTX: R_1 = $-C_4H_9$, R_2 = $-C_5H_{11}$

The histrionicotoxins represent the first occurrence of acetylenes or allenes in the animal kingdom. The spiro ring system also has no precedent. Histrionicotoxins and pumiliotoxins occur together in some species of Dendrobates, and may share a common precursor, perhaps an unsaturated disubstituted piperidine which could undergo cyclization to either ring system.

Histrionicotoxins were interesting and challenging molecules to synthetic organic chemists because of the spiro ring system and the four asymmetric carbons. In 1975 syntheses were reported for H_{12}-HTX (Corey et al., 1975a, 1975b; Aratani et al., 1975; Fukuyama et al., 1975) and octahydrohistrionicotoxin (H_8-HTX) (Fukuyama et al., 1975).

The first electrophysiological studies were reported in 1973 by Albuquerque and his coworkers (Albuquerque et al., 1973a, 1973b). Neither H_{12}-HTX nor HTX by itself depolarized the postsynaptic membrane. Both compounds blocked postsynaptic depolarization produced by indirect stimulation or by microiontophoretically applied AcCh and also AcCh induced extrajunctional depolarization of denervated muscle. H_{12}-HTX was slightly more potent. The onset of blockade was dependent on the rate of application of AcCh. When AcCh was applied in pulses of 1/sec after preincubation with 15 μM H_{12}-HTX, complete blockade was produced after the fifth stimulus. If stimulation was interrupted for several minutes and then resumed, the same pattern was repeated. Blockade was reversible. Washing resulted in complete recovery of end plate potential.

Recent work showed that the H_{12}-HTX concentrations required for half-maximal blockade of rat muscle endplate potentials and extrajunctional AcCh sensitivity were 8.4 μM and 2.4 μM, respectively (Dolly et al., 1977). Concentrations of H_{12}-HTX required to block α-BuTx binding were over two orders of magnitude higher, and the block was non-competitive. Other studies have elaborated on these observations (Albuquerque et al., 1975; Lapa et al., 1975).

These results were confirmed by the work of another group (Glavinovic et al., 1974; Kato et al., 1974; Kato and Changeux, 1976). In addition HTX was shown to block the spontaneous and glutamate evoked discharges of spinal and cortical neurons in the cat central nervous system. HTX also blocked the steady-state depolarization of the Electrophorus electroplaque produced by bath applied Carb.

Certain local anesthetics such as SKF 525A and dimethisoquin show effects which are very similar to the effects of HTX described above. One possible explanation for these effects is that HTX and local anesthetic are metaphilic agents which greatly accentuate the process of desensitization (Magazanik and Vyskocil, 1973; Kato and Changeux, 1976). Another hypothesis is that these agents interact with the open channel form of the AcChR, binding to the ionophore or to an entity which regulates the ionophore in such a way as to cause blockage of the ion channel (Albuquerque et al., 1973a, 1973b).

The object of the present study has been the biochemical characterization of HTX action. This has included (1) the preparation and characterization of a tritiated derivative, [^{3}H]H_{12}-HTX, (2) use of this derivative and also the fluorescent probe, ethidium bromide, to

characterize the binding of HTX to AcChR enriched membrane fragments and the effects of other ligands on this binding, (3) determination of the effects of HTX on the agonist affinity state of the receptor, and (4) the determination of the conditions under which HTX binding can be solubilized from membrane fragments and the development of new purification techniques to localize the site of HTX binding.

II. EXPERIMENTAL

1. Materials

Lyophilized crude Bungarus multicinctus venom was obtained from Sigma Chemical Co. H_{12}-HTX and H_8-HTX were gifts of Dr. Y. Kishi, who produced them synthetically (Aratani et al., 1975; Fukuyama et al., 1975), and H_{10}-HTX was provided by Richard Cherpeck. Tritiation of H_8-HTX was performed at ICN Pharmaceuticals, Inc., Irvine, California. Carb was obtained from Aldrich Chemical Co. and AcCh from Sigma Chemical Co. [^{3}H]Carb was prepared by Dr. Yuan Chao and [^{3}H]AcCh was purchased from ICN Pharmaceuticals. Ethidium bromide was obtained from Calbiochem, procaine from Sigma Chemical Co., dibucaine and tetracaine from ICN Pharmaceuticals, and lidocaine from Pfaltz and Bauer. BrAcCh was prepared by Moore and Raftery as previously described (Moore and Raftery, 1979a). Sodium cholate was obtained from Schuchardt Co. All other chemicals were of the highest purity commercially available.

DEAE discs were from Whatman, Ltd. Scintillation counting was done in a Packard Tricarb Liquid Scintillation Spectrometer Model 3375 using Aquasol (New England Nuclear) or 25% v/v Triton X-100, 0.55% Permablende III (Packard) in toluene as scintillation fluid.

Torpedo californica were obtained locally and maintained in a seawater tank at 16°C for no more than 7 days or the excised electric organs were quick-frozen in liquid nitrogen and stored at -90° C.

2. Preparation of [^{125}I]α-BuTx

α-BuTx was purified from crude Bungarus multicinctus venom by cation exchange chromatography (Clark et al., 1972) and iodinated with ^{125}I, which adds to a tyrosine residue. The monoiodo derivative was purified from other labeled species by CM-Sephadex chromatography and usually had specific activities of 1-3 Ci/nmole with biological activity indistinguishable from unlabeled toxin (Blanchard et al., 1979a).

3. Preparation of [^{3}H]H_{12}-HTX

[^{3}H]H_{12}-HTX was prepared at ICN Pharmaceuticals, Irvine, California, by the catalytic reduction of H_8-HTX with carrier free [$^{3}H_2$] over Pd/C as previously described (Tokuyama et al., 1975). The product was purified by preparative thin layer chromatography on silica gel in 9:1 chloroform:methanol. The [^{3}H]H_{12}-HTX was routinely checked for purity by analytical thin layer chromatography and was found to be stable for extended periods up to one year following initial purification.

4. AcChR Enriched Membrane Preparations

Approximately 450 g of Torpedo electric organ was minced into small pieces and an equal volume of cold buffer [10 mM NaCl, 5 mM EDTA, 0.02% NaN_3, 5 mM iodoacetamide, 10 mM PMSF] was added. After a 2-min initial grind at high speed in a commercial Waring blendor the homogenate was reground in small portions for 4 periods of 30 sec at 30,000 rpm in a Virtis "60". Connective tissue and other large particles were pelleted by centrifugation of the homogenate at 5000 rpm

for 10 min in a Sorvall GSA rotor. The supernatant was passed through two layers of cheesecloth and centrifuged at 16,000 rpm for 1 hour in a Beckman Type 35 rotor at 2°C. The pellet was resuspended for 2 periods of 30 sec at 30,000 rpm in the Virtis homogenizer in 10 mM NaP_i (pH 7.4), 1 mM EDTA, 0.02% NaN_3. Sucrose and NaCl were then added to final concentrations of 30% w/w and 400 mM, respectively.

10 Ml per tube of this sample were layered atop discontinuous sucrose gradients consisting of 5 ml 50% w/w, 5 ml 39% w/w, 12 ml 35% w/w sucrose in 0.4 M NaCl, 10 mM NaP_i (pH 7.4), 1 mM EDTA, 0.02% NaN_3. The samples were overlayed with the above buffer containing no sucrose to fill the tubes to 34 ml. The tubes were centrifuged at 45,000 rpm for one hour in a Beckman VTi50 vertical rotor using a Beckman model L-5 centrifuge with slow acceleration and deceleration in the range of 0-1000 rpm. Identical results were obtained using a Sorvall TV-850 rotor. Following centrifugation three light scattering bands were observed: heavy bands at the top and middle of the gradients and a lighter one near the bottom. The bands were collected by aspiration, diluted two fold into buffer containg no sucrose and centrifuged for 1 hour at 30,000 rpm in a Beckman Type 35 rotor. The membranes were resuspended on the Virtis homogenizer and assayed for [^{125}I] α-BuTx binding (Schmidt and Raftery, 1973a) and protein concentration (Lowry et al., 1951).

5. Base Treatment of AcChR Enriched Membranes

Base treatment of the membranes was performed essentially as described by Steck and Yu (1973) for erythrocyte ghosts. For routine

preparations membranes containing approximately 60 mg of protein were pelleted and then resuspended in 32 ml ice cold distilled water. The pH was adjusted to 11.0 with 0.5% NaOH, and the membranes were immediately pelleted for 30 min at 30,000 rpm in a Type 35 rotor. The pellet was resuspended in buffer at pH 7.4.

6. Fractionation of the Protein Components of Enriched Membranes

The membranes were dissolved in 2% sodium cholate containing 10 mM NaP_i (pH 7.4), 400 mM NaCl, 1 mM EDTA, 0.02% NaN_3 by stirring on ice for 30 min, followed by centrifugation at 100,000 g for 1 hour at 0°. 1 Ml of the supernatant containing 10 mg protein was applied to a 13 ml 5 - 20% w/v sucrose gradient containing 2% cholate. The gradients were centrifuged 18.5 hour at 40,000 rpm in a Beckman SW 41 rotor at 0°C. Approximately 13 1-ml fractions were collected and assayed for protein and [^{125}I] α-BuTx sites.

7. Treatment of Membranes with Alkylating or Acetylating Agents

Covalent labeling of reduced AcChR with BrAcCh was carried out as follows: dithiothreitol to a final concentration of 40 μm was added to membranes (2 μM in α-BuTx sites) which had been pretreated with the esterase inhibitor diethylnitrophenylphosphate (DNPP). Reduction was performed at room temperature for 1 hr and BrAcCh (2 μM) was then added and the reaction allowed to continue for another hour at 4° C. The membranes were then dialysed overnight at 2° C against two changes of buffer (20 mM Hepes (pH 7.4), 250 mM NaCl, 5 mM KCl, 0.02% w/v NaN_3) to remove excess reagents. Under these

conditions essentially all of the specific BrAcCh sites were covalently labeled (Moore and Raftery, 1979a).

For chemical modification, membrane suspensions of AcChR containing 1-2 μM α-BuTx sites in Hepes buffer (250 mM NaCl, 5 mM KCl, 20 mM Hepes (pH 7.4), 0.02% NaN_3) were allowed to react with 1 mM dithiothreitol. The reaction vials were flushed with argon and allowed to stand at room temperature for one hour. Alkylation of reduced AcChR was accomplished by adding 3 mM iodoacetamide to the reduced membrane preparation, and the reaction was allowed to continue for another 2 hours at 4° C. The membrane fragments were then pelleted, washed, and resuspended in fresh Torpedo Ringers. Stock solutions of reagents were made in 200 mM Tris buffer, pH 8.5.

Treatment of AcChR enriched membranes with $(CH_3)_3O^+BF_4^-$ was performed as described by Chao et al. (1975) with slight modifications. Membrane fragments containing 20 mg protein were suspended in 5 ml of 10 mM NaP_i (pH 7.4). 30 Mg $(CH_3)_3O^+BF_4^-$ was added in 5 mg aliquots as the reaction mixture was stirred on ice and the pH was maintained between 6.5 and 7.0 with 0.1 M NaOH. The reaction was allowed to continue for 30 min, and then the reaction mixture was dialysed overnight at 2° C against 2 × 500 ml Hepes buffer.

8. Solubilized AcChR

Detergent extracts of AcChR enriched membrane fragments were prepared as follows: The detergent and membranes (5-10 μM in α-BuTx sites) were stirred for 30-60 min on ice. Insoluble material was then pelleted by centrifugation at 40,000 rpm in a Beckman Type

65 rotor (100,000 g) for 1 hour.

AcChR was purified by affinity chromatography as previously described (Schmidt and Raftery, 1972, 1973b; Vandlen et al., 1976, 1979). Briefly, the membranes were prepared by homogenization and low speed centrifugation as was described above for AcChR enriched membrane preparations. They were then dissolved in Triton X-100 and chromatographed on an affinity column consisting of $-NH(CH_2)_5CONH(CH_2)_3N(CH_3)_3Cl$ coupled to Sepharose.

9. [^{125}I]α-BuTx Binding Assays

The time course of [^{125}I] α-BuTx-AcChR complex formation was followed by a modification of the DEAE disc method (Schmidt and Raftery, 1973a). For each time point a 0.1 ml aliquot of the reaction mixture was pipetted onto a Whatman DE-81 filter paper disc, thus quenching the reaction (Blanchard et al., 1979a). The discs were then washed in three changes of buffer containing 100 mM NaCl, 0.1% (v/v) Triton X-100, 10 mM NaP_i, pH 7.4. Radioactivity was determined using a Packard liquid scintillation spectrometer (Model 3375) or a Beckman 4000 gamma counter. The rate of [^{125}I]α-BuTx binding to the receptor was determined from the slope of a plot of ln ($C_\infty - C_t$) versus time where C_t was the cpm bound to the disc at time t and C_∞ was the cpm bound at completion of the reaction (typically measured 3-5 hours after the start of the reaction). This method has been shown to give an accurate measurement of the rate of [^{125}I]α-BuTx-AcChR complex formation (Blanchard et al., 1979a).

For measurement of the rate of the receptor transition from the low to high affinity state for agonists, membranes were incubated with H_{12}-HTX for 15-20 minutes at room temperature followed by addition of Carb (1 μM final concentration). After a given time, [^{125}I]α-BuTx was added and the rate of toxin-receptor complex formation, k_{app}, was determined as described above. The variation of k_{app} versus time of incubation was fit to an exponential decay of the form:

$$k_{app}(t) = (k_0 - k_\infty)e^{-k't} + k_\infty \qquad (1)$$

where k_0 was the rate of toxin binding in the presence of 1 μM Carb when all of the receptor was in the high affinity state (for the purposes of these experiments, this condition was fulfilled after 15 minutes preincubation), and k' was the rate of the transition in receptor affinity for agonists. Estimates for k' were obtained from a linear least squares fit of the data to the logarithmic form of equation 1. Conversely, the rate of receptor recovery from the high affinity to the low affinity state was determined as above by monitoring the initial rate of [^{125}I]α-BuTx binding in the presence of 1 μM Carb at given time intervals after dilution out of 1 μM Carb.

10. Ligand Binding Assays

[^{3}H] H_{12}-HTX binding to AcChR enriched membrane fragments was measured by a centrifugation assay and by equilibrium dialysis. In the centrifugation assay 150 μl samples containing membrane fragments, [^{3}H] H_{12}-HTX and any other desired ligands were incubated for 20 minutes at room temperature. The membranes in Torpedo Ringers

were then pelleted by centrifugation for 20 minutes at 30 psi (g_{av} = 132,000) in a Beckman Airfuge. 10 μl samples from the top of each centrifuge tube (in triplicate) were counted before and after centrifugation in 5 ml Triton-Toluene scintillation fluid (in a Packard Model 3375 Scintillation Spectrometer). Since the cellulose nitrate tubes normally supplied with the Airfuge were found to adsorb large amounts of [^{3}H]H_{12}-HTX, polyethylene tubes were substituted. Binding to detergent extracts was measured by equilibrium dialysis. 0.5 Ml of the sample plus [^{3}H]H_{12}-HTX was dialysed against 1.5 ml of Ringers plus [^{3}H]H_{12}-HTX for 16 hours at 4°C with gentle shaking. Results obtained for membrane fragments in the centrifugation assay were the same as those obtained by equilibrium dialysis. Unless otherwise stated, all binding experiments were performed in Torpedo Ringers (250 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 4 mM $CaCl_2$, 20 mM Hepes pH 7.4, 0.02% NaN_3). Scatchard plots (Scatchard, 1949) of binding data were fit using an unweighted linear, least squares fit.

[^{3}H] Carb and [^{3}H] AcCh binding to AcChR enriched membrane fragments were measured by centrifugation assays. The ligand was incubated with the membranes for 30 min, and then the membranes were pelleted in the Airfuge in the case of [^{3}H] Carb or in a Beckman Type 65 rotor at 40,000 rpm for 30 min in the case of [^{3}H] AcCh. Duplicate 50 μl ([^{3}H]Carb) or 1 ml ([^{3}H] AcCh) samples were withdrawn before and after centrifugation and these were counted in 10 ml Aquasol.

11. Fluorescence Experiments

Ethidium bromide fluorescence experiments were performed at 25°C on a Perkin Elmer MPF-4 fluorimeter. The wavelength for excitation was 483 nm for experiments involving AcChR enriched membrane fragments and 490 nm for those using solubilized purified AcChR; in both cases emission was monitored at 610 nm. Gain and slit widths were optimized for each experiment.

12. Gel Electrophoresis

8.75% polyacrylamide SDS gels were run using the buffer system of Laemmli (1970). The gels were stained for protein with 0.05% (w/v) CBB in 10% acetic acid, 25% methanol and destained in the same solution without the dye. Densitometer scans were at 550 nm using a Gilford 240 with linear-transport accessory.

13. Binding of AcChR to α-BuTx-Sepharose

α-BuTx was coupled to CNBr-activated Sepharose 2B as described by Porath et al. (1973). The amount of α-BuTx coupled was determined by inclusion of a [^{125}I]α-BuTx tracer. The amount of this which was active was determined from the ability of the resin to remove purified AcChR from solution. In general approximately 20% of the coupled α-BuTx was active, and typical activities obtained for the resin were 0.02 μM/g dry weight.

III. BIOCHEMICAL STUDIES ON PERHYDROHISTRIONICOTOXIN FUNCTION

1. Characterization of [^{3}H]H_{12}-HTX

After purification, the [^{3}H]H_{12}-HTX was homogeneous by thin layer chromatography and cochromatographed with unlabeled H_{12}-HTX (Figure 1). The purified product bound to membrane fragments and was displaced by increasing amounts of cold H_{12}-HTX with I_{50} equal to approximately 0.5 μM (Figure 2). For use in binding assays the [^{3}H]H_{12}-HTX was isotopically diluted approximately 100 fold with unlabeled H_{12}-HTX and the specific activity was therefore defined by the known concentration of the unlabeled toxin. Two dilutions were used for the work reported here with specific activities of 1.03 and 1.38 Ci per millimole. The dissociation constants for binding to Torpedo californica membrane fragments obtained with the two dilutions were the same within experimental error, (K_D = 0.57 ± 0.06 μM). In order to verify the specific activity of the [^{3}H]H_{12}-HTX dilutions, inhibition of binding by two concentrations of unlabeled H_{12}-HTX was measured (Figure 3). This experiment yielded a K_D for [^{3}H]H_{12}-HTX of 0.2 μM and K_I for unlabeled H_{12}-HTX of 0.3 μM.

2. Characterization of [^{3}H]H_{12}-HTX Binding to Torpedo californica Membranes

As has been previously reported (Elliott and Raftery, 1977, 1979) the binding of [^{3}H]H_{12}-HTX to membrane fragments could be resolved into two components, a hyperbolic saturable binding isotherm super-

FIGURE 1: Thin Layer Chromatography of Purified [^{3}H]H_{12}-HTX in 9:1 Chloroform:Methanol on a Silica Plate: The plate was cut into pieces approximately 1/8 inch wide which were counted in 5 ml scintillation fluid. The [^{3}H]H_{12}-HTX comigrated with authentic H_{12}-HTX which was detected by staining the plate with iodine.

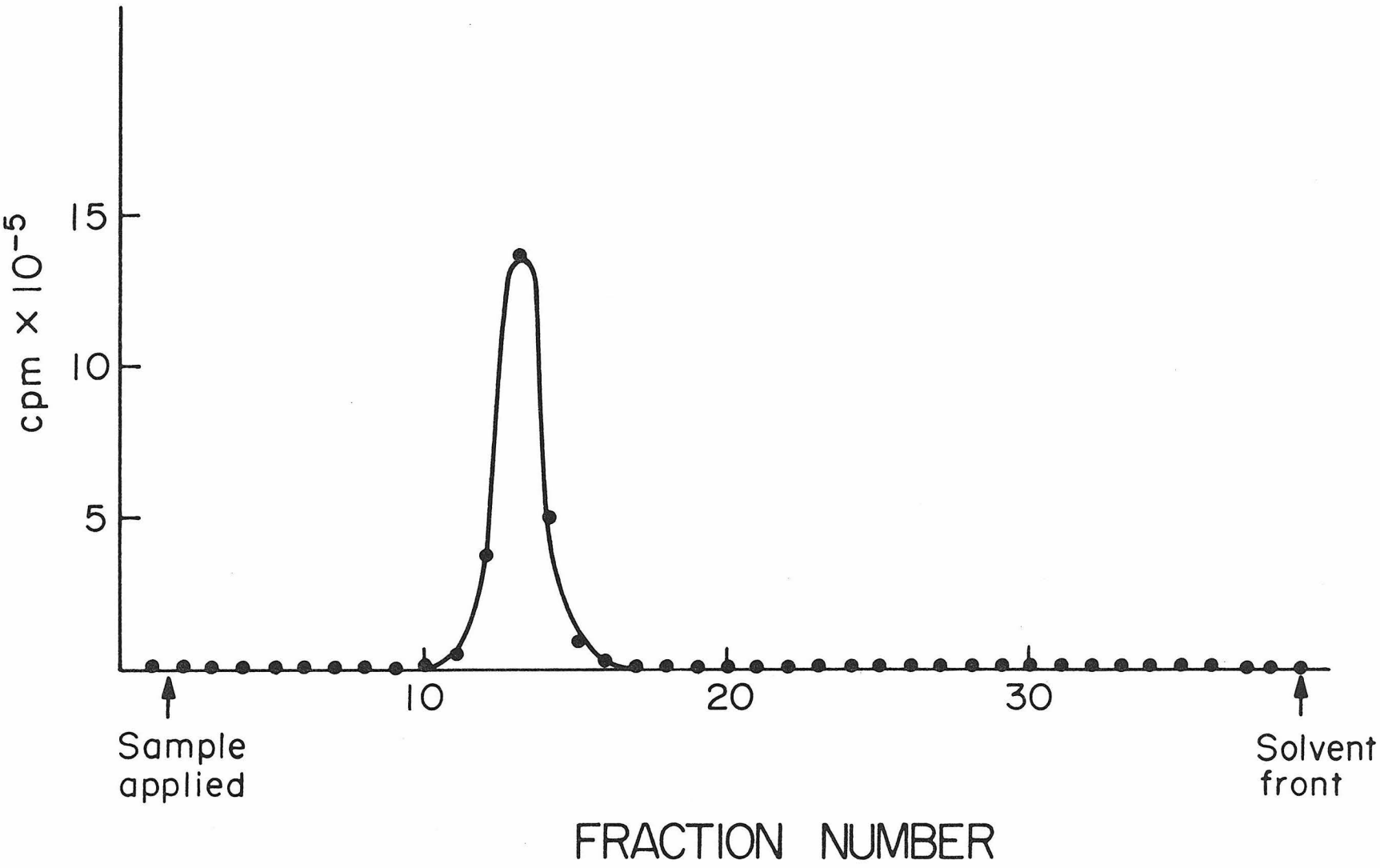

cpm X 10⁻⁵
15
10
5
10
20
30
Sample applied
Solvent front
FRACTION NUMBER

FIGURE 2: Displacement of Undiluted [^{3}H]H_{12}-HTX by Cold H_{12}-HTX:

Binding to membrane fragments, having a concentration of 0.5 μM [^{125}I]α-BuTx sites, was measured by centrifugation assay in order to show that all [^{3}H]H_{12}-HTX binding could be displaced by cold H_{12}-HTX. The concentration of the [^{3}H]H_{12}-HTX before isotopic dilution was not determined.

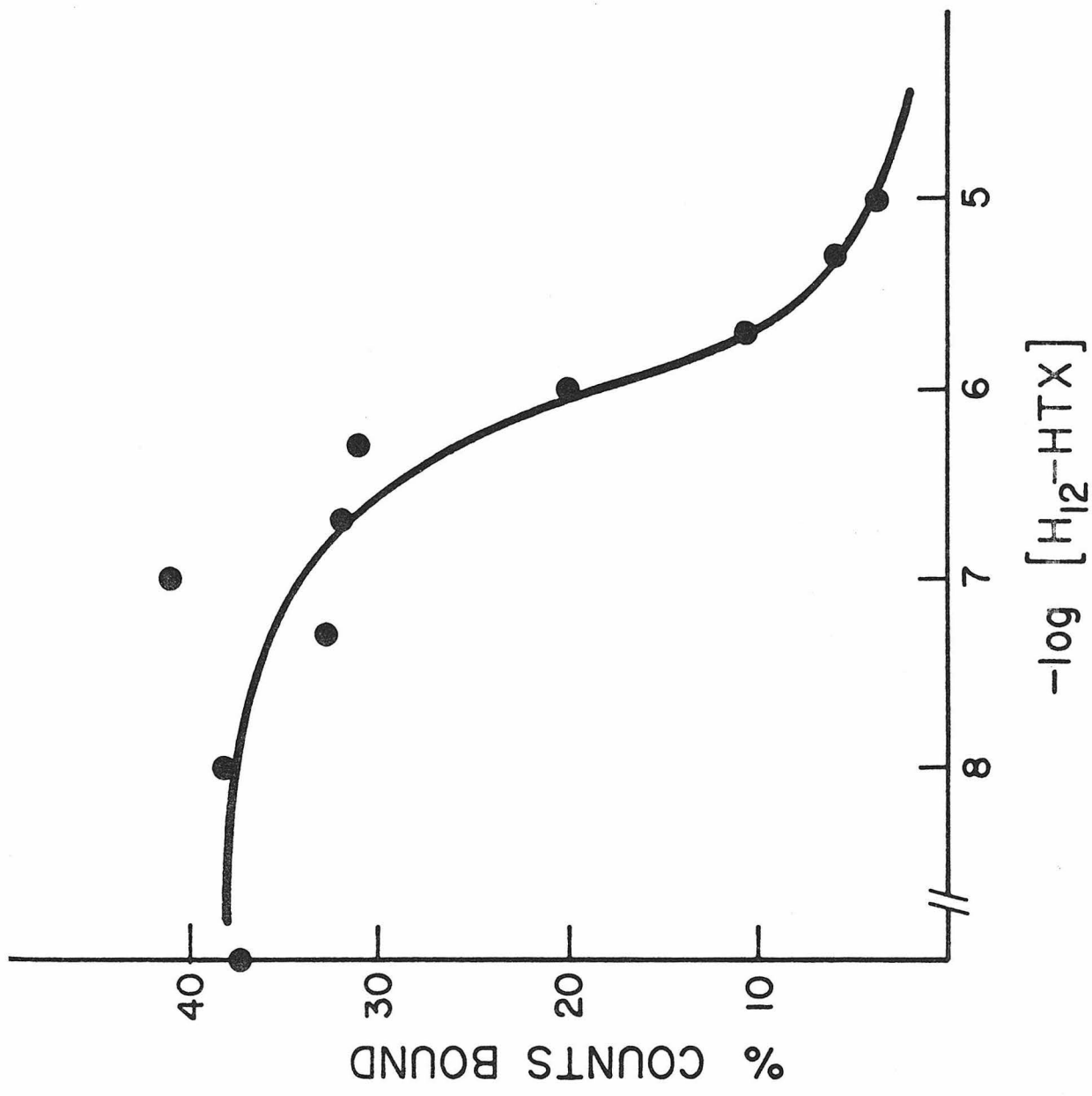
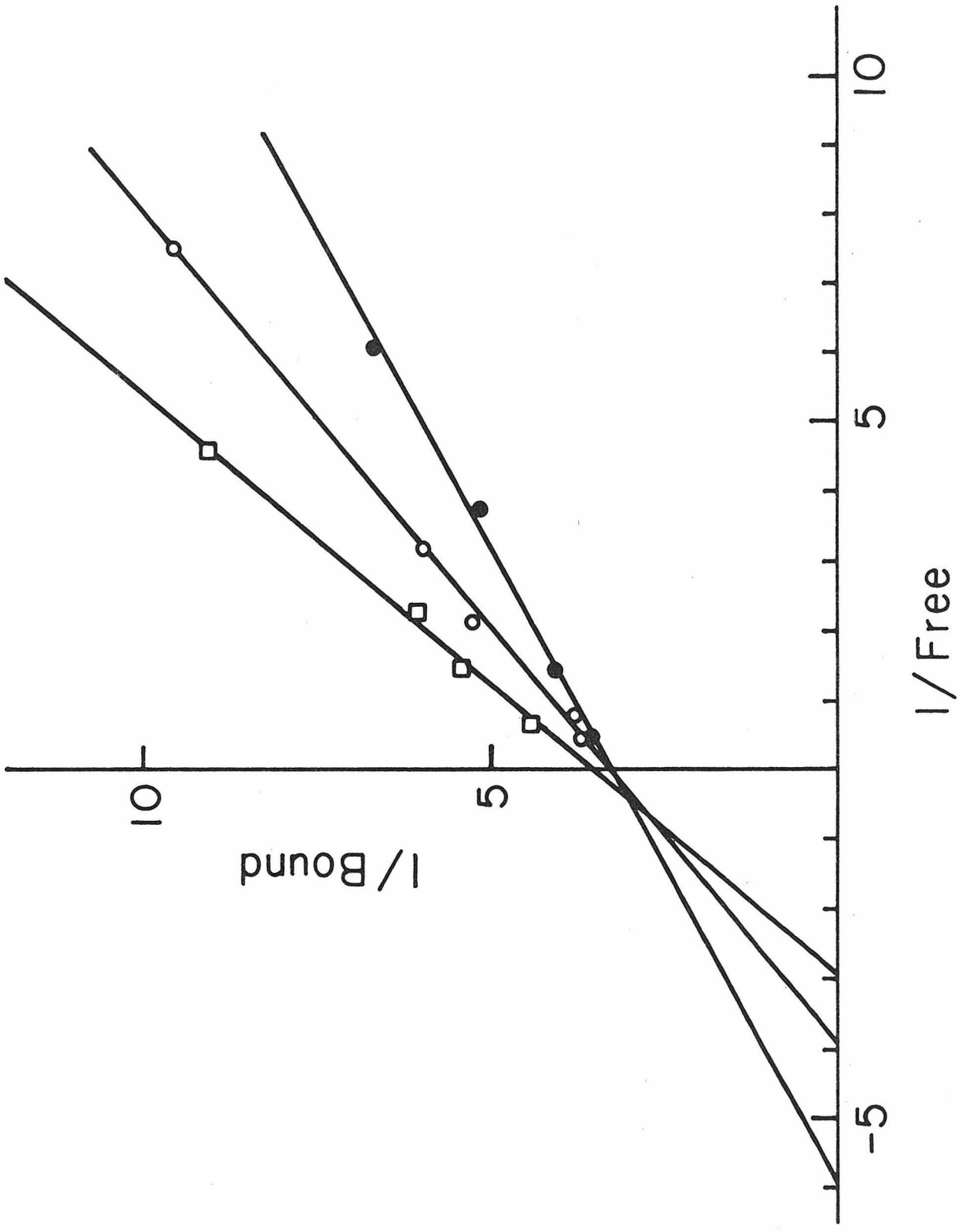

% COUNTS BOUND
40
30
20
10
5
6
7
8
−log [H12−HTX]

FIGURE 3: Inhibition of the Binding of the Isotopically Diluted [^{3}H]H_{12}-HTX by Cold H_{12}-HTX: This experiment was a check on the specific activity of the isotopically diluted [^{3}H]H_{12}-HTX which was used for all further binding experiments. The concentration of the membrane fragments was 1.19 μM in [^{125}I]α-BuTx sites. (●) concentration of cold H_{12}-HTX was zero; (○) concentration of cold H_{12}-HTX was 0.22 μM; (□) concentration of cold H_{12}-HTX was 0.44 μM. The cold H_{12}-HTX concentrations were corrected for nonspecific binding. The units of bound and free ligand are μM.


10
5
1/ Bound
10
5
-5
1/ Free

imposed on a linear, nonsaturable uptake. The saturable component was completely inhibited by competition with 40 μM unlabeled H_{12}-HTX and in this way the linear component was determined and subtracted from the total binding to yield the saturable component alone. This component followed a normal hyperbolic curve characteristic of a single class of non-interacting binding sites (Figure 4). The number of binding sites was found to equal one quarter of the number of [^{125}I]α-BuTx binding sites present in the preparation.

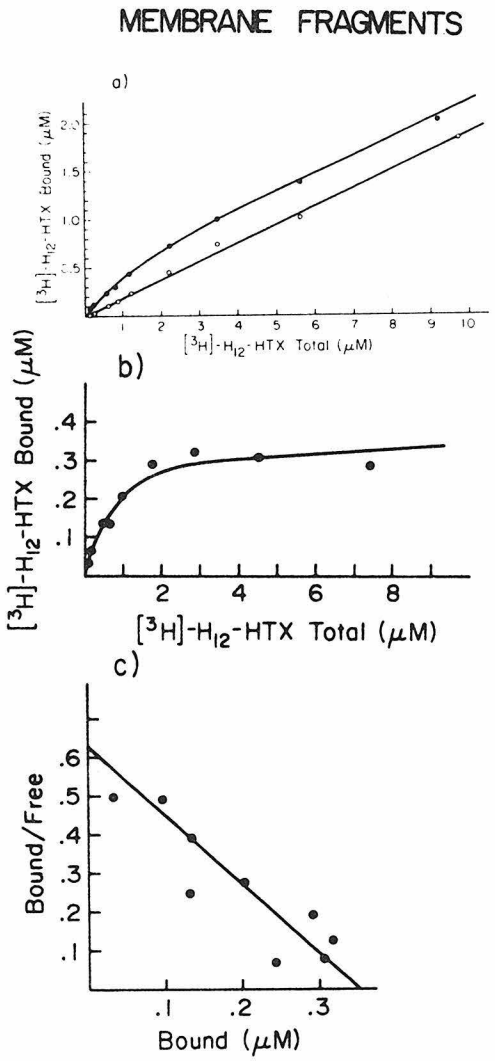
Therefore the numbers of H_{12}-HTX and agonist binding sites relative to α-BuTx binding sites are 1:2:4 (Raftery et al., 1975, 1979; Elliott and Raftery, 1977, 1979). Our lowest value for Torpedo californica AcChR molecular weight is 270 ± 30,000 daltons (Martinez-Carrion et al., 1975) and our most recent estimates of specific activity (Vandlen et al., 1979) for α-BuTx binding indicate that two toxin molecules bind per AcChR monomer (S value ~9, Raftery et al., 1972). Recent evidence also indicates that in membrane preparations the AcChR of Torpedo californica occurs mainly as a dimer (S value ~13.5, Raftery et al., 1972) specifically linked by means of a disulfide bond(s) involving 65,000 dalton subunits (Hucho et al., 1978; Chang and Bock, 1977; Hamilton et al., 1977; Witzemann and Raftery, 1978). This membrane-bound dimeric form would therefore bind four [^{125}I]α-BuTx molecules, two agonist molecules with high affinity, and one [^{3}H]H_{12}-HTX molecule. Thus H_{12}-HTX may block conduction by binding in a one-to-one ratio with AcChR dimers.

FIGURE 4: [^{3}H]H_{12}-HTX Binding by AcChR Enriched Membrane Fragments: The buffer was Torpedo Ringers and the concentration of α-BuTx sites was 1.45 μM.

A: (●) Binding by membrane fragments, (○) binding by membrane fragments + 40 μM unlabeled H_{12}-HTX.

B: Binding curve of (A) corrected for non-specific binding.

C: Scatchard plot of the corrected data.


MEMBRANE FRAGMENTS
a)
[3H]-H12-HTX Bound (μM)
[3H]-H12-HTX Total (μM)
b)
[3H]-H12-HTX Bound (μM)
[3H]-H12-HTX Total (μM)
c)
Bound/Free
Bound (μM)

α-BuTx has been shown to bind to a site distinct from the H_{12}-HTX binding site (see below; Dolly et al., 1977; Elliott and Raftery, 1977; 1979). However, the presence of saturating amounts of α-BuTx had an interesting effect on the observed binding of [^{3}H]H_{12}-HTX; the dissociation constant and number of binding sites were unaffected but the amount of non-specific binding was very nearly doubled (Figure 5).

3. Coupling Between the H_{12}-HTX Binding Site and the Agonist Recognition Site

The HTX binding site, although distinct, is conformationally linked to the AcCh recognition site. HTX and H_2-HTX were reported to increase the affinity of membrane-bound AcChR for [^{3}H]AcCh with the maximal effects occurring at approximately 20 μM HTX and 5 μM (Kato and Changeux, 1976). H_{10}-HTX also enhanced [^{3}H]AcCh binding (Figure 6). However, at the concentrations tested (up to 50 μM) a maximum in the effect was not observed.

[^{3}H]H_{12}-HTX bound to AcChR enriched membranes with slightly lower affinity in the absence of divalent cations than in Torpedo Ringers. In both cases the addition of 10 μM Carb resulted in an approximately two-fold lowering of the K_D without affecting the total number of H_{12}-HTX binding sites (Figure 7). In Torpedo Ringers K_D was 0.6 μM in the absence of Carb and 0.3 μM with Carb added; in EDTA containing buffer the K_D's were 0.9 μM and 0.5 μM, respectively. It should be noted that this concentration of Carb was sufficient to completely convert the AcChR to the conformational state having high affinity for agonists (Lee, et al., 1977).

FIGURE 5: The Effect of Saturating α-BuTx on [^{3}H]H_{12}-HTX Binding to Membrane Fragments. The concentration of the membrane fragments was 1.15 μM in [^{125}I] α-BuTx sites.

A: (●) no α-BuTx present, total binding; (○) no α-BuTx present, binding in the presence of 40 μM H_{12}-HTX; (■) membranes which have been incubated with 2.3 μM α-BuTx, total binding: (□) 2.3 μM α-BuTx, binding in the presence of 40 μM H_{12}-HTX.

B: Specific Component of Binding Only: (●) no α-BuTx; (■) 2.3 μM α-BuTx.

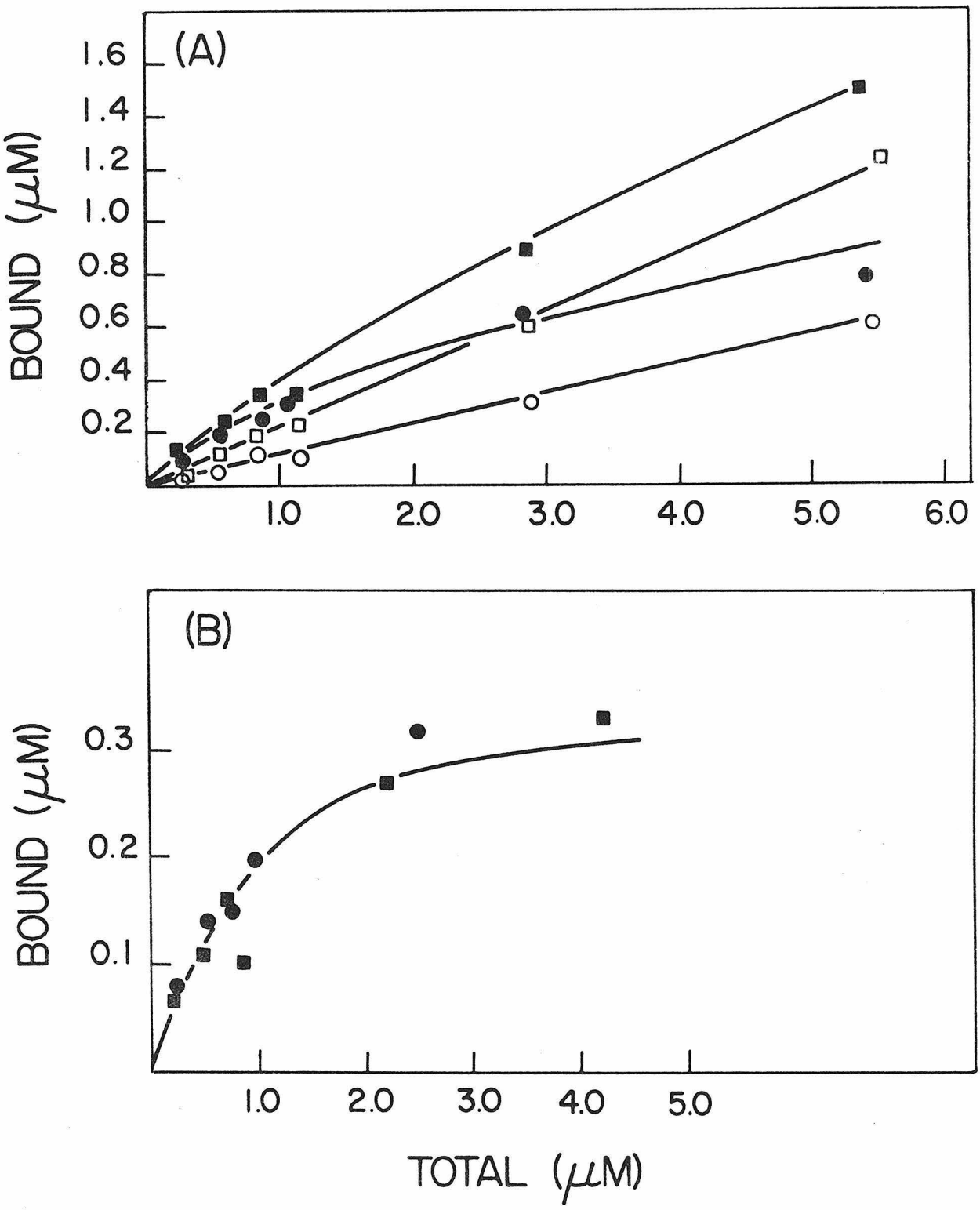

(A)
BOUND (μM)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
1.0
2.0
3.0
4.0
5.0
6.0
(B)
BOUND (μM)
0.3
0.2
0.1
1.0
2.0
3.0
4.0
5.0
TOTAL (μM)

FIGURE 6: The Effect of H_{10}-HTX on the Binding of [^{3}H]AcCh to Membrane Fragments: The concentration of the membranes was 20 nM in α-BuTx binding sites; the concentration of [^{3}H]AcCh was 20 nM. The buffer was Torpedo Ringers; the incubation time was 30 min.

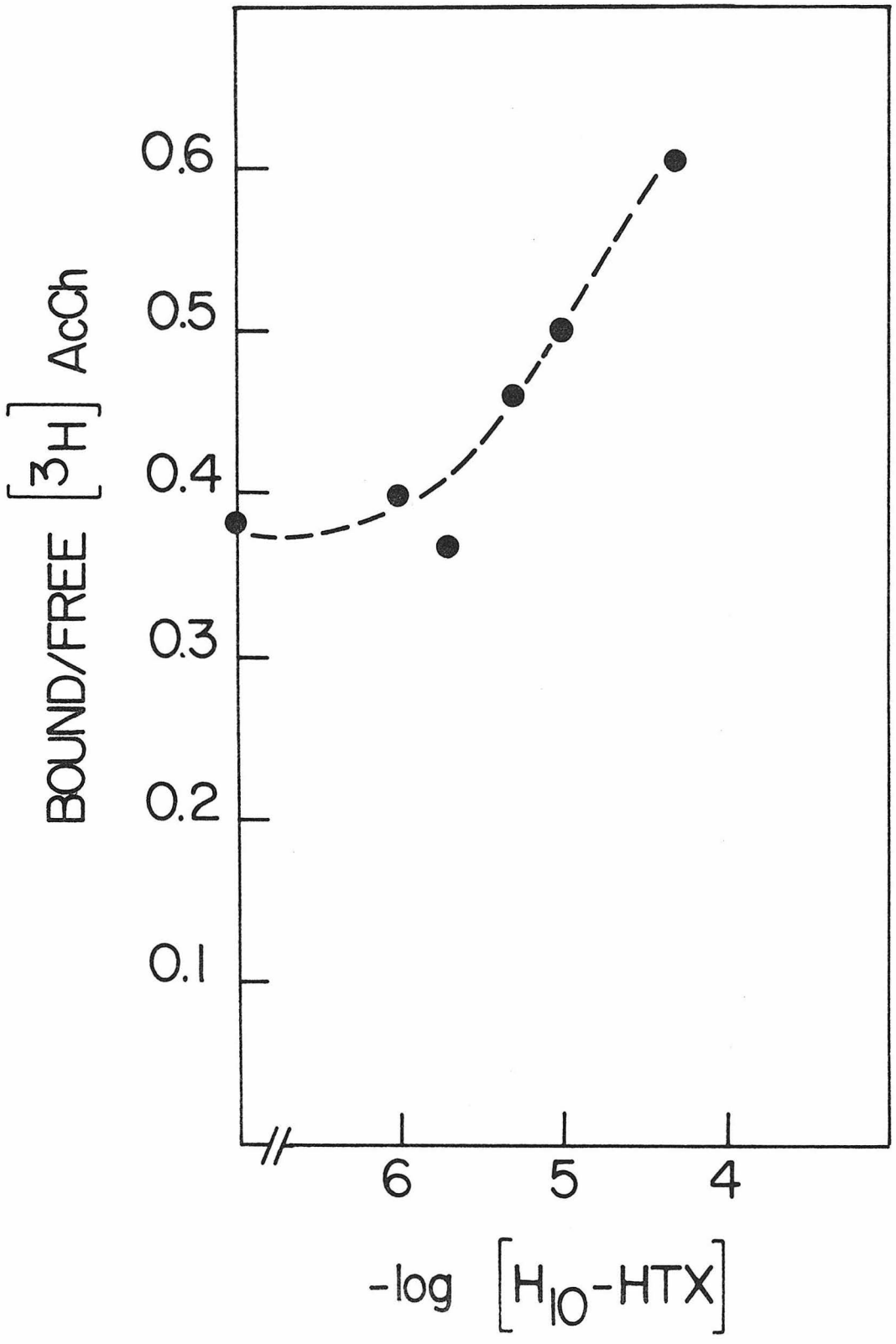
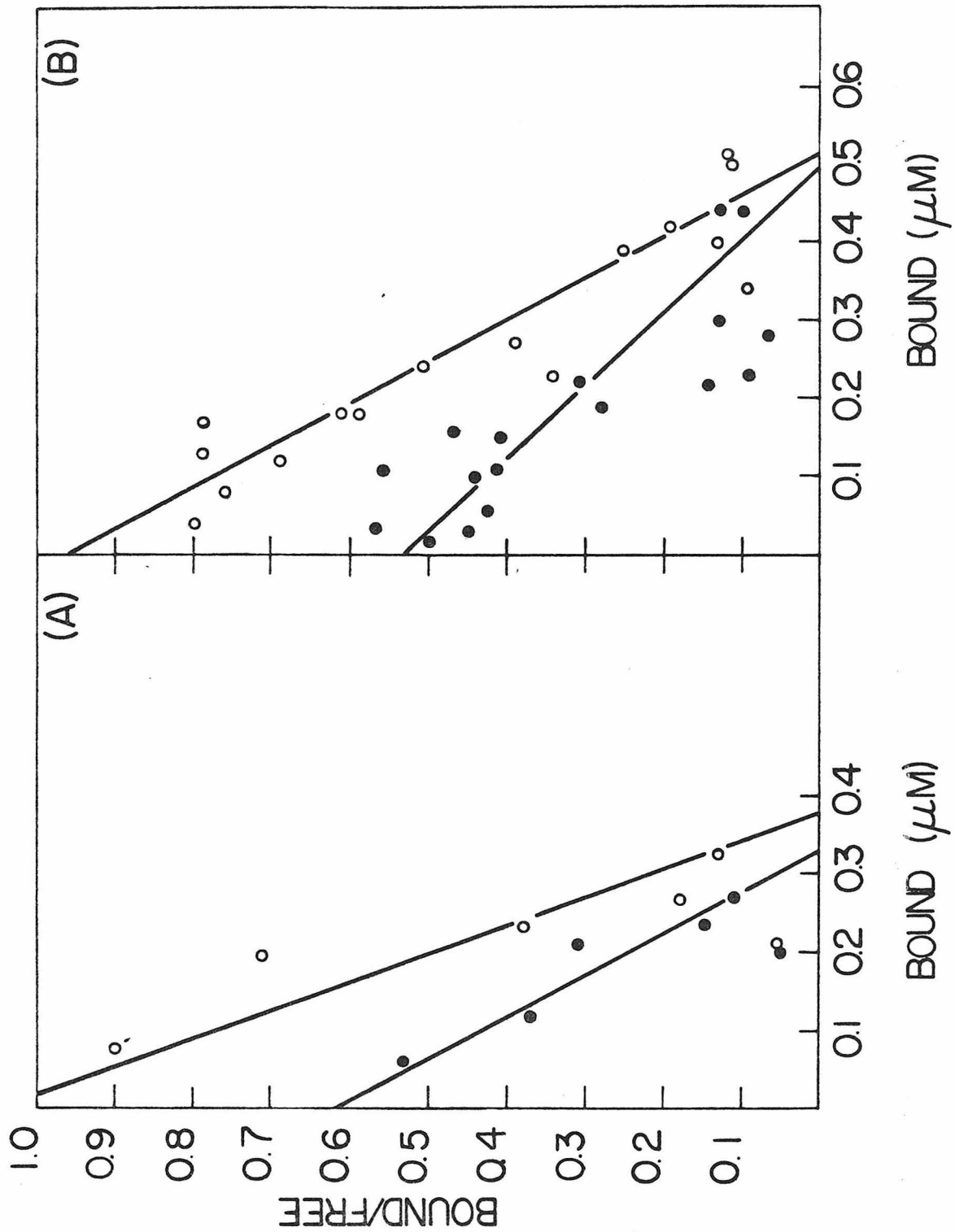

BOUND/FREE [3H] AcCh
0.6
0.5
0.4
0.3
0.2
0.1
6
5
4
-log [H10-HTX]

FIGURE 7: A: The Effect of Carb on [^{3}H]H_{12}-HTX Binding to Membrane Fragments. The concentration of [^{125}I]α-BuTx sites was 1.3 μM. The buffer was Torpedo Ringers. (●) no Carb present; (○) Carb concentration was 10 μM;

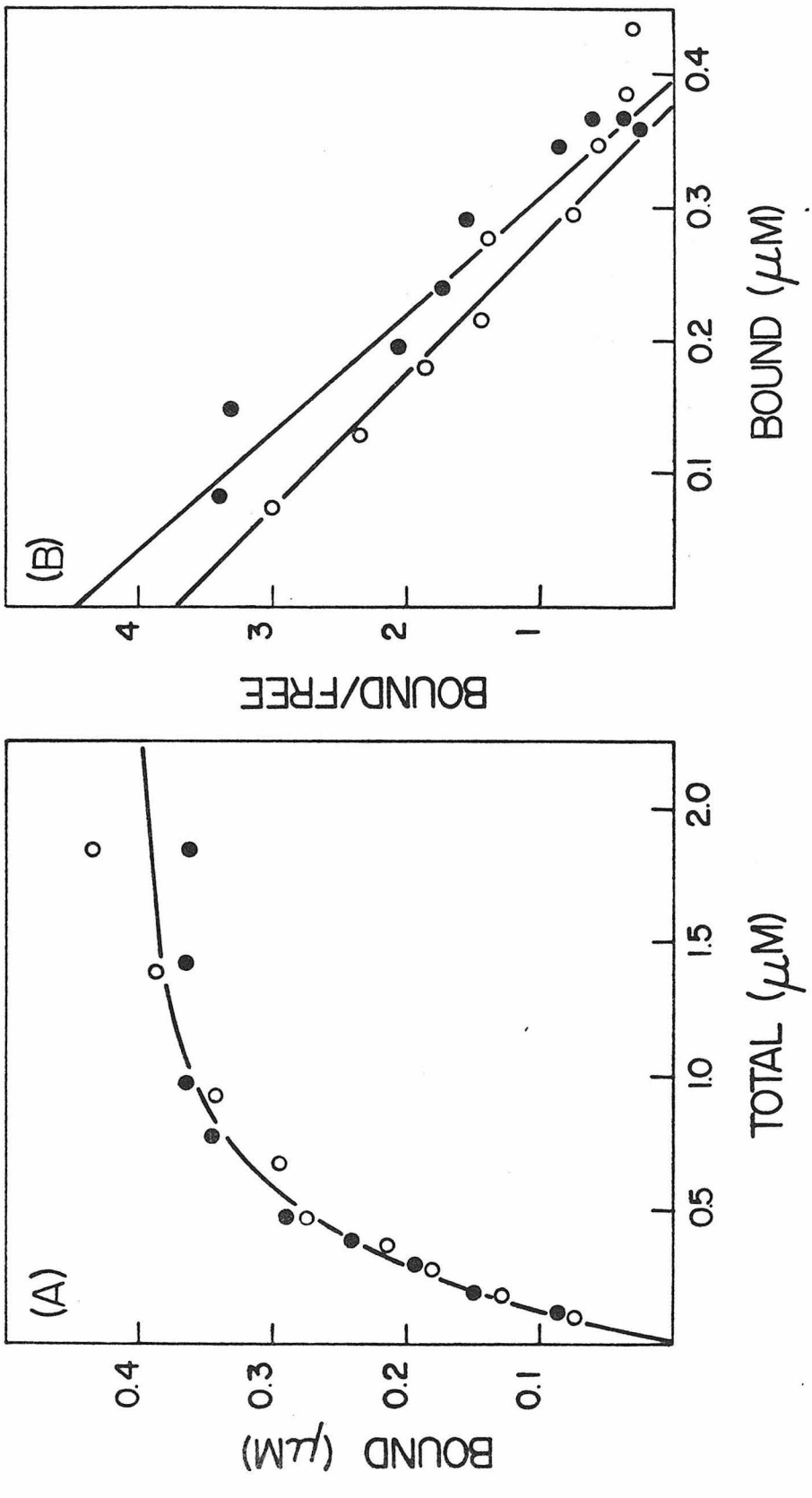
B: [^{3}H]H_{12}-HTX Binding to Membrane Fragments in the Presence of 2 mM EDTA: The concentration of [^{125}I] α-BuTx sites was 1.84 μM. The buffer was 250 mM NaCl, 5 mM KCl, 20 mM Hepes, pH 7.4, 2 mM EDTA 0.02% NaN_3. (●) no Carb present; (○) Carb concentration was 10 μM.


(A)
(B)
BOUND/FREE
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.1 0.2 0.3 0.4
0.1 0.2 0.3 0.4 0.5 0.6
BOUND (μM)
BOUND (μM)

The presence of 5 μM H_{12}-HTX did not significantly affect [^{3}H]Carb binding to membrane fragments (Figure 8). The dissociation constant for [^{3}H]Carb was 0.09 ± 0.01 μM in the absence of H_{12}-HTX; in the presence of H_{12}-HTX the value obtained was 0.10 ± 0.01 μM. The number of [^{3}H]Carb sites in the first case was 0.48 of the α-BuTx sites and 0.46 of the α-BuTx sites in the second experiment. Since Carb is a strong effector the fraction of Carb bound at equilibrium and the extent of conversion to the high affinity state are very nearly the same (Lee et al., 1977; Quast et al., 1978b), so that any change in equilibria between states induced by H_{12}-HTX would only be detected at extremely low [^{3}H]Carb concentrations. That this effect might be small can be inferred from the finding that 10 μM Carb, which would have completely converted the membranes to the high affinity state, caused only a two-fold increase in affinity for [^{3}H]H_{12}-HTX.

The effect of H_{12}-HTX on the affinity of Torpedo membranes for agonists was also tested by an [^{125}I]α-BuTx binding kinetic assay. In this assay the extent of conversion to the high affinity state was monitored by the extent to which 1 μM Carb added simultaneously with [^{125}I]α-BuTx to membranes slowed the initial rate of [^{125}I]α-BuTx–AcChR complex formation. In the low affinity state (Carb $K_D \cong 50$ μM (Popot et al., 1976)) slowing of the initial rate was not detectable; in the high affinity state (Carb $K_D \cong 50$ nM (Raftery et al., 1975)) the rate was slowed very considerably. This assay has been used extensively for the study of the in vitro affinity change, or "desensitization" (Lee et al., 1977; Weiland et al., 1977; Quast et al., 1978b).

FIGURE 8: A: <u>The Effect of H_{12}-HTX on [^{3}H]Carb Binding to Membrane Fragments:</u> The membranes were 0.83 μM in [^{125}I]α-BuTx sites. (●) no H_{12}-HTX present; (○) H_{12}-HTX concentration as 5 μM.
B: <u>Scatchard Plot of [^{3}H] Carb Binding Data:</u> (●) no H_{12}-HTX present; (○) H_{12}-HTX concentration was 5 μM.


(A)
BOUND (μM)
0.4
0.3
0.2
0.1
0.5
1.0
1.5
2.0
TOTAL (μM)
(B)
BOUND/FREE
4
3
2
1
0.1
0.2
0.3
0.4
BOUND (μM)

Preincubation with 3 μM H_{12}-HTX did not slow the rate of [^{125}I]α-BuTx binding to membrane bound AcChR either in the presence or absence of 1 μM Carb when the Carb was added simultaneously with the [^{125}I]α-BuTx (Figure 9). This confirmed that H_{12}-HTX was not competitive with α-BuTx and indicated that H_{12}-HTX at this concentration ($\sim$ 6 fold above its K_D) did not shift the AcChR to the high affinity form for Carb as did preincubation with Carb alone. This was also found to be the case when the concentration of H_{12}-HTX was increased to 30 μM (not shown).

Since H_{12}-HTX did not directly convert the membrane bound AcChR to the high affinity state, the rate of the agonist induced conversion was determined. In the absence of H_{12}-HTX the half-time for Carb-induced conversion to the high affinity state was 80 s (Lee et al., 1977); in the presence of 3 μM H_{12}-HTX the half-time was 73 s (Figure 10), which was not significantly different. The rate of recovery of the membranes to the low affinity state was also not significantly affected by 3 μM H_{12}-HTX; in the absence of H_{12}-HTX the half-time was 132 s and in the presence of 3 μM H_{12}-HTX it was 125 s (Figure 11). When the concentration of H_{12}-HTX was increased to 30 μM, the half-time for desensitization determined with receptor in excess over [^{125}I]α-BuTx (Quast et al., 1978b) was 29 s; the control valve was 40 s. This was not a large effect; for example, the detergent Triton X-100 at only 3 μM lowered the half-time to approximately 2 s (see below).

The preceding results and the relatively small effect of 10 μM Carb on the [^{3}H]H_{12}-HTX K_D suggested that the primary mode of

FIGURE 9: [^{125}I]α-BuTx Binding by Torpedo Membrane Fragments in the Presence of 3 μM H_{12}-HTX: R refers to AcChR and CC to carbamylcholine. The concentration of α-BuTx sites was 0.05 μM, [^{125}I]α-BuTx 0.5 μM. (Δ) Membrane fragments, ($\blacktriangle$) membrane fragments + 3 μM H_{12}-HTX, ($\square$) 1 μM Carb and toxin added simultaneously to membrane fragments, ($\bigcirc$) 1 μM Carb and toxin added simultaneously to membranes preincubated in 3 μM H_{12}-HTX, ($\bullet$) membrane fragments preincubated with 1 μM Carb.

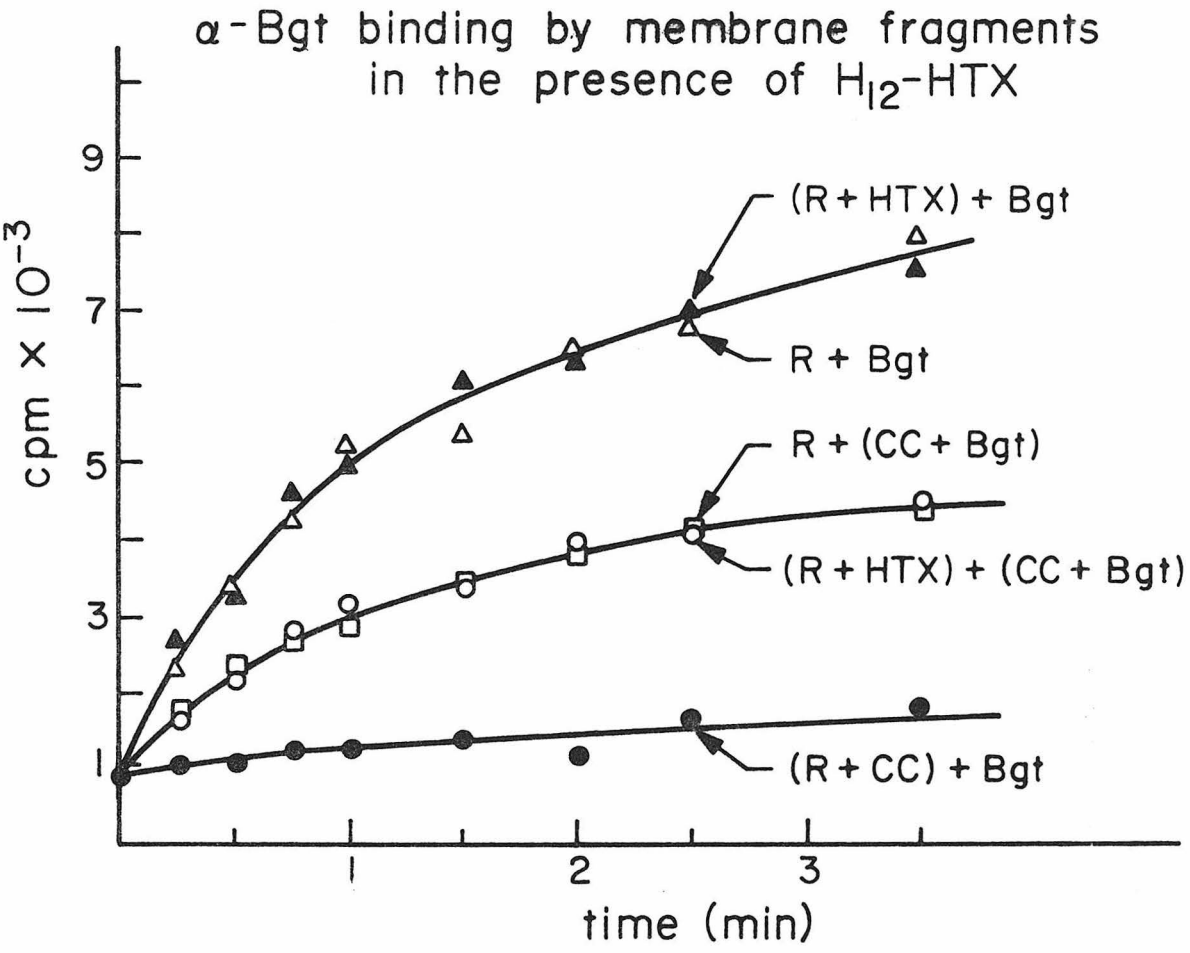

α-Bgt binding by membrane fragments
in the presence of H_{12}-HTX
cpm x 10^{-3}
9
7
5
3
1
1
2
3
time (min)
(R + HTX) + Bgt
R + Bgt
R + (CC + Bgt)
(R + HTX) + (CC + Bgt)
(R + CC) + Bgt

FIGURE 10: The Rate of Conversion of Membrane Bound AcChR to the High Agonist Affinity State: The concentration of α-BuTx sites was 0.06 μM, [^{125}I]α-BuTx was 0.6 μM, and Carb was 1 μM. The initial rate of toxin-receptor complex formation is plotted vs. time of incubation in 1 μM Carb. The rate of conversion was obtained as described in part 9 of the Experimental section.

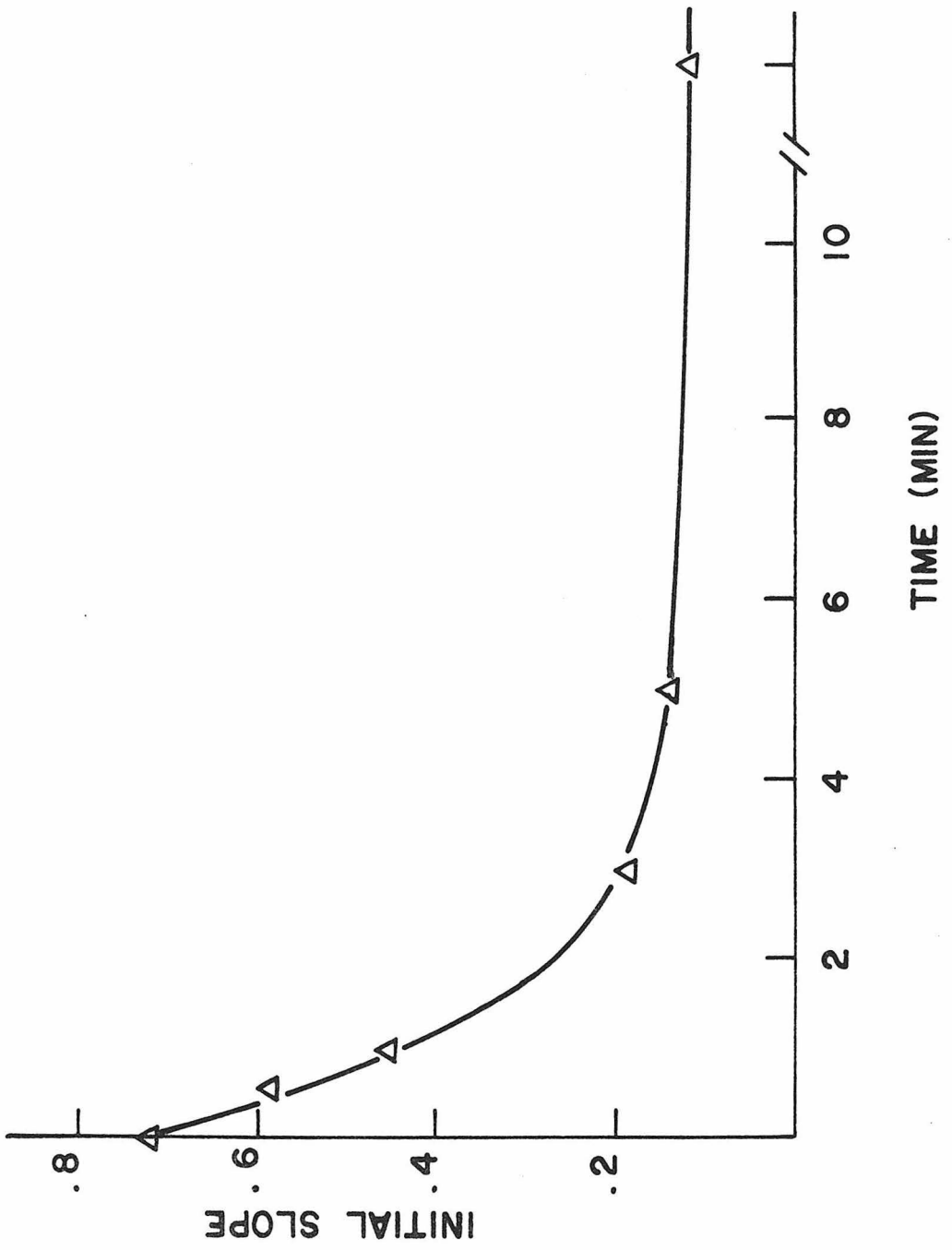
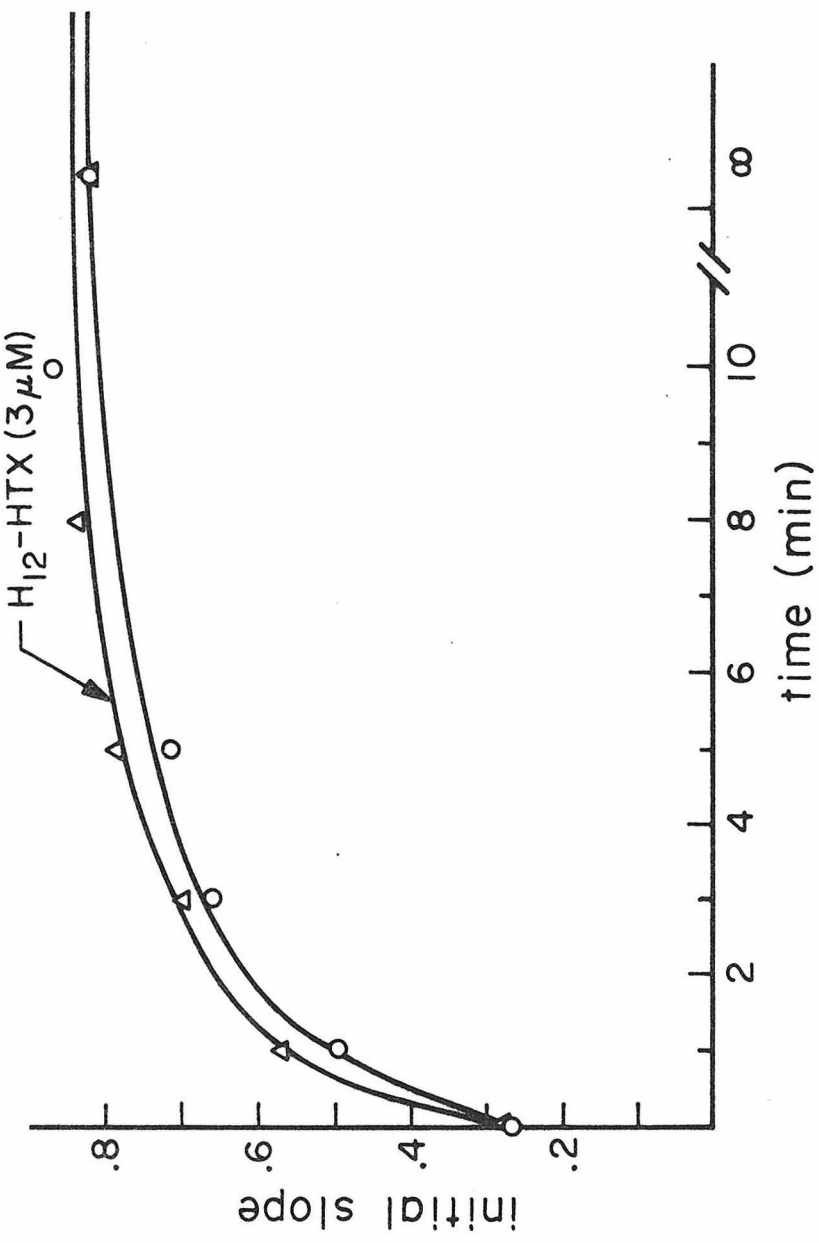

INITIAL SLOPE
.8
.6
.4
.2
2
4
6
8
10
TIME (MIN)

FIGURE 11: The Rate of Recovery of Membrane Bound AcChR to the Low Affinity State: Membrane fragments 5.6 μM in α-BuTx sites were incubated 60 min in 5 μM Carb. The incubation mixture was diluted 100 fold into Torpedo Ringers at time t = 0, and 1 μM Carb and 0.6 μM [^{125}I] α-BuTx added together at various times after dilution. The initial rate of toxin-receptor complex formation is plotted vs. time after dilution.


initial slope
.8
.6
.4
.2
2
4
6
8
10
8
time (min)
H_{12}-HTX (3 μM)

H_{12}-HTX action in this system did not involve an effect on agonist induced desensitization. Burgermeister et al. (1977), recently reported that HTX induced conversion of an AcChR preparation to a state of high ligand affinity. However, the embryonic chick muscle cell preparation used may not be comparable to Torpedo electroplax membrane preparations since it seems to have very different affinity for agonists; 20 μM Carb was reported not to cause "desensitization" whereas this concentration would have completely converted Torpedo electroplax membrane AcChR extremely rapidly to the high affinity state ($t_{\frac{1}{2}} <$ 30 sec) (see Lee et al., 1977). Thus using the in vitro Torpedo AcChR membrane system there is no conclusive evidence that the mechanism of HTX receptor blockage is by conversion of the system to the high affinity state induced by agonists. Furthermore kinetic evidence (Schimerlik et al., 1979b) supports the notion that the final receptor conformations induced by cholinergic agonists on the one hand and by HTX on the other are indeed different. Thus a more direct effect on channel conductance may better explain H_{12}-HTX action.

In order to investigate the affinity for [^{3}H]H_{12}-HTX of AcChR enriched membranes in the activated or open-channel form, the membranes were treated with BrAcCh. Electrophysiological studies showed that BrAcCh reversibly depolarized the non-reduced electroplax of the electric eel, Electrophorus electricus. Reduction of the electroplax followed by BrAcCh addition resulted in an irreversible activation of the cell, presumably due to the covalent alkylation of the AcChR by BrAcCh (Karlin, 1969). Moore and Raftery (1979a) showed that BrAcCh bound to AcChR enriched membranes competitively with AcCh

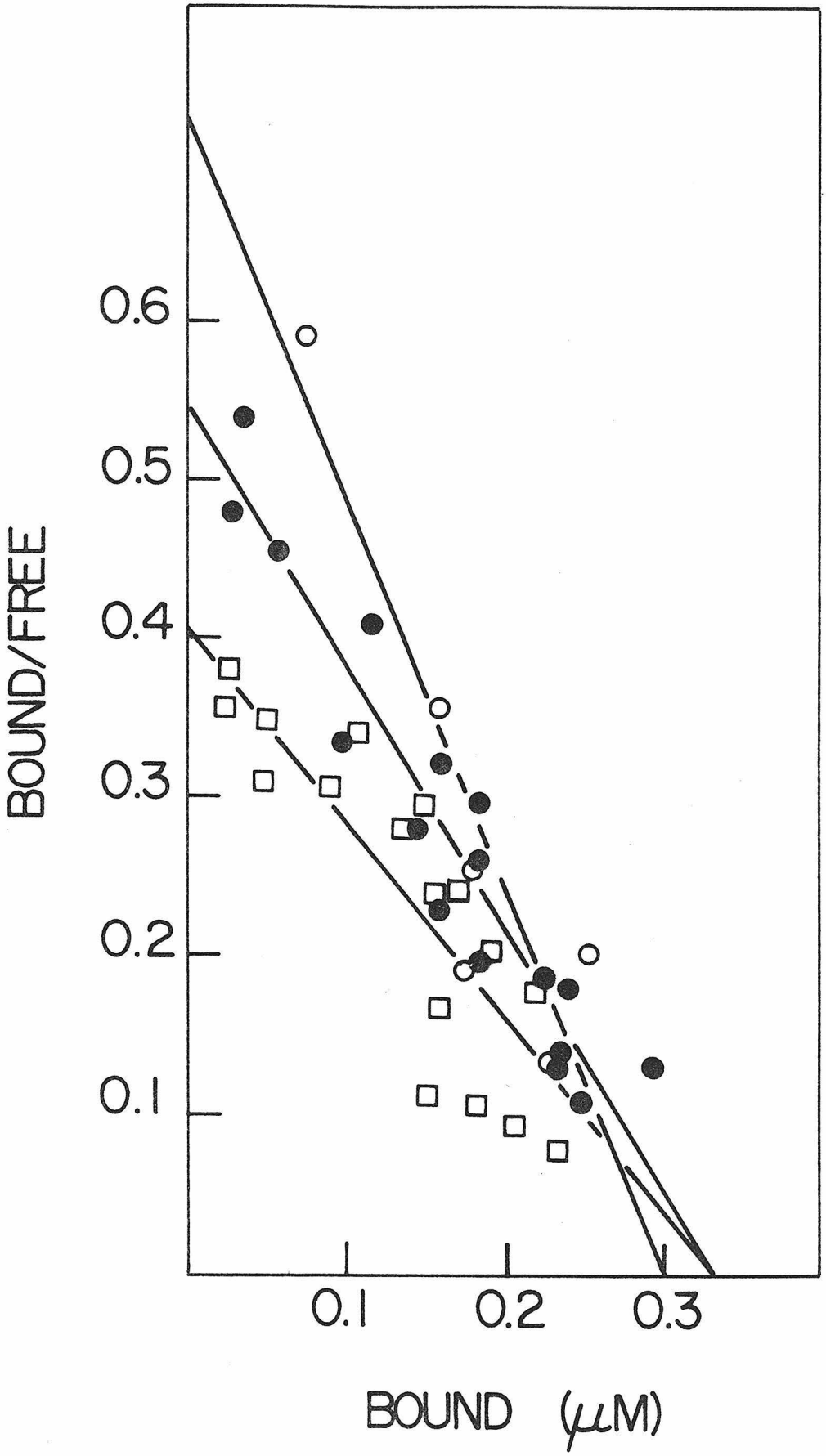
and irreversibly alkylated the 40,000 dalton AcChR subunit. It has not yet been shown that Torpedo electroplax are, like Electrophorus electroplax, irreversibly activated by BrAcCh, though this is not unlikely.

AcChR enriched membranes were reduced with dithiothreitol and treated with BrAcCh under conditions where essentially all of the AcChR was labeled. [^{3}H]H_{12}-HTX bound to these membranes with affinity intermediate (K_D = 0.6 μM) between binding to control membranes (K_D = 0.8 μM) and binding to control membranes in the presence of 10 μM Carb (K_D = 0.4 μM). Figure 12 shows Scatchard plots of the specific binding component in each case. Thus if these membranes were indeed in the activated state, then H_{12}-HTX binds to this state more tightly than to low affinity membranes and not yet as tightly as to high affinity or "desensitized" membranes.

4. Interactions of Local Anesthetics with the H_{12}-HTX Binding Site

In vivo, local anesthetics, like H_{12}-HTX, block the normal response of the AcChR to the binding of agonists. Electrophysiological studies of the actions of local anesthetics on the AcChR (Ruff, 1976; Neher and Sakmann, 1976; Steinbach, 1977) have led to proposals that these compounds bind to the open channel form of the AcChR and act to decrease ionic conductance. Additionally, some local anesthetics increase the rate of pharmacologic desensitization (Magazanik and Vyskocil, 1973; Magazanik, 1976), thus facilitating the conversion of the receptor to a non-conducting state.

FIGURE 12: The Effects of BrAcCh Treatment on [^{3}H]H_{12}-HTX Binding to Membrane Fragments: The concentration of α-BuTx binding sites in each experiment was 1.3 μM. Membranes were treated with BrAcCh as described in part 7 of the Experimental section. Control membranes were treated only with the 40 μM dithiothreitol and allowed to reoxidize during the overnight dialysis. (○) 10μM Carb; (●) BrAcCh treated; (□) control membranes.


BOUND/FREE
0.6
0.5
0.4
0.3
0.2
0.1
0.1
0.2
0.3
BOUND (μM)

Blanchard et al. (1979b) determined K_Is for the inhibition of [^{125}I]α-BuTx binding by local anesthetics; these are listed in Table 1. By working at local anesthetic concentrations below the K_I they were able to use the [^{125}I]α-BuTx binding kinetic assay to determine the effects of local anesthetics on the conversion of membrane-bound AcChR to the high affinity state much as was described for H_{12}-HTX in the preceding section. No local anesthetics directly converted the receptor to the form having high affinity for agonists. However, dibucaine and lidocaine increased the rate of the agonist induced conversion; tetracaine slowed this rate and procaine had little effect (Table 1).

The ability of these local anesthetics to displace [^{3}H]H_{12}-HTX bound to AcChR enriched membrane fragments in the presence and absence of 10 μM Carb was tested. Only dibucaine and tetracaine were effective in the micromolar concentration range (Figure 13). In the presence of 10 μM Carb, the effective concentrations at which local anesthetics displaced [^{3}H]H_{12}-HTX were shifted fromthose observed in the absence of Carb (Figure 13; Table 1). Because this concentration of Carb was sufficient to completely convert the AcChR to the high affinity state for agonists (Quast et al., 1978b), the different apparent K_Is in the presence and absence of Carb reflected the effectiveness with which a particular compound displaced [^{3}H]H_{12}-HTX bound to the high and low affinity states of the AcChR. Figure 14 shows a double reciprocal plot of bound versus free [^{3}H]H_{12}-HTX at various tetracaine concentrations. This plot indicates that, for tetracaine, the assumption of competitive inhibition which was used to

Table I: Effects of Local Anesthetics on Properties of AcChR Membrane Fragments

Local Anesthetic	Inhibition of $[^{125}I]\alpha$-BuTx Binding K_I, (μM)	Displacement of $[^3H]H_{12}$-HTX specifically bound K_I, (μM)		Effect on Rate of Carb Induced Affinity Change	
		(-)carb	(+)carb	effect[1]	Concentration Tested(μM)
dibucaine	56± 6	80	7.5	++	1-5
tetracaine	800± 100	1.5	30	-	50
lidocaine	1100± 200	2500	600	+	150
procaine	930± 60	3000	3000	0	150

[1] + = increase rate; - = decrease rate; 0 = no effect

FIGURE 13: Displacement by Local Anesthetics of [^{3}H]H_{12}-HTX Specifically Bound to AcChR Containing Membranes: The displacement of [^{3}H]H_{12}-HTX by increasing concentrations of local anesthetics was measured as described in the text. Displacement was measured in the absence of Carb (●) and in the presence of 10 μM Carb (○), which insured conversion of the receptor to its high affinity state for agonists. The increased amount of [^{3}H]H_{12}-HTX bound to the membranes in the presence of Carb was a reflection of the somewhat smaller K_d for this compound to high affinity membranes (0.35 μM) as compared to low affinity membranes (0.55 μM). The curves were calculated using the K_Is listed in Table I. The concentration α-BuTx sites was 1.2 μM and the total concentration of [^{3}H]H_{12}-HTX present was 0.3 μM. (A) tetracaine; (B) lidocaine; (C) dibucaine; (D) procaine.

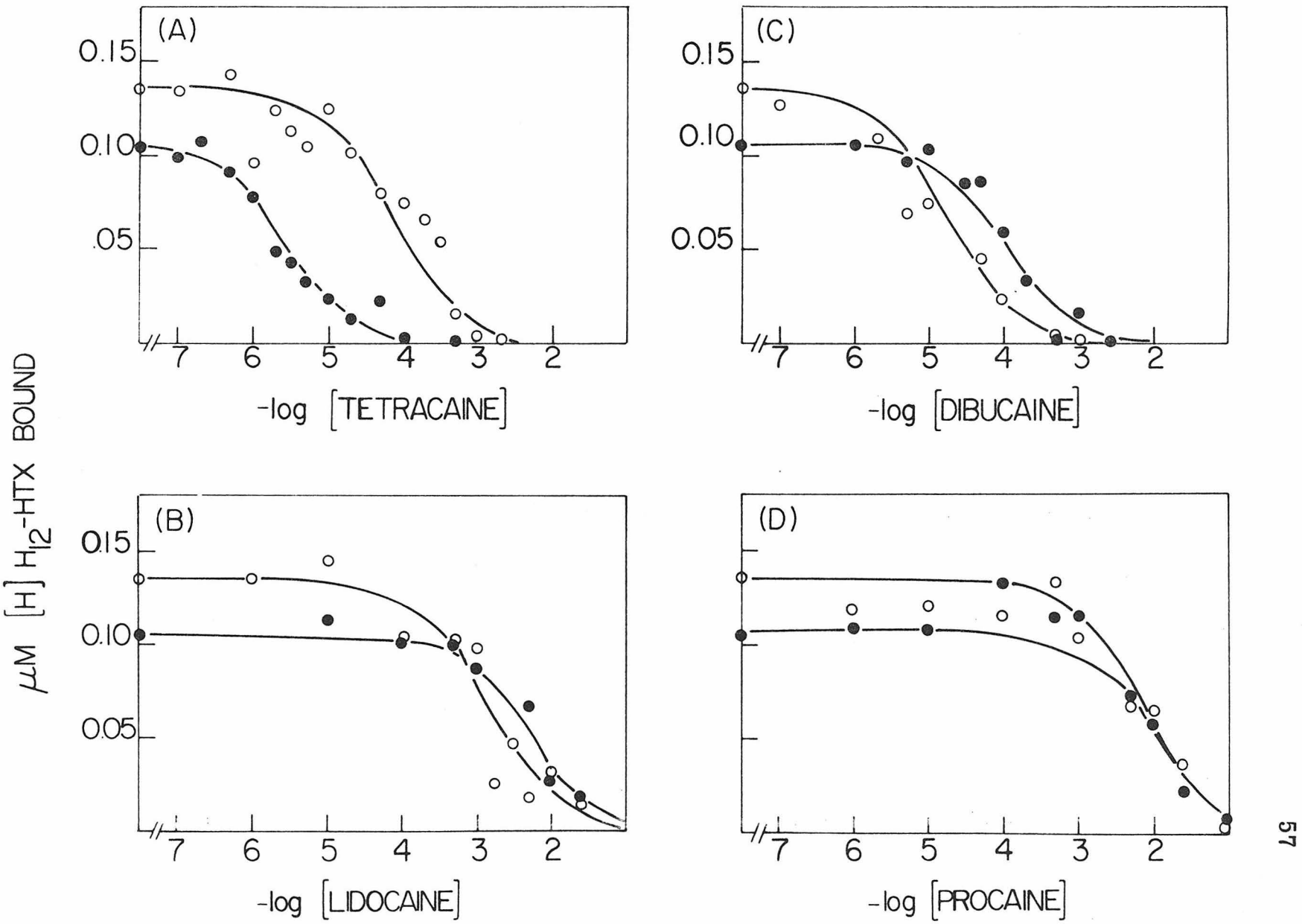
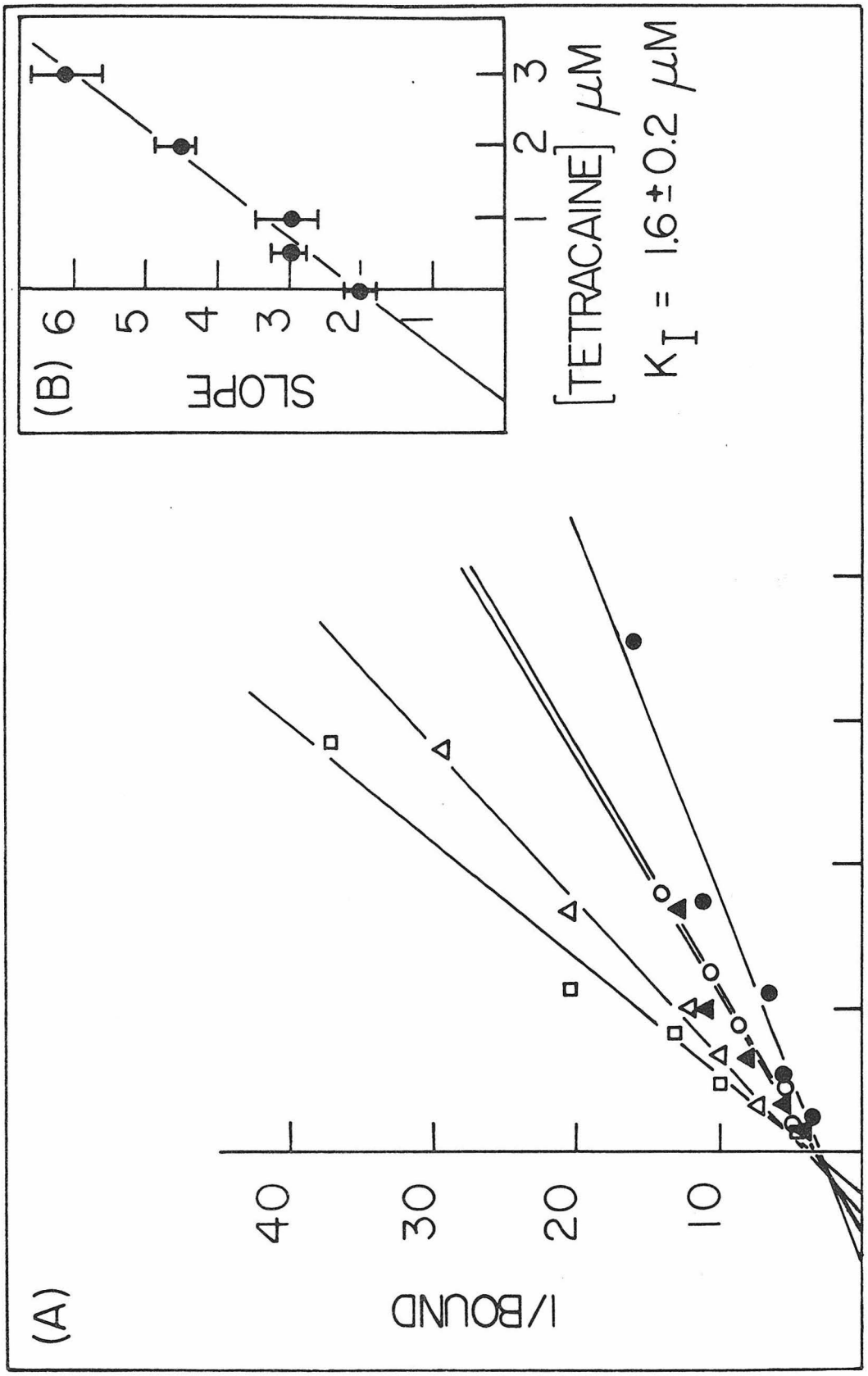

(A)
(B)
(C)
(D)
0.15
0.10
.05
0.05
7
6
5
4
3
2
-log [TETRACAINE]
-log [LIDOCAINE]
-log [DIBUCAINE]
-log [PROCAINE]
μM [H] H12-HTX BOUND

FIGURE 14: (A). Double Reciprocal Plots of [^{3}H]H_{12}-HTX Binding to Membranes in the Presence of Various Tetracaine Concentrations: The concentration of α-BuTx sites was 1.2 μM. (●) no tetracaine present, (○) [tetracaine] = 0.5 μM, (▲) [tetracaine] = 1.0 μM, (△) [tetracaine] = 2.0 μM, (□) [tetracaine] = 3.0 μM.

(B) Replot of the slopes obtained from the double reciprocal plots versus tetracaine concentration to yield K_I, which equals - (x intercept).

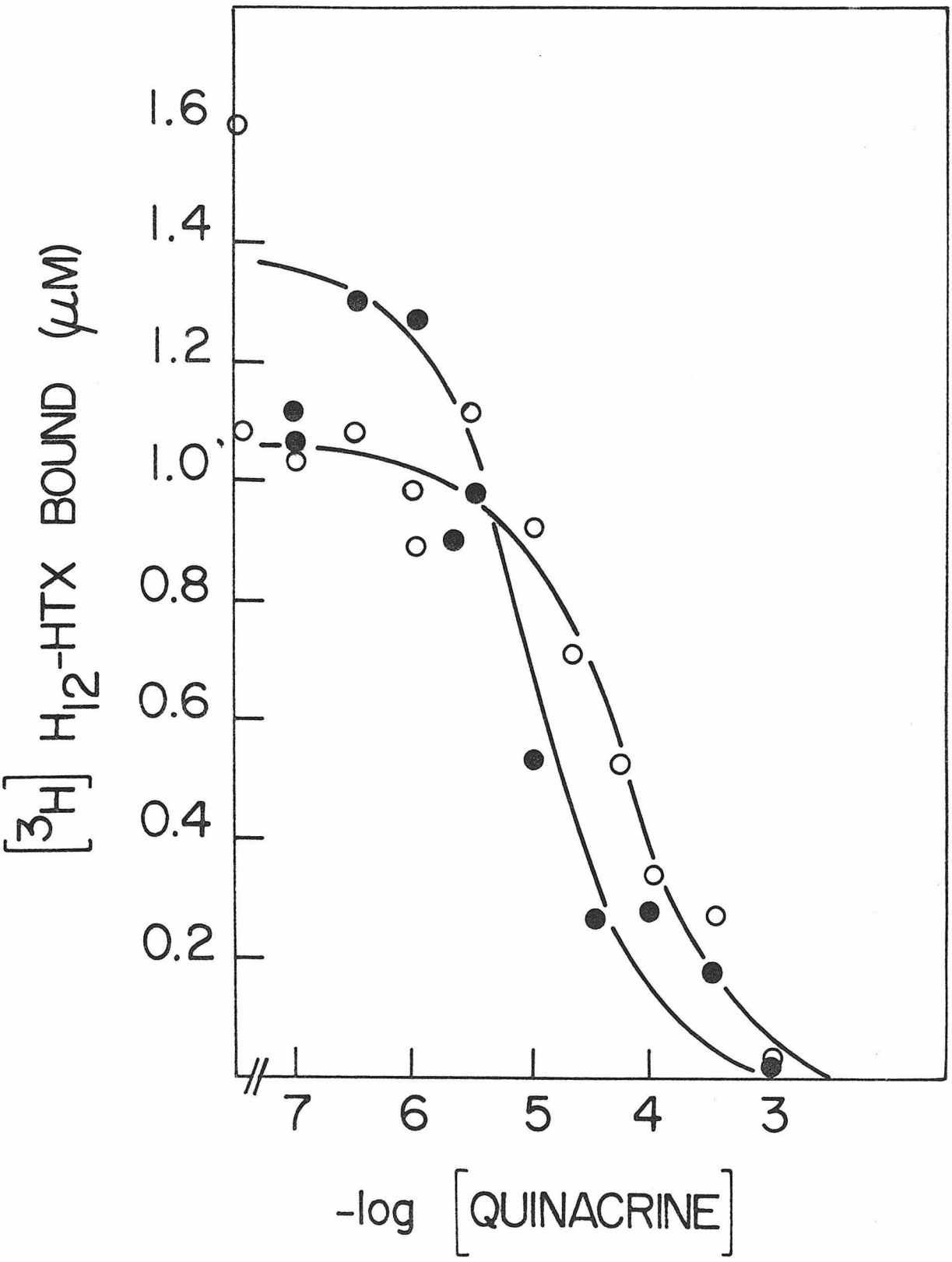

(A)
1/BOUND
40
30
20
10
2
4
6
8
1/FREE
(B)
SLOPE
6
5
4
3
2
1
1
2
3
[TETRACAINE] μM
K_I = 1.6±0.2 μM

calculate the apparent K_Is was reasonable. The fluorescent local anesthetic and antigiardiasis agent quinacrine, which has been used as a probe of AcChR function (Grünhagen and Changeux, 1976), displaced bound [^{3}H]H_{12}-HTX at relatively low concentrations (Figure 15). Its apparent K_I was 30 μM in the absence of Carb and 4 μM in 10 μM Carb.

As can be seen in Table 1, the effects of local anesthetics on the rate of the Carb induced increase in receptor affinity for agonists roughly paralleled the differences in their affinities for the low affinity and high affinity states of the receptor as reflected by [^{3}H]H_{12}-HTX displacement in the presence and absence of 10 μM Carb. Dibucaine, which effected the largest increase in the rate of transition, displaced bound [^{3}H]H_{12}-HTX at $\sim$10 fold lower concentrations in the presence of 10 μM Carb than in its absence. A particularly interesting case was that of tetracaine. This compound decreased the rate of the Carb induced affinity change and displaced [^{3}H]H_{12}-HTX 20 fold more effectively in the absence of Carb.

A possible explanation for the parallel in these effects was that the rate of the agonist induced transition in receptor affinity was modulated by compounds bound to the [^{3}H]H_{12}-HTX binding site with the magnitude of the effect determined by the ratio of the K_Is to the two states. Compounds such as dibucaine that bound more tighly to the high affinity state would cause an increased rate of transition and those such as tetracaine which bound more tightly to the low affinity state would decrease the rate of transition. This explanation is also consistent with the observation that compounds such as H_{12}-HTX (Elliott and Raftery, 1979) which did not appreciably change the rate of the

FIGURE 15: Displacement by Quinacrine of [^{3}H]H_{12}-HTX Specifically Bound to AcChR Containing Membranes: Displacement was measured in the absence of Carb (○) and in the presence of 10 μM Carb (●) under the same conditions as described in Figure Legend 13.


[3H] H12-HTX BOUND (μM)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
7
6
5
4
3
-log [QUINACRINE]

receptor transition also showed little difference in their apparent affinities for the low and high affinity states. That local anesthetics might bind specifically to the H_{12}-HTX site seems not unlikely since they are also relatively small, positively charged, and hydrophobic.

Another possible explanation of the effects of local anesthetics on the Carb-induced increase in AcChR affinity is that these compounds perturb the resting conformation of the receptor in a nonspecific manner, perhaps by disturbing the lipid annulus surrounding the receptor. Such a mechanism has been previously suggested as a possible explanation of the effects of both local (Weiland et al., 1977) and general anesthetics (Young et al., 1978) on membrane bound AcChR from Torpedo, as well as of the effects of anesthetics on proteins in general (Richards et al., 1978). The fact that local anesthetics displace specifically bound [^{3}H]H_{12}-HTX does not rule out this explanation: following dissolution of AcChR enriched membranes in the nonionic detergent Triton X-100 both the ability to undergo the agonist mediated transition in affinity (V. Witzemann and M. Raftery, unpublished) and specific [^{3}H]H_{12}-HTX binding (Elliott and Raftery, 1977) were lost. In addition, 2% (w/v) sodium cholate extracts of AcChR enriched membranes from Torpedo californica showed no specific binding of [^{3}H]H_{12}-HTX (Elliott and Raftery, 1977) but following dilution of the detergent to 0.5% (w/v) binding similar to that seen for intact membranes was recovered (Elliott and Raftery, 1979). Considering the sensitivity of the specific binding of [^{3}H]H_{12}-HTX to perturbations of the membrane environment of the AcChR, binding of local anesthetics to sites other than the specific [^{3}H]H_{12}-HTX binding

might result in displacement of specifically bound H_{12}-HTX by means of changes in receptor conformation.

With respect to this second hypothesis, the effects of the detergent Triton X-100 on the Carb induced affinity change and [^{3}H]H_{12}-HTX binding were investigated. Triton X-100 (Octyl Phenoxyl Polyethoxyethanol) is dissimilar in structure to H_{12}-HTX and local anesthetics; though hydrophobic it is large and uncharged. This detergent was found to accelerate the agonist induced change in affinity at micromolar concentrations (Figure 16). It also displaced bound [^{3}H]H_{12}-HTX at micromolar concentrations, and the displacement curve was shifted to lower concentrations in the presence of 10 μM Carb (Figure 17). Both of these effects occurred at Triton concentrations well below those at which membranes begin to dissolve (Raftery et al., 1973). It is difficult to conceive of Triton X-100 binding specifically to the H_{12}-HTX site, and therefore the effects described above might be the result of nonspecific perturbation of the membrane around the AcChR.

Local anesthetics are a very diverse class of compounds and they may not all act on the AcChR via the same mechanism. They also may act by a combination of specific and non-specific effects.

FIGURE 16: The Effect of Triton X-100 on the Carb Induced AcChR Affinity Change: The halftime of the affinity change is plotted versus the Triton X-100 concentration. The halftimes were determined as described by Quast et al. (1978b) with receptor in excess over toxin. The concentration of α-BuTx sites was 0.7 μM, [^{125}I]α-BuTx was 0.07 μM, and Carb was 5 μM.

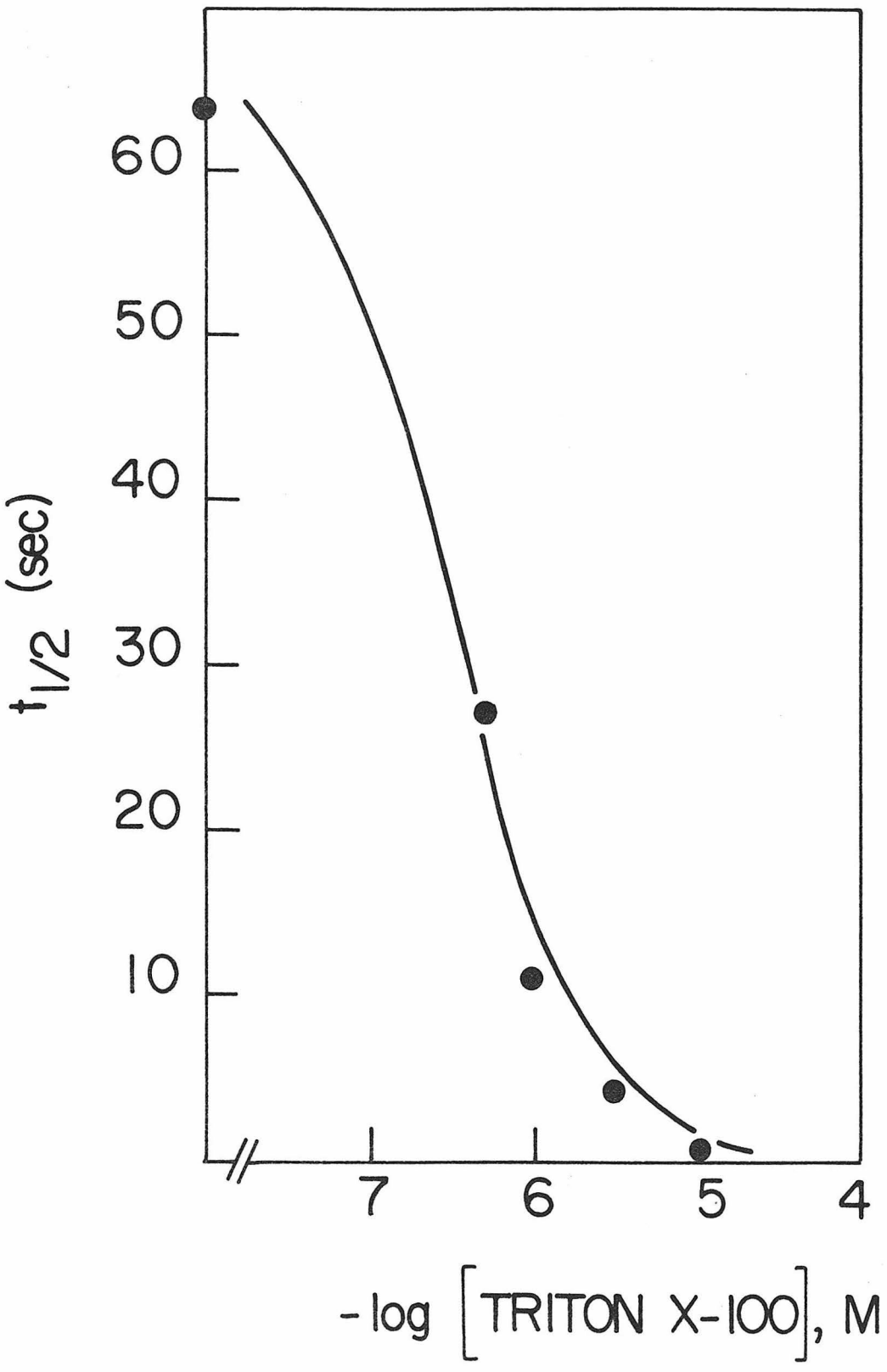
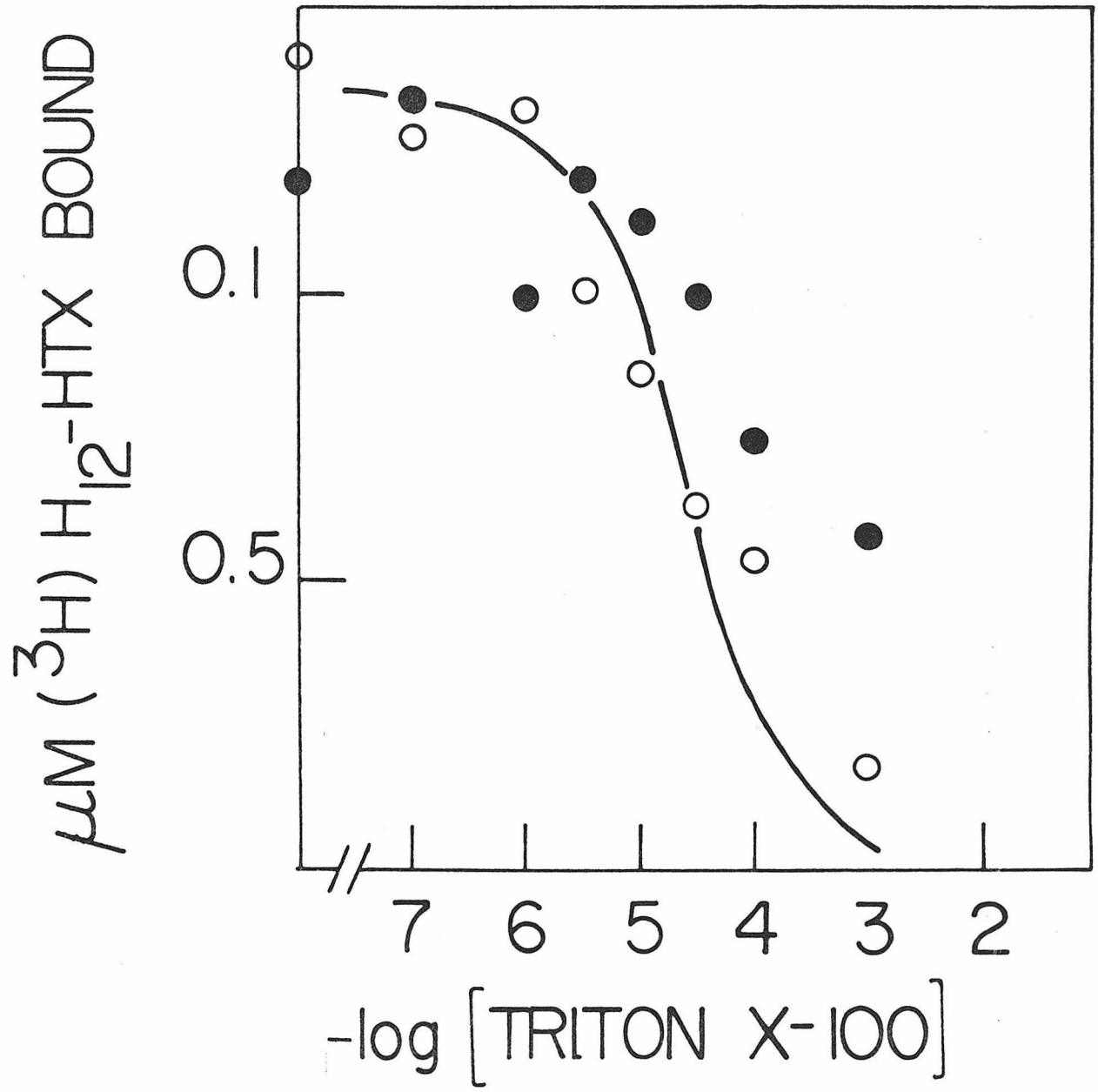

60
50
40
30
20
10
$t_{1/2}$ (sec)
7
6
5
4
- log [TRITON X-100], M

FIGURE 17: Displacement by Triton X-100 of [^{3}H]H_{12}-HTX Specifically Bound to AcChR Containing Membranes: Displacement was measured in the absence of Carb (●) and in the presence of 10 μM Carb (○) under the same conditions as described in Figure Legend 13.


μM (^{3}H) H_{12}-HTX BOUND
0.1
0.5
7
6
5
4
3
2
-log [TRITON X-100]

5. Ethidium Bromide Fluorescence

The fluorescent dye ethidium bromide has been shown to bind to Torpedo californica AcChR enriched membrane fragments (Schimerlik and Raftery, 1976) and to respond to cholinergic ligand binding by a change in quantum yield of the bound dye (Schimerlik et al., 1979a). Specific ethidium fluorescence, defined as the difference in fluorescence in the presence and absence of saturating α-BuTx (Schimerlik and Raftery, 1976) was quenched by H_{12}-HTX both in the presence and absence of 40 μM Carb (Figure 18). The plots of specific fluorescence versus H_{12}-HTX concentration were well fit by simple titration curves with midpoints at 0.25 μM in the absence of Carb and 0.36 μM in the presence of Carb. The larger amount of initial specific fluorescence in the second case was due to the ethidium fluorescence enhancement caused by the addition of the agonist Carb (Schimerlik et al., 1979a).

That the decrease in fluorescence caused by H_{12}-HTX occurred by means of a change in the environment of the bound ethidium rather than by a direct displacement was indicated by the following evidence: H_{12}-HTX in concentrations up to 30 μM did not displace bound [^{3}H]ethidium (Schimerlik et al., 1979a). Ethidium displaced [^{3}H]H_{12}-HTX binding to membranes with an apparent K_I of about 25 μM (Figure 19), whereas the K_D determined directly for [^{3}H] ethidium binding was 3.2 μM (Schimerlik et al., 1979a). Ethidium inhibited the initial rate of [^{125}I]α-BuTx-AcChR complex formation; H_{12}-HTX did not (see Figure 9 above). Preincubation with sufficient H_{12}-HTX to theoretically (on the basis of the fluorescence quenching results) displace 75% of the bound ethidium afforded no protection against this inhibition (Figure 20),

FIGURE 18: Ethidium Bromide Fluorescence: The difference in fluorescence in the presence and absence of a five-fold molar excess of α-BuTx is plotted vs. the H_{12}-HTX concentration. The concentration of α-BuTx sites was 0.31 μM, the concentration of ethidium was 0.5 μM, and the concentration of Carb was zero (○) or 40 μM (●).

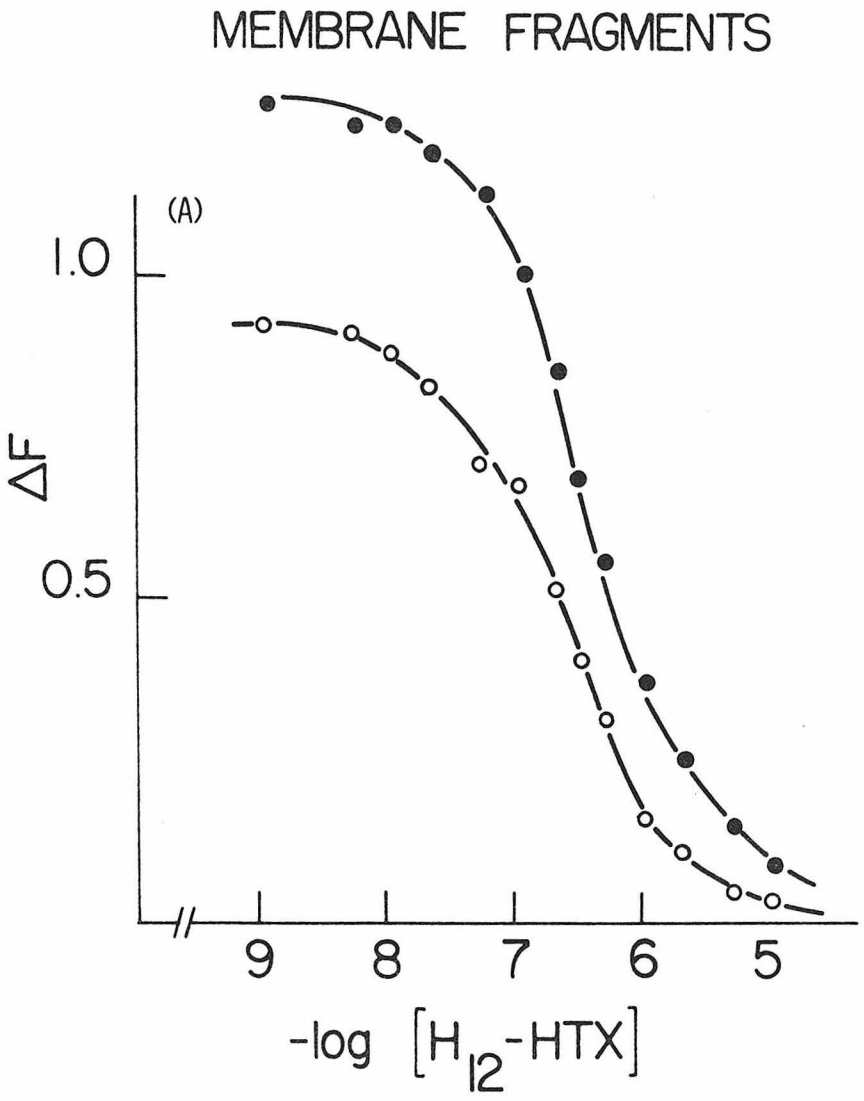

MEMBRANE FRAGMENTS
(A)
1.0
0.5
ΔF
9
8
7
6
5
-log [H12-HTX]

FIGURE 19: The Displacement of [^{3}H]H_{12}-HTX Bound to AcChR Enriched Membranes by Ethidium: The concentration of α-BuTx sites was 1.6 μM and [^{3}H]H_{12}-HTX was 0.4 μM.

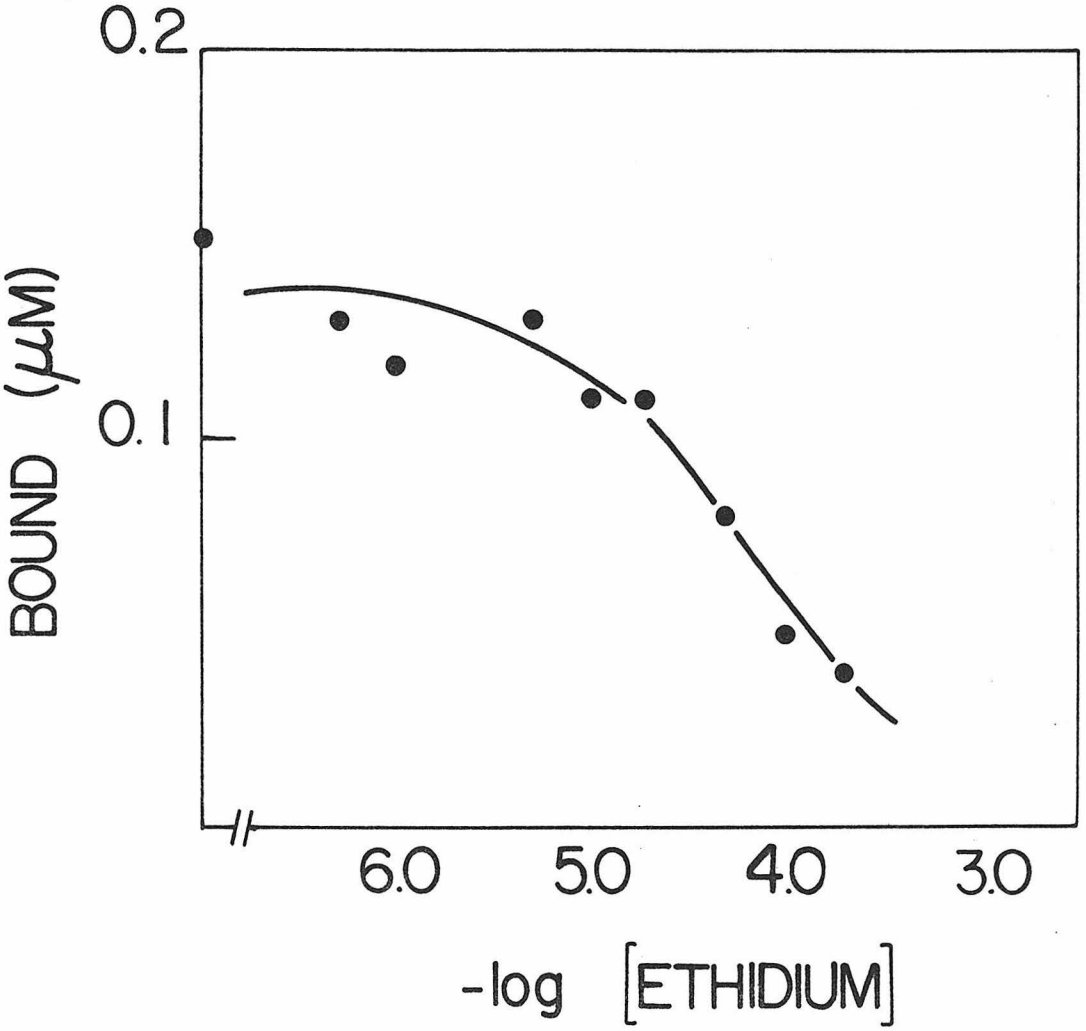
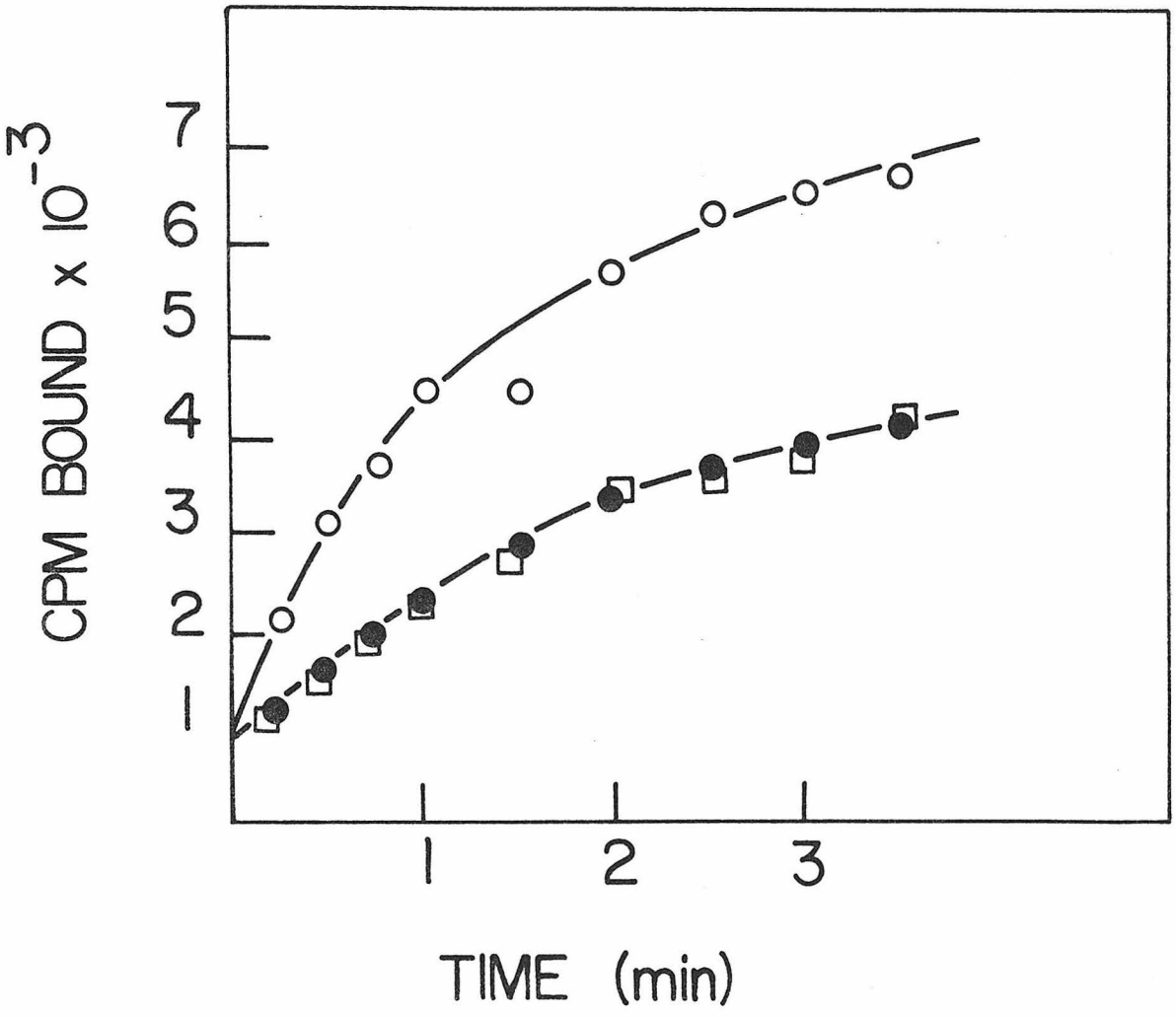

0.2
0.1
BOUND (μM)
6.0
5.0
4.0
3.0
-log [ETHIDIUM]

FIGURE 20: [^{125}I]α-BuTx Binding to Membranes in the Presence of Ethidium Bromide and H_{12}-HTX: The buffer was Torpedo Ringers. The concentration of α-BuTx sites was 0.05 μM and the concentration of [^{125}I]α-BuTx was 0.3 μM. (○) [^{125}I]α-BuTx added to membranes. (●) [^{125}I]α-BuTx added to membranes which had been preincubated for 15 min in 10 μM ethidium. (□) Membranes were incubated for 20 min in 3 μM H_{12}-HTX, 10 μM ethidium was added and incubated for an additional 15 min before addition of toxin.


CPM BOUND x 10^{-3}
7
6
5
4
3
2
1
1
2
3
TIME (min)

indicating that the ethidium was not actually displaced.

These results suggested that the binding of H_{12}-HTX produced a conformational change in the AcChR-ethidium complex which was reflected by fluorescence changes in the bound ethidium. Since the sign of the fluorescence change was different (decrease instead of increase), the final state of the AcChR-Ethidium-H_{12}-HTX complex was different than that of the receptor with cholinergic agonists or antagonists.

IV. THE LOCATION OF THE PERHYDROHISTRIONICOTOXIN BINDING SITE

1. Detergent Extraction of H_{12}-HTX Binding Activity from Membranes

The non-ionic detergent in which the AcChR was originally isolated by affinity chromatography, Triton X-100, proved to be unsuitable for the extraction and characterization of H_{12}-HTX binding activity. Only linear, nonspecific binding could be detected in 1% Triton X-100 extracts of AcChR enriched membrane fragments (Elliott and Raftery, 1977). This finding contradicted results reported by Eldefrawi et al. (1977), who chromatographed a 1% Triton extract of Torpedo ocellata membranes on a Sephadex G-200 column and observed [^{3}H]H_{12}-HTX binding activity which separated from the peak of [^{3}H]AcCh binding activity. However, this solubilized binding component was not shown to be a protein, nor was the observed binding demonstrated by controls to be specific in nature. It was not unlikely that lipid-detergent micelles accounted for this apparently low molecular weight binding peak. Chromatography of 2% cholate extracts on columns of Sepharose 6B and Sephadex G150 showed similar peaks of binding activity, not associated with [^{125}I]α-BuTx binding activity or bulk protein, which could be duplicated by chromatographing a sample containing only several lipids known to be present in Torpedo electroplax: phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, cholesterol, and lysolecithin (not shown, Elliott and Raftery, 1977).

Sobel et al. (1978) reported that upon extraction of AcChR

enriched membranes with a mixture of Triton X-100 and Berol 043 an insoluble precipitate resulted which was composed primarily of a polypeptide of apparent molecular weight 43,000 on SDS gels. In experiments using quinacrine fluorescence, it appeared that after removal of detergents HTX bound to this material, though with an extremely slow time course. This led to the conclusion that the HTX binding site was located on a polypeptide of molecular weight 43,000 which could be separated from the AcChR "sensu strictor," and that this separable component might be the AcChR ionophore.

A specific [^{3}H]H_{12}-HTX binding component could be solubilized from membrane fragments by the anionic detergent sodium cholate (Elliott and Raftery, 1979). Above 0.6% cholate, which is near the critical micelle concentration of the detergent, specific binding rapidly fell off (Figure 21A). This loss of binding activity was reversible, since a 2% cholate extract could be diluted to 0.5% cholate with complete restoration of binding activity to the level of one quarter of the α-BuTx sites present, the same ratio found in membrane fragments.

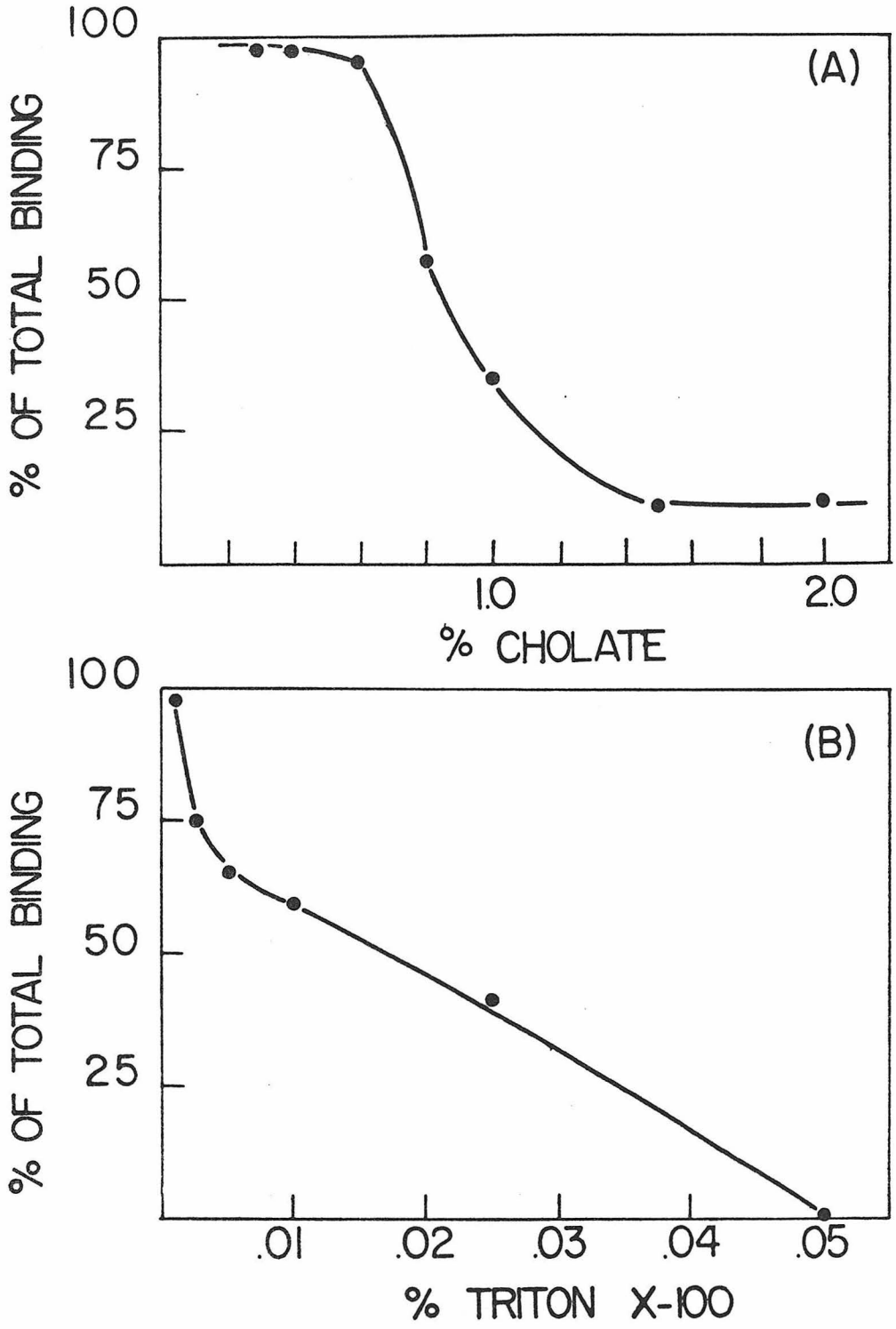
This reconstitution of [^{3}H]H_{12}-HTX binding activity was accompanied by aggregation, which was manifested by a large increase in light scattering of the sample. If a 2% cholate extract was diluted to 0.5% cholate and again centrifuged at 100,000g for 1 hour, approximately half the protein and [^{125}I]α-BuTx binding activity were lost in the pellet.

Typically 50-65% of the total protein and [^{125}I]α-BuTx binding activity were solubilized upon treatment of membrane fragments with 2% cholate. Binding of [^{3}H]H_{12}-HTX to the extract was measured by

FIGURE 21: Effect of Detergents on [^{3}H] H_{12}-HTX Binding:

A: A 2% cholate extract was diluted to various final cholate concentrations. The concentration of [^{125}I] α-BuTx sites was constant at 1.07 μM in one experiment and 0.8 μM in another and results were combined and normalized. The percent of the total binding was plotted as a function of cholate concentration.

B: A 2% cholate extract was diluted to a final cholate concentration of 0.5% cholate and small amounts of Triton X-100 were added. The concentration of [^{125}I] α-BuTx sites was 0.81 μM. Percent of total binding was plotted as a function of Triton concentration.


100
75
50
25
% OF TOTAL BINDING
(A)
1.0
2.0
% CHOLATE
100
75
50
25
% OF TOTAL BINDING
(B)
.01
.02
.03
.04
.05
% TRITON X-100

equilibrium dialysis after dilution in Ringers to 0.4% cholate (Figure 22). The residue, which was not solubilized upon treatment of membrane fragments with 2% cholate, was washed several times by resuspension in cholate free Ringers and was recovered by recentrifugation. Specific [^{3}H]H_{12}-HTX binding to this material could be measured by centrifugation assay (Figure 23). The partitioning of binding into specific and nonspecific components in these two preparations resembled the results with membrane fragments (see Figure 4). The ratio of [^{3}H]H_{12}-HTX binding sites to [^{125}I]α-BuTx sites did not change significantly while the dissociation constant was higher for the extract and residue; the K_D for the membranes in this experiment was 0.56 μM, for the extract 1.6 μM, and for the residue 0.86 μM. The ratio of [^{3}H]H_{12}-HTX binding sites to [^{125}I]α-BuTx sites was 0.25 for membranes, 0.26 for the cholate extract, and 0.26 for the washed residue.

Specific ethidium bromide fluorescence was quenched by H_{12}-HTX in a 2% cholate extract which had been diluted to 0.4% cholate (Figure 24). Whereas the plot of specific fluorescence versus H_{12}-HTX concentration was a simple titration curve for membrane fragments (see Figure 18), for the cholate extract the plot was much steeper and did not level off at zero specific fluorescence. In four experiments with cholate extracts the amount of specific fluorescence quenched ranged from 69% to 90% and averaged 78% of the total specific fluorescence. The percent quenched did not correlate with any immediately obvious variables such as age or specific activity of the membrane preparation used. The I_{50} for fluorescence quenching was 0.45 μM in the absence

FIGURE 22: [^{3}H]H_{12}-HTX Binding to a Cholate Extract of Membranes

The cholate concentration was 0.4% and the concentration of α-BuTx sites was 1.38 μM.

A: Total binding in the absence (●) and presence (○) of 40 μM unlabeled H_{12}-HTX.

B: The specific component of binding.

C: Scatchard plot of the specific component of binding.

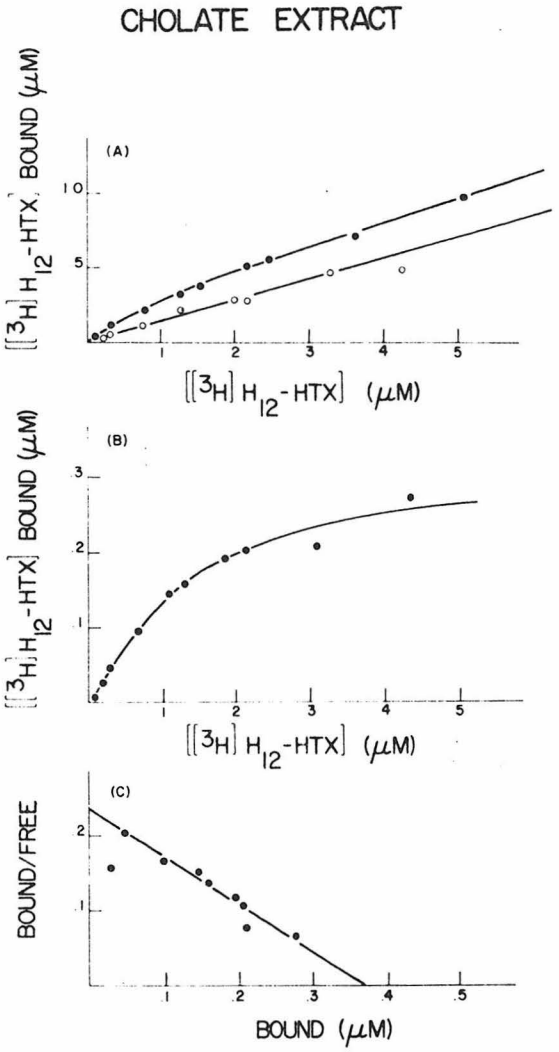

CHOLATE EXTRACT
(A)
[[3H] H12-HTX], BOUND (μM)
10
5
1
2
3
4
5
[[3H] H12-HTX] (μM)
(B)
[[3H] H12-HTX] BOUND (μM)
3
2
1
1
2
3
4
5
[[3H] H12-HTX] (μM)
(C)
BOUND/FREE
2
1
.1
.2
.3
.4
.5
BOUND (μM)

FIGURE 23: [^{3}H] H_{12}-HTX Binding to the Insoluble Residue after Cholate Extraction: The concentration of [^{125}I] α-BuTx binding sites was 1.18 μM.

A: Total binding in the absence (●) and presence (○) of 40 μM unlabeled H_{12}-HTX.

B: The specific component of binding.

C: Scatchard plot of the specific component of binding.

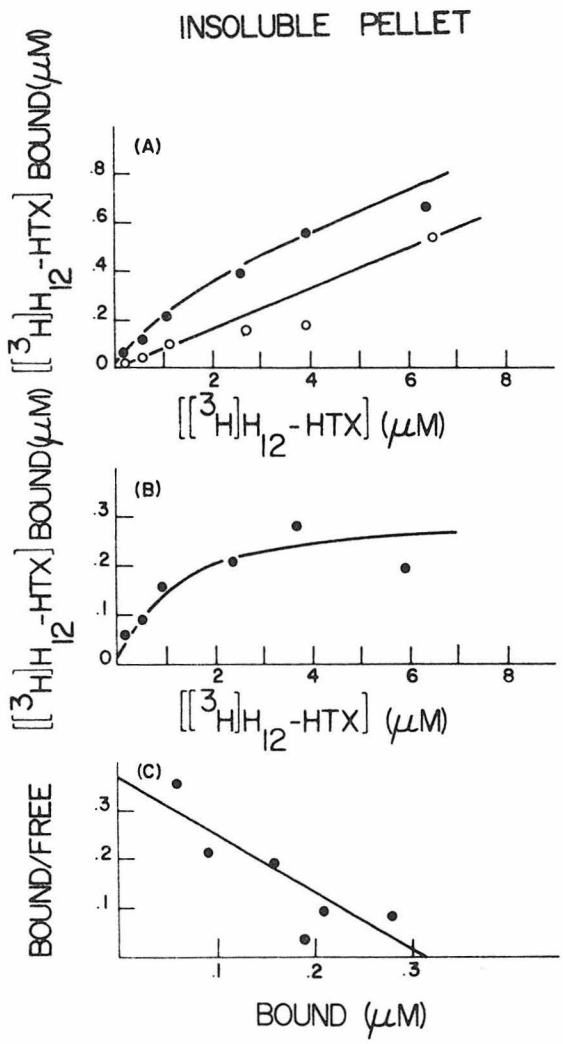
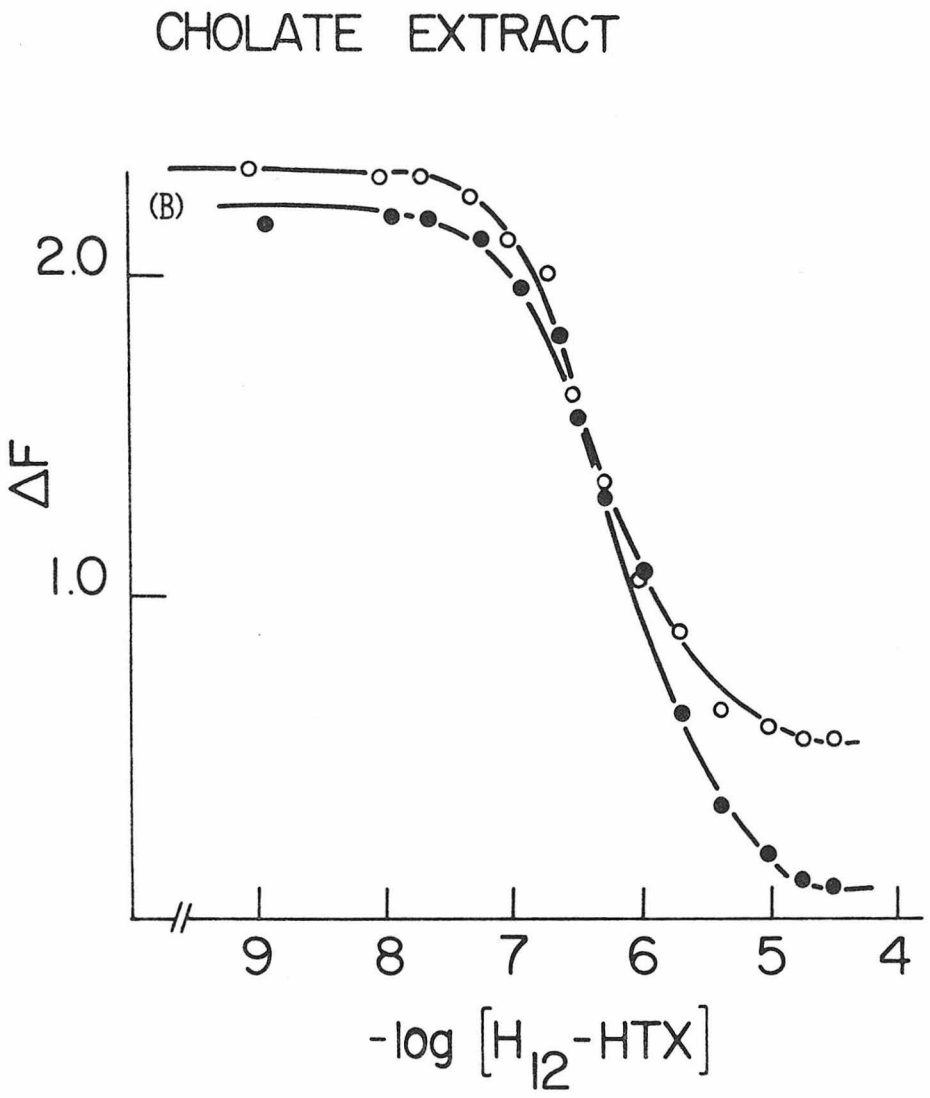

INSOLUBLE PELLET
[[3H]H12-HTX] BOUND(μM)
(A)
0
.2
.4
.6
.8
2
4
6
8
[[3H]H12-HTX] (μM)
[[3H]H12-HTX] BOUND(μM)
(B)
0
.1
.2
.3
2
4
6
8
[[3H]H12-HTX] (μM)
BOUND/FREE
(C)
.1
.2
.3
BOUND (μM)

FIGURE 24: Ethidium Bromide Fluorescence with a Cholate Extract: The difference in fluorescence in the presence and absence of a five-fold molar excess of α-BuTx is plotted vs. the H_{12}-HTX concentration. The 2% cholate extract was diluted to final cholate concentration of 0.4% and final concentration of [^{125}I] α-BuTx sites of 1.1 μM. Ethidium concentration was 1.16 μM. (○) no Carb present; (●) Carb concentration was 40 μM.

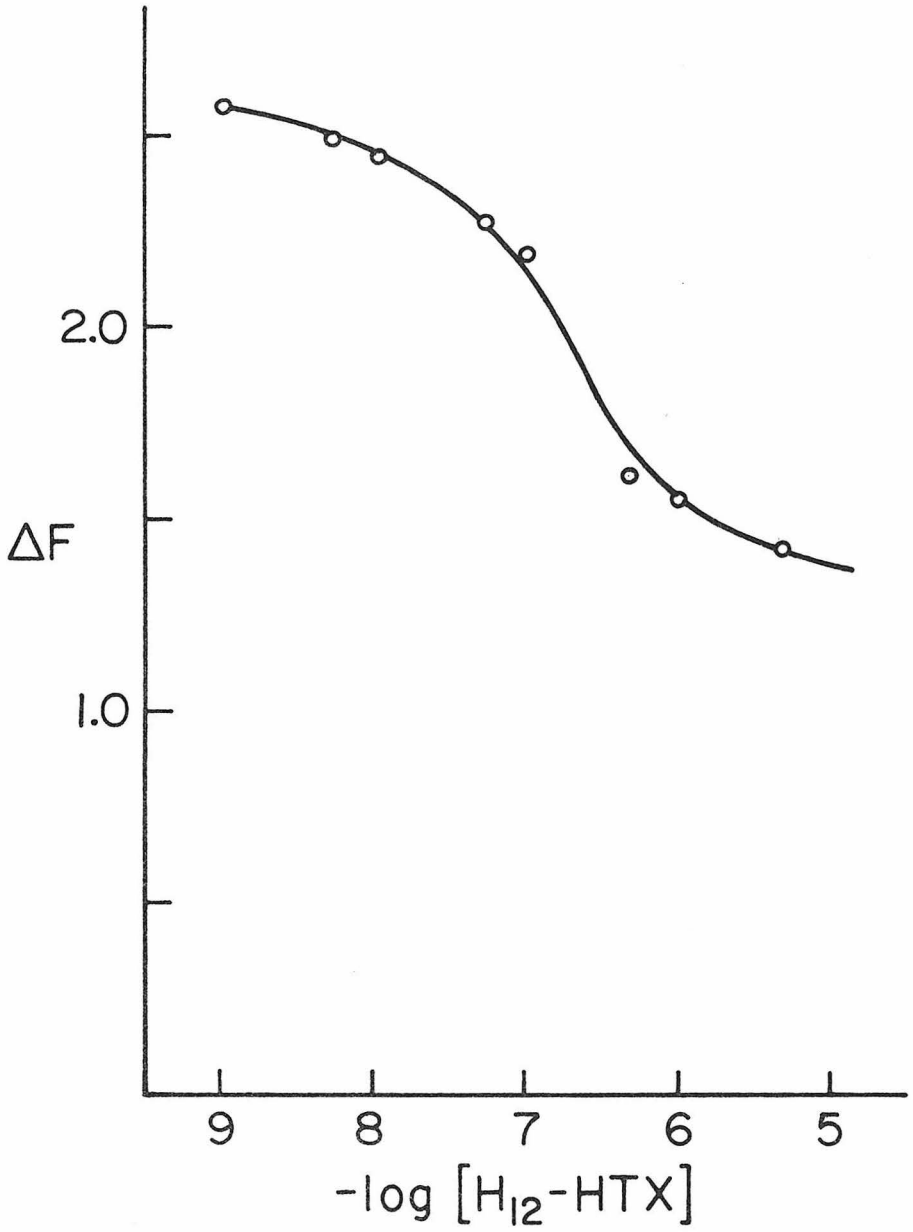

CHOLATE EXTRACT
(B)
2.0
1.0
ΔF
9
8
7
6
5
4
-log [H12-HTX]

of Carb and 0.71 μM in the presence of 40 μM Carb. It is worth noting that the fluorescence enhancement seen upon the addition of Carb to membrane fragments was not observed with the cholate extracts.

The addition of small amounts of Triton X-100 to a cholate extract diluted to 0.5% cholate obliterated the specific components of binding (Figure 21B). In light of the results of Sobel et al. (1978) it is interesting to note that no visible precipitate was observed in samples to which Triton X-100 was added. The ability of low levels of Triton X-100 to block [^{3}H]H_{12}-HTX binding to the diluted cholate extract was similar to its effect on binding to membranes reported above (Figure 17).

A great many attempts were made by the ethidium fluorescence quenching technique to measure H_{12}-HTX binding to AcChR purified by affinity chromatography. The 0.1% Triton X-100 in which the receptor was purified was removed by extensive dialysis or by detergent exchange for cholate via chromatography on DE 52 anion exchange resin. The results of these experiments were very inconsistent and in no case was complete binding activity observed. In most experiments no binding activity was recovered. The experiment in which the largest amount of specific ethidium fluorescence was quenched is shown in Figure 25. The midpoint of this curve occurred at about 0.3 μM H_{12}-HTX, suggesting that some H_{12}-HTX binding activity had been recovered. The fact that any [^{3}H]H_{12}-HTX binding at all could be recovered to purified AcChR argued against the specific binding site being located on the 43,000 polypeptide.

FIGURE 25: Ethidium Bromide Fluorescence with Solubilized Purified AcChR: The buffer is 10 mM Na-phosphate, pH 7.4. Fluorescence is plotted as in Figure 24. The concentration of α-BuTx sites was 0.5 μM, ethidium was 1.0 μM, and there was no Carb present.

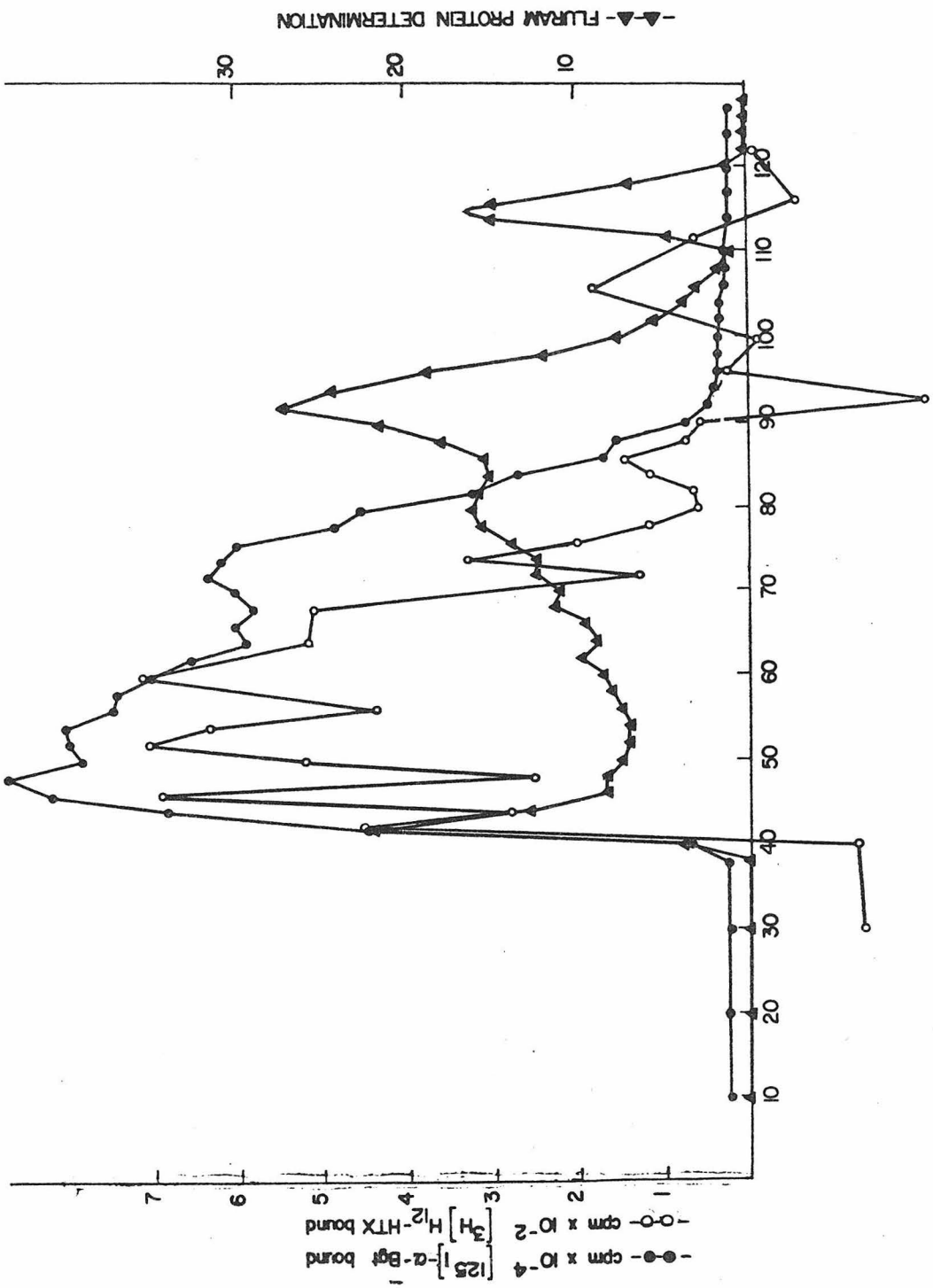

2.0
1.0
ΔF
9
8
7
6
5
-log [H12-HTX]

2. Fractionation of the Protein Components of Detergent Extracts

Some of the results reported above suggested that the HTX binding site is identical with the AcChR, in disagreement with the proposals of Eldefrawi et al. (1977) and Sobel et al. (1978). The constancy of the ratio of [^{3}H]H_{12}-HTX sites to [^{125}I]α-BuTx sites in membranes, upon solubilization with 2% cholate, and in the insoluble residue argued in favor of this view, as did the apparent recovery of partial H_{12}-HTX binding activity in some purified AcChR preparations, which contained only the four AcChR subunits of molecular weights 40,000, 50,000, 60,000 and 65,000. In order to settle this point, it was necessary to develop a purified preparation of some sort which would show full H_{12}-HTX binding activity. Unfortunately the anionic detergent sodium cholate, most suitable for the study of H_{12}-HTX binding activity, is ill adapted for use in established methods of affinity chromatographic purification of AcChR. This may be due to the fact that it binds to the positively charged cholinergic ligands coupled to the column support material. Efforts to develop an affinity chromatographic technique effective in cholate solution have to date been unsuccessful (Miller and Raftery, unpublished results). Thus more traditional protein purification techniques were utilized in an attempt to specifically identify the HTX binding component.

When very concentrated 2% cholate extracts of membrane fragments were chromatographed on a Sepharose 4B column, most of the [^{125}I]α-BuTx binding activity occurred in peaks of very high apparent molecular weigth (Figure 26). These were probably large aggregates or protein-lipid-detergent micelles. The majority of the protein separated from the [^{125}I]α-BuTx binding peak. In addition, there was

FIGURE 26: Sepharose 4B Chromatography of a Cholate Extract: The buffer was 250 mM NaCl, 5 mM KCl, 20 mM Hepes (pH 7.4), 0.4% cholate. The fraction size was 1 ml. The void volume occurred at 41 ml and the column volume eluted at 118 ml. (▲) Total protein determined by Fluram assay. (●) [^{125}I]α-BuTx binding. (○) [^{3}H]H_{12}-HTX binding.


-▲-▲- FLURAM PROTEIN DETERMINATION
-●-●- cpm x 10⁻⁴ [125I]-α-Bgt bound
-○-○- cpm x 10⁻² [3H] H12-HTX bound

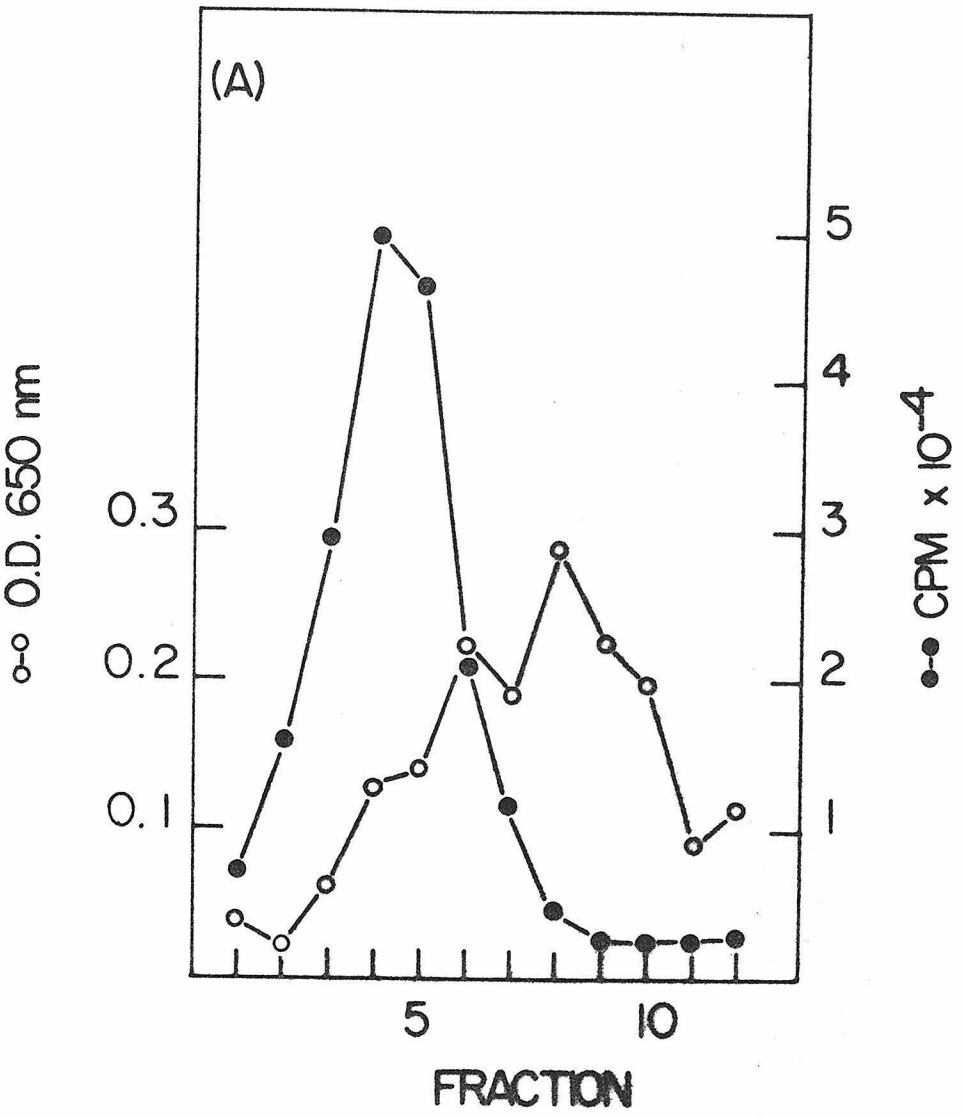
a "protein" peak at the column volume, which was probably phospholipids which gave a false positive protein assay or free amino acids. Specific [^{3}H]H_{12}-HTX binding activity eluted under the [^{125}I]α-BuTx binding peaks, especially the peaks of very high molecular weight. These experiments suggested that HTX binding was closely associated with the AcChR, particularly in its more aggregated forms.

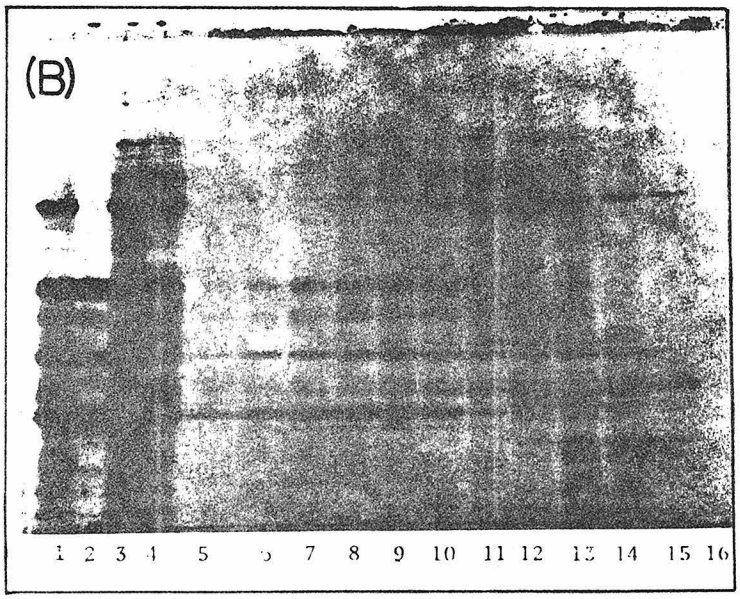
Another effective method of fractionation was centrifugation of 2% cholate extracts of membrane fragments on sucrose density gradients. When extracts were run on 5-20% sucrose gradients in 2% cholate the AcChR moved as the heaviest protein. Figure 27A shows a profile of a typical gradient, and Figure 27B shows SDS gel electrophoresis of the resulting fractions. The tubes near the bottom of the gradient contained nearly pure AcChR by this criterion. The 90,000 and 43,000 dalton bands peaked relatively close to one another, and resolution was not as good but they were reasonably well separated from the AcChR. These proteins migrated with apparent sedimentation coefficients 8.2 S and 6.5 S respectively. For comparison, the AcChR sedimented as one peak having a value of 13.2 S. This value compares favorably with values previously obtained for the dimeric form (Raftery et al., 1972; Reynolds and Karlin, 1978). SDS gel electrophoresis of this purified AcChR preparation showed that the predominant polypeptide species present were the four AcChR subunits (Figure 27B). The small band migrating slightly faster than the 40,000 dalton band is thought to be a degradation product; it is found in some but not all preparations of membranes and purified AcChR (unpublished results, C. D. Strader). The separation of the AcChR from other proteins in this case was

FIGURE 27: Sucrose Gradient Fractionation of a Cholate Extract:

(A). Profiles of [^{125}I] α-BuTx binding (●) and protein measured by Lowry assay (○) of a 5-20% (w/v) surcrose gradient fractionation of a 2% cholate extract made from AcChR enriched membrane fragments. The buffer was 10 mM NaP_i, pH 7.4, 400 mM NaCl, 1 mM EDTA, 2% Na cholate, 0.02% NaN_3.

(B). SDS gel electrophoresis of the fractions resulting from sucrose gradient fractionation of the cholate extract. Wells #5-16 are the fractions from the sucrose gradient, starting at the bottom of the gradient with fraction # 1, wells # 1 and # 3 are AcChR enriched membrane fragments, # 2 is AcChR purified by affinity chromatography, and # 4 is the cholate extract applied to the gradient.


(A)
o-o O.D. 650 mm
•-• CPM x 10⁻⁴
0.3
0.2
0.1
5
4
3
2
1
5
10
FRACTION


(B)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

dependent on the predomination of the dimeric form (see Raftery et al., 1972; Suarez-Isla 1977; Chang and Bock, 1977; Hamilton et al., 1977; Witzemann and Raftery, 1978) which resulted from the iodoacetamide treatment during the initial homogenization of the electroplax as described in the experimental section. The 9 S form, which is the predominant species found without iodoacetamide treatment, would not be well separated from the other components.

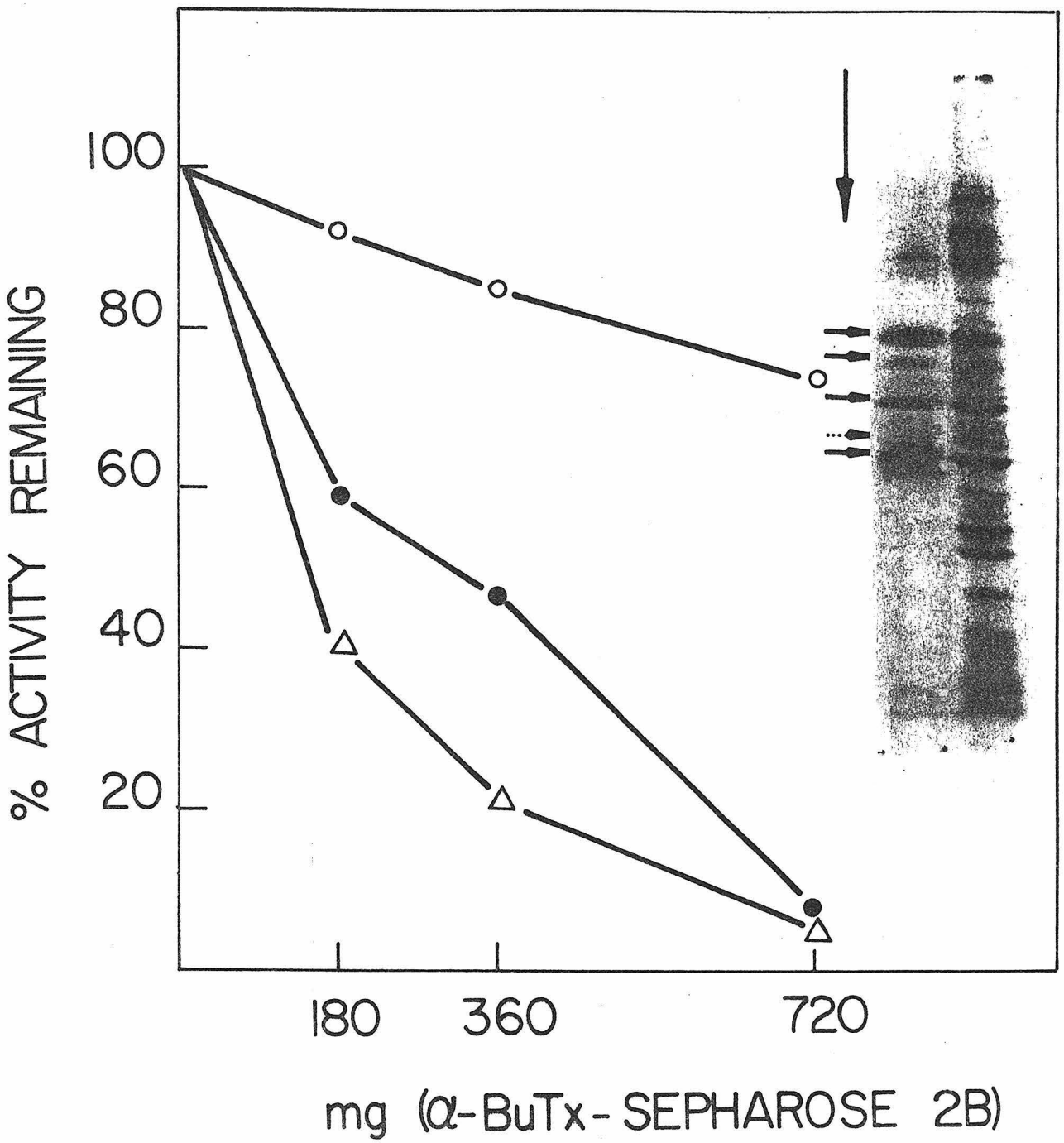
After centrifugation the concentration of protein in the fractions was too dilute to allow a direct measurement of [^{3}H]H_{12}-HTX binding. Control experiments with unfractionated cholate extracts showed that methods of concentration such as vacuum dialysis and membrane ultrafiltration resulted in a loss of specific [^{3}H]H_{12}-HTX binding activity. This may have been due to an effect of contact with the surfaces of the membranes or from a loss of critical lipid, since some [^{3}H]phosphatidylcholine tracer was lost in these experiments. Thus after fractionation by this method it was not possible to distinguish which fraction contained the [^{3}H]H_{12}-HTX binding activity.

Treatment of a 2% cholate extract with a resin consisting of α-BuTx coupled to Sepharose 2B resulted in loss of specific [^{3}H]H_{12}-HTX binding (measured by equilibrium dialysis following dilution of the extract to 0.5% cholate) as well as loss of [^{125}I]α-BuTx binding activity (Figure 28). Most of the protein in the extract, however, was not bound to the resin. After rinsing the resin with the cholate buffer used in the experiment (2% cholate, 250 mM NaCl, 5 mM KCl, 20 mM Hepes pH 7.4, 0.02% NaN_3) or with cholate free Torpedo Ringers it was treated with 8 M urea, 1% SDS, 0.05 M Tris pH 8.8. The material

FIGURE 28: The Effects of Treatment of a 2% Cholate Extract with α-BuTx-Sepharose 2B: Weighed amounts of α-BuTx-Sepharose 2B were added to 1 ml of a cholate extract initially containing 1.9 μM α-BuTx sites, 2.6 mg/ml protein, and binding 0.18 μM [^{3}H]H_{12}-HTX after four-fold dilution to 0.5% cholate. After gentle shaking at 4°C for 48 hours, the resin was pelleted by centrifugation and the supernatant was assayed. Both α-BuTx binding and [^{3}H]H_{12}-HTX binding were removed more rapidly than bulk protein.

Insert: SDS Gels of the Material that Remained Bound to α-BuTx-Sepharose 2B After Rinsing with Buffer: Left, material remaining bound to the resin after extensive rinsing with cholate buffer (2% cholate, 250 mM NaCl, 5 mM KCl, 20 mM Hepes pH 7.4, 0.02% NaN_3); Right, the membrane fragments used in this experiment.

(○) Protein; (●) [^{3}H] H_{12}-HTX binding; (△) [^{125}I]α-BuTx binding.


% ACTIVITY REMAINING
100
80
60
40
20
180
360
720
mg (α-BuTx-SEPHAROSE 2B)

thus removed from the resin was analyzed by SDS gel electrophoresis (Figure 28, insert). The four bands identified as constituent polypeptides of the purified AcChR of molecular weigth 40,000, 50,000, 60,000 and 65,000 daltons were the major components present in the material that bound strongly to the resin. Most of the 43,000 dalton band frequently observed in Torpedo membrane preparations (Sobel et al., 1978; Witzemann and Raftery, 1978) appeared in the supernatant which did not bind to the resin. Since both [^{3}H]H_{12}-HTX and [^{125}I]α-BuTx binding activities were removed from the cholate extract by the α-BuTx-Sepharose 2B resin, [^{3}H]H_{12}-HTX binding activity is most likely associated with one or more of the AcChR subunits rather than the 43,000 dalton component.

3. Extraction of AcChR Enriched Membranes with Base

It was recently reported by Neubig et al. (1979) that AcChR enriched membranes from Torpedo could be selectively depleted of the 43,000 M_r polypeptide and some other non-AcChR polypeptides by treatment with base. These treated membranes contained three of the four polypeptides (M_r 40,000, 50,000 and 66,000) characteristic of the isolated purified AcChR. These membranes bound a ^{14}C labeled local anesthetic analog specifically, and this binding was displaced by micromolar concentrations of H_{12}-HTX.

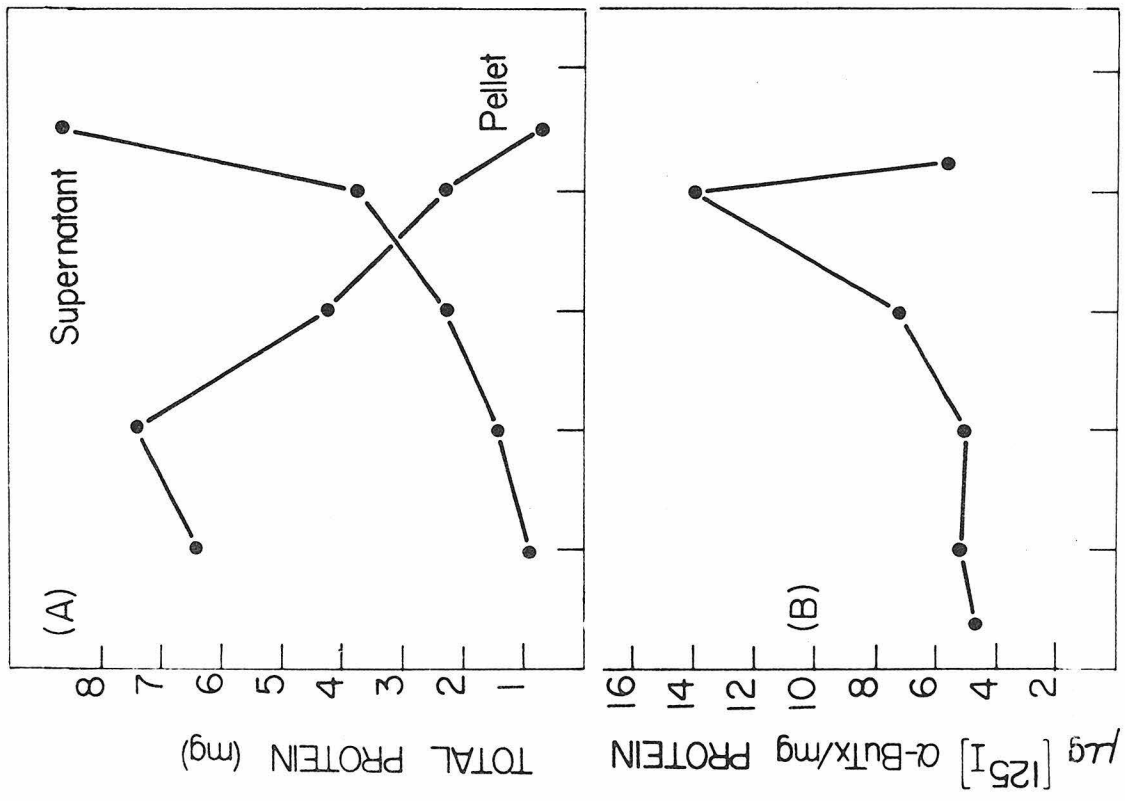
AcChR enriched membranes were treated by increasing the pH at low ionic strength. Figure 29 shows plots of protein and α-BuTx binding activity extracted at pH values ranging from 7.4 to 11.5. Below pH 11, extraction of protein was not efficient, and above this pH α-BuTx

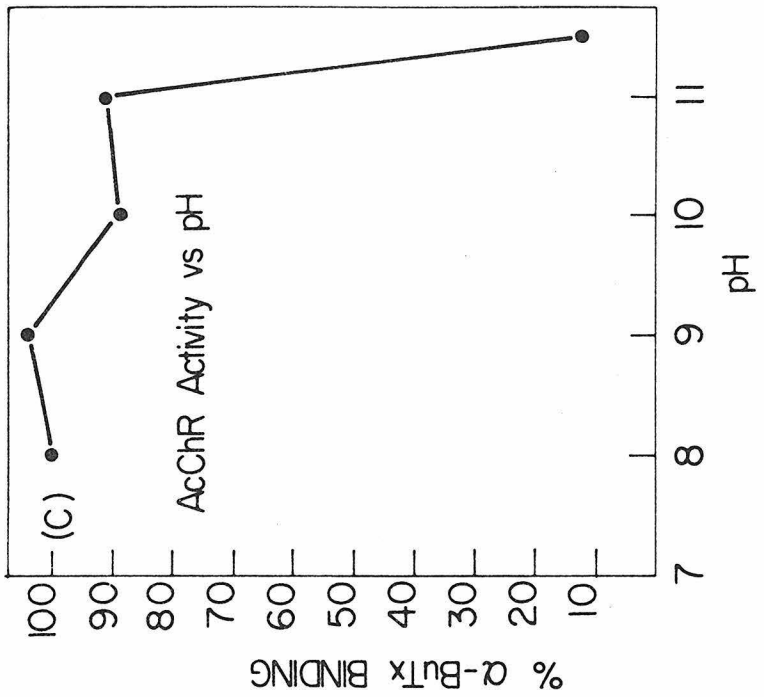
FIGURE 29: Effects of Base Treatment of AcChR Enriched Membranes

(A). Protein recovery for membranes treated at varying pH values as follows: 6.4 ml samples of AcChR enriched membranes suspended in distilled water, containing 8.5 mg protein and 33 nmoles [^{125}I] α-BuTx binding sites, were adjusted to various final pH values with 0.5% NaOH.

(B). Specific activity of these membranes after pH treatment.

(C). [^{125}I] α-BuTx binding activity remaining in the membranes after pH treatment.


(A)
Supernatant
Pellet
TOTAL PROTEIN (mg)
(B)
μg [125I] α-BuTx/mg PROTEIN
pH


(C)
AcChR Activity vs pH
% α-BuTx BINDING
pH

binding activity fell off rapidly. Maximal specific activity was obtained by treatment at pH 11. In this experiment, protein recovery in the pelleted membranes was 38% and recovery of [^{125}I]α-BuTx sites was 91%. Typical experiments yielded 75% recovery of toxin sites, although lower recoveries were occasionally obtained.

Figure 30 shows SDS gel electrophoresis of the membranes and supernatants after treatment at increasing pH. At pH 11 essentially all of the 43,000 polypeptide and most of 90,000 polypeptide were found in the supernatant fraction, along with several minor components. The membranes contained the four polypeptides identified as subunits of the AcChR (Raftery et al., 1974; Weill et al., 1974; Raftery et al., 1975; Karlin et al., 1975; Vandlen et al., 1976; Witzemann and Raftery, 1978), having molecular weights of 40,000, 50,000, 60,000 and 65,000 daltons as well as minor bands of molecular weight significantly smaller than 40,000 daltons and a band at 90,000 daltons.

At the electron microscope level, the base-treated membrane fragments were virtually indistinguishable from the untreated ones. Both preparations consisted primarily of spherical vesicles ranging in diameter from 1000 to 10,000 Å, many of which were covered with the 60 to 70 Å diameter rosettes characteristic of AcChR-containing membranes (Elliott et al., 1979a).

[^{3}H]H_{12}-HTX bound to the treated membranes lacking the 43,000 polypeptide with unchanged stoichiometry and dissociation constant (Figure 31). The ability of Carb to regulate the affinity of the membranes for [^{3}H]H_{12}-HTX was unchanged; the K_D for untreated membranes was lowered from 1.4 μM to 0.77 μM in the presence of 10 μM

FIGURE 30: SDS Polyacrylamide Gel Electrophoresis of Base Treated Membranes: Left to right, 1, Purified AcChR; the following pairs are treated membranes and supernatants at given pH values: 2,3, pH 7.4; 4,5, pH 8.0; 6,7, pH 9.0; 8,9, pH 10.0; 10,11, pH 11.0; 12,13, pH 11.5; 14, purified AcChR; 15, AcChR enriched membranes; 16, membranes which had lower density than AcChR enriched membranes in the membrane purification step.

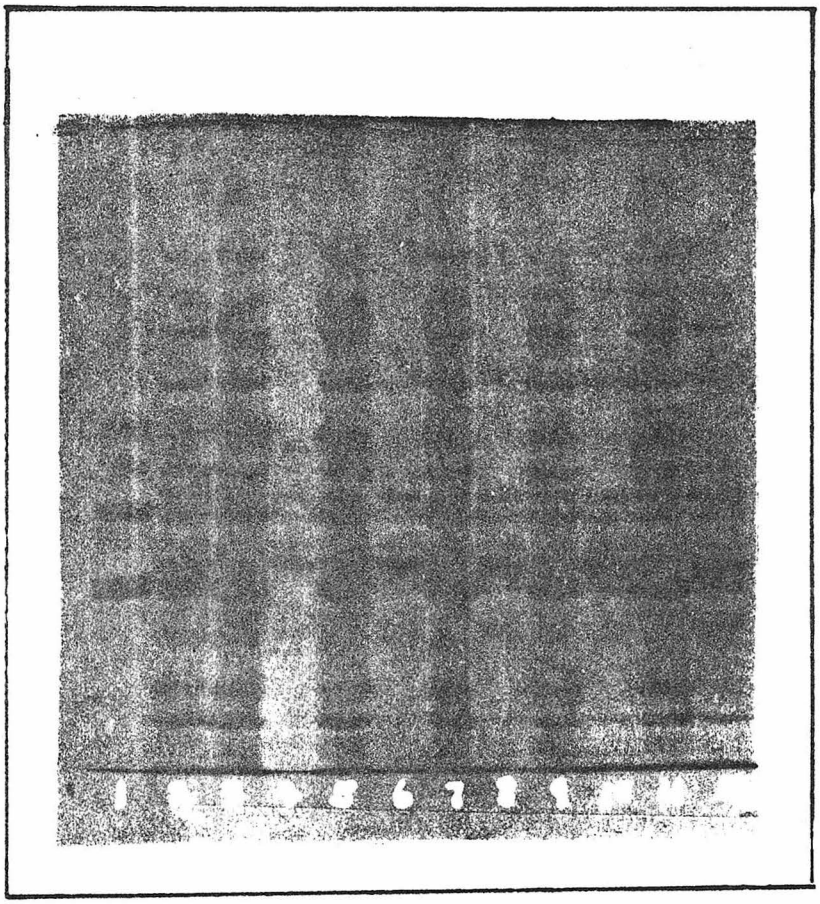
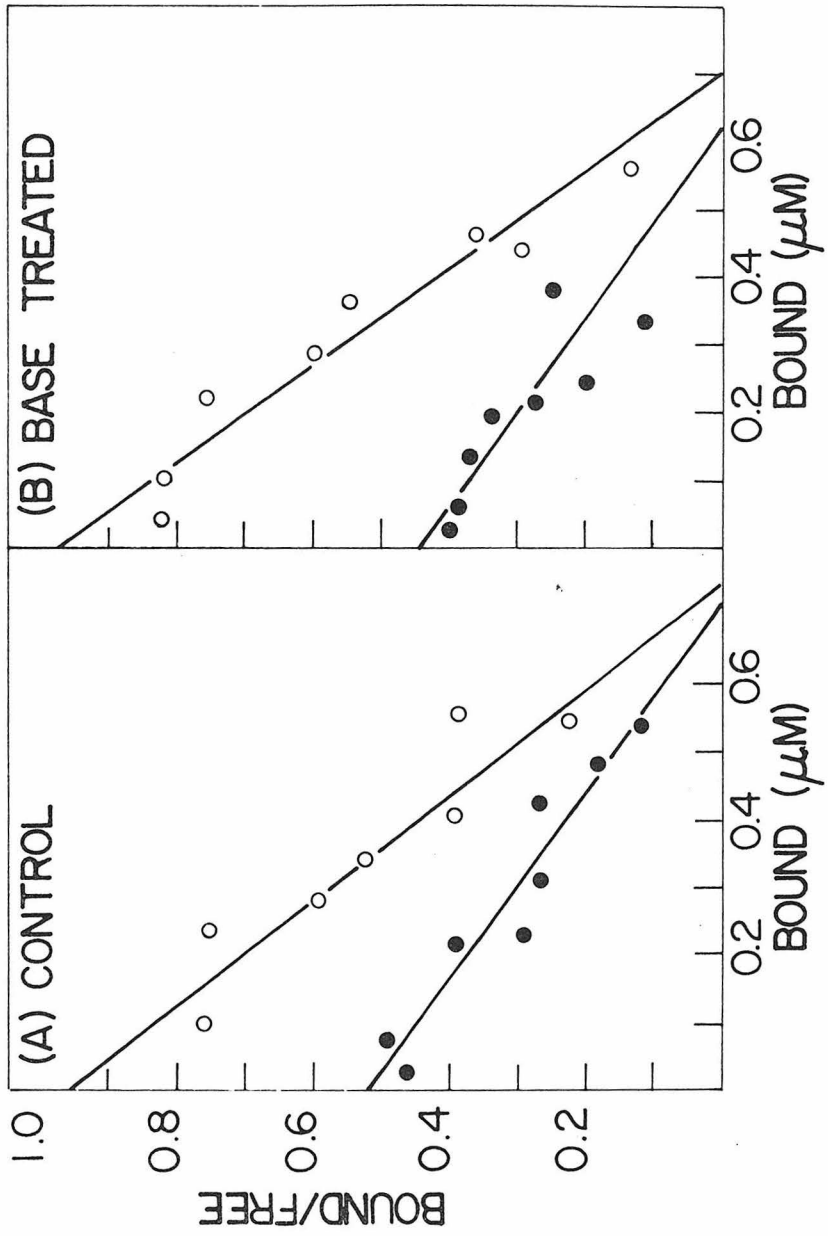


FIGURE 31: Scatchard Plots of [^{3}H]H_{12}-HTX Binding to AcChR Enriched Membranes Before and After Treatment at pH 11: Left, pH 7.4; Right, pH 11.0. (●) no Carb present; (○) 10 μM Carb. In both experiments the concentration of [^{125}I] α-BuTx sites was 1.9 μM and the buffer was 20 mM Hepes (pH 7.4), 250 mM NaCl, 5 mM KCl, 0.02% NaN_3. The plots were made using a linear least-squares fit of the data.

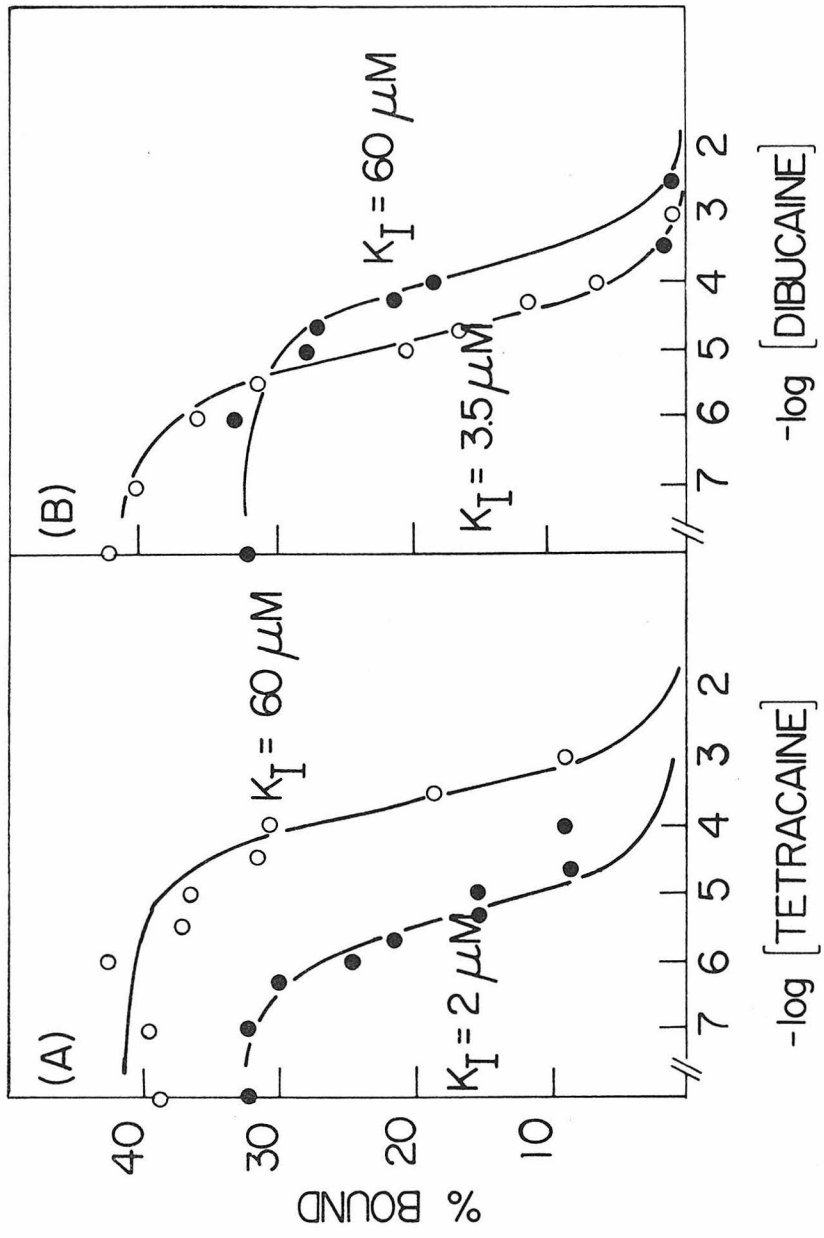

(A) CONTROL
(B) BASE TREATED
BOUND/FREE
BOUND (μM)
1.0
0.8
0.6
0.4
0.2
0.2
0.4
0.6

Carb while for base treated membranes it changed from 1.4 μM to 0.73 μM. The values of K_D previously reported for membranes in these buffer conditions (no Ca^{++} or Mg^{++} present) are 0.9 μM in the absence and 0.5 μM in the presence of 10 μM Carb (see Figure 7 above; Elliott and Raftery, 1979). The kinetic mechanism of H_{12}-HTX binding, determined by stopped flow kinetics using the extrinsic fluorescence probe ethidium, was unchanged after base treatment (Elliott et al., 1979b).

The local anesthetics dibucaine and tetracaine displaced [^{3}H]H_{12}-HTX bound to base treated membranes with apparent K_Is not significantly different from those determined for untreated membranes (Figure 13 above; Blanchard et al., 1979). The apparent K_I for dibucaine was 60 μM and for tetracaine 2 μM (Figure 32). Carb regulated the affinity of the base treated membranes for these local anesthetics. 10 μM Carb increased the apparent affinity of the H_{12}-HTX site for dibucaine approximately 17-fold (K_I = 3.5 μM). The reverse effect was seen with tetracaine; its apparent affinity was decreased $\sim$ 30 fold in the presence of 10 μM Carb (K_I = 60 μM) (Figure 32). It should be noted that at this concentration of Carb the membrane bound AcChR was essentially fully converted to the conformational state(s) having high affinity for agonists (Quast et al., 1978b).

Since the 43,000 polypeptide was not necessary for specific binding of H_{12}-HTX, or for binding of the local anesthetics tested, to membrane fragments or for Carb regulation of those binding activities, we investigated the possibility that it has a structural role. We examined this possibility by determining whether [^{3}H]H_{12}-HTX binding

FIGURE 32: Displacement of [^{3}H] H_{12}-HTX Bound to Base Treated Membranes by Tetracaine and Dibucaine: The concentration of [^{125}I] α-BuTx sites was 1.33 μM, [^{3}H] H_{12}-HTX was 0.44 μM, and the buffer was Torpedo Ringers. The H_{12}-HTX dissociation constants used to draw the theoretical curves for competitive inhibition were 0.2 μM in the presence of 10 μM Carb (○) and 0.4 μM in its absence (●). Left, tetracaine: (○) K_I = 60 μM, (●) K_I = 2 μM. Right, dibucaine: (○) K_I = 3.5 μM, (●) K_I = 60 μM.


(A)
(B)
K_I = 60 μM
K_I = 2 μM
K_I = 60 μM
K_I = 3.5 μM
% BOUND
40
30
20
10
7 6 5 4 3 2
7 6 5 4 3 2
-log [TETRACAINE]
-log [DIBUCAINE]

could be reconstituted after dissolution of the membranes in 2% cholate. The extract of base treated membranes was diluted from 2% to 0.5% cholate, a concentration at which [^{3}H]H_{12}-HTX binding was previously shown to be completely recovered in extracts of untreated membranes (Figure 21 above). The extract bound [^{3}H]H_{12}-HTX with $K_D \sim 2$ μM and 0.18 [^{3}H]H_{12}-HTX sites per [^{125}I]α-BuTx site (Figure 33). The K_D and sites ratio for [^{3}H]H_{12}-HTX binding to cholate extracts reported above were 1.6 μM and 0.26 respectively. Thus [^{3}H]H_{12}-HTX binding was largely, though not completely, recovered. The binding of [^{3}H]H_{12}-HTX to extracts of membranes not treated with base was strongly dependent on cholate concentration (Figure 21 above). It has not yet been shown whether such concentration dependence is the same for base treated membranes. Thus the incomplete recovery of H_{12}-HTX binding sites ($\sim$70% recovered) might be a function of a different dependence on cholate concentration. The effect of base treatment on the lipid composition of the membranes has not yet been investigated, and so with base treated membranes the lipid:detergent ratio may be significantly different.

When a cholate extract of the pH 11 treated membrane preparation was centrifuged on a 5-20% sucrose gradient containing 2% cholate, a small amount of protein separated from the [^{125}I] α-BuTx binding peak. A profile of a gradient is shown in Figure 34A. SDS gel electrophoresis of the peak tube from the region of [^{125}I] α-BuTx binding activity showed that it contained only the four AcChR polypeptides (Figure 34B). This preparation of solubilized AcChR was as pure according to this criterion as the most highly purified AcChR obtained by affinity chromatographic

FIGURE 33: Reconstitution of [^{3}H] H_{12}-HTX Binding: Binding of [^{3}H] H_{12}-HTX to a 2% cholate extract of base treated membrane fragments diluted to 0.5% cholate in the presence (○) and absence (●) of 40 μM unlabeled H_{12}-HTX. The concentration of [^{125}I] α-BuTx sites was 1.9 μM. The bottom curve (○) shows a fairly large component of non-specific binding.

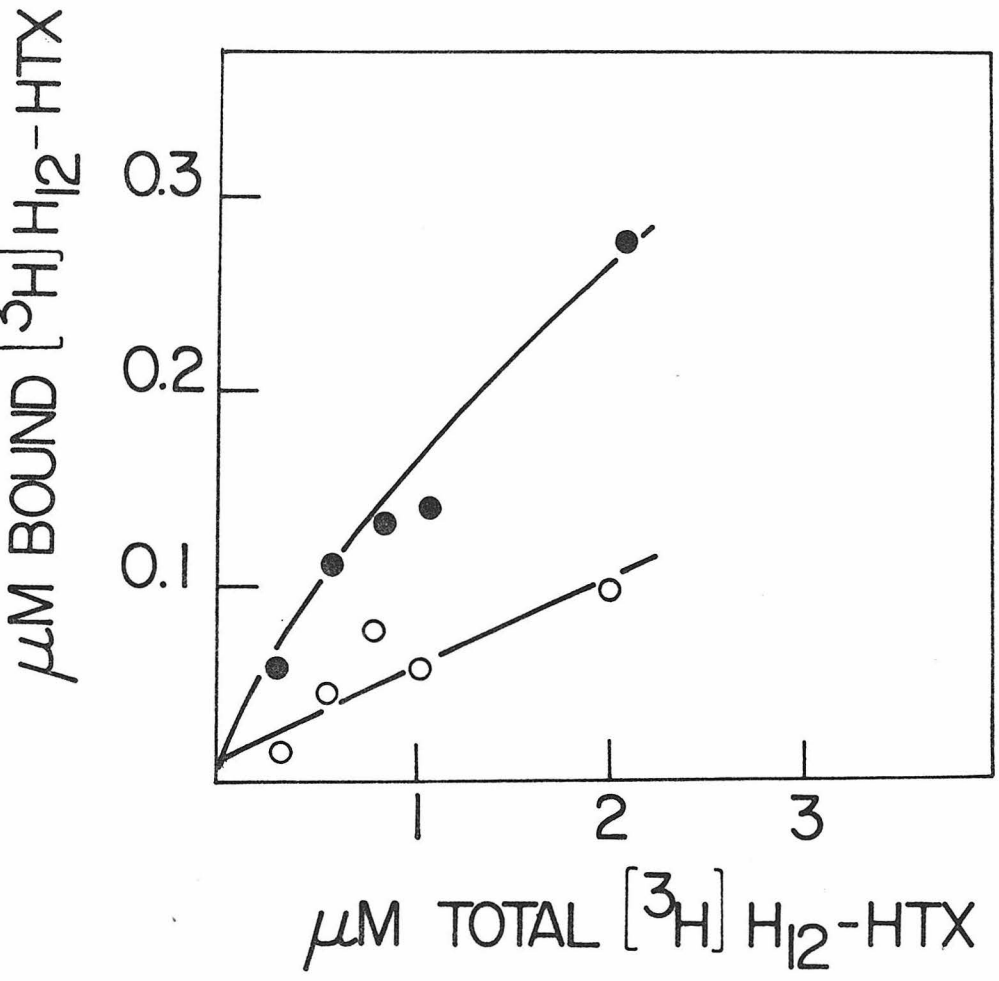
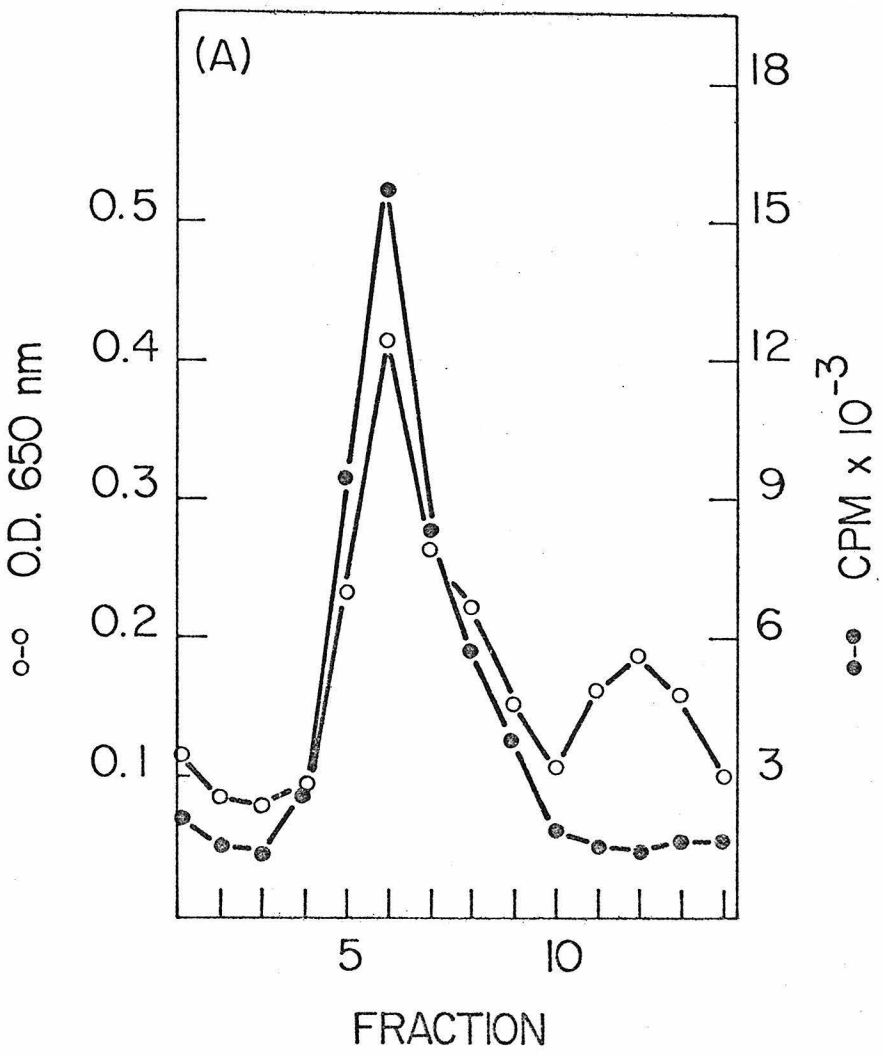
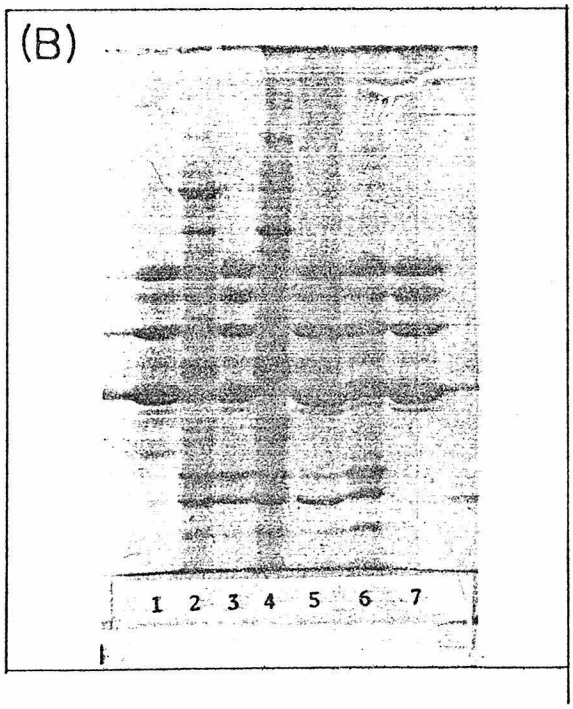

μM BOUND [3H] H12-HTX
0.3
0.2
0.1
1
2
3
μM TOTAL [3H] H12-HTX

FIGURE 34: Purification of AcChR from Base Treated Membranes:
(A). Profiles of [^{125}I]α-BuTx binding (●) and protein measured by Lowry assay (○) of a 5-20% (w/v) sucrose gradient fractionation of a 2% cholate extract of pH 11 treated membrane fragments. Buffer is the same as in Figure 27.
(B). SDS gel electrophoresis. 1. AcChR purified by affinity chromatography; 2. The membrane fragments used in this experiment; 3. The membrane fragments after treatment at pH 11; 4. The supernatant containing polypeptides extracted at pH 11; 5. The 2% cholate extract of the pH 11 treated membranes; 6. The pellet containing material not solubilized by 2% cholate; 7. Fraction # 6 (the peak tube) from the sucrose gradient centrifugation.


(A)
0.5
0.4
0.3
0.2
0.1
O.D. 650 nm
18
15
12
9
6
3
CPM x 10^{-3}
5
10
FRACTION


(B)
1 2 3 4 5 6 7

procedures (Raftery, 1975; Vandlen et al., 1976).

The results reported above eliminate the possibility that the 43,000 polypeptide contains the specific binding site for H_{12}-HTX of $K_D \sim 0.5\ \mu M$ and for some local anesthetics such as dibucaine and tetracaine. Base treated membranes retained the capacity to bind [^{3}H] H_{12}-HTX and the local anesthetics tested, and the regulation of this binding by Carb and the kinetic mechanism of H_{12}-HTX binding were unchanged. Additionally, [^{3}H] H_{12}-HTX binding was largely reconstituted from 2% cholate extracts of base treated membranes. Thus the H_{12}-HTX binding site appears to be located on one or more of the AcChR polypeptides, and the reconstitution of that binding site from detergent extracts does not require the presence of a 43,000 M_r polypeptide.

4. Some Characteristics of the H_{12}-HTX Binding Site

Moore and Raftery (1979b) showed that treatment of AcChR enriched membranes with the reducing agent dithiothreitol blocked agonist induced conversion of the receptor from the low affinity to the high affinity state for Carb. Alkylation by iodoacetamide after reduction permanently "locked" the membrane bound AcChR in the low affinity state. Recent unpublished work showed that this treatment could reduce the number of α-BuTx sites available in the membrane, while not affecting the number of Carb or AcCh sites (Dunn and Raftery, unpublished). Usually the stoichiometry of α-BuTx sites to agonist sites changed from 2:1 to approximately 1:1. The number of α-BuTx sites lost could vary from 0 to 65% of the starting value with respect

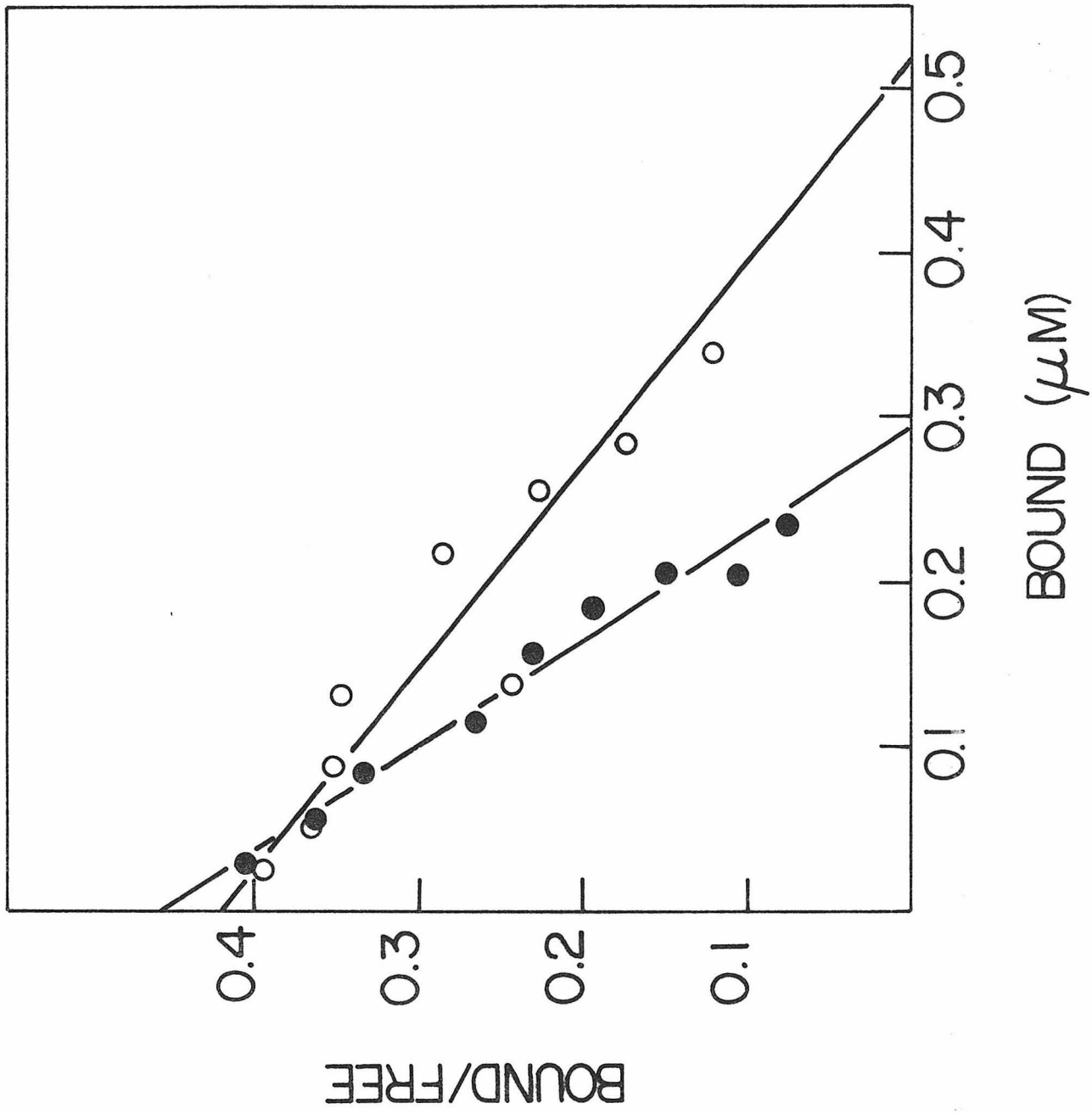
to total protein. In all cases the ratio of [^{3}H]H_{12}-HTX sites to total protein remained approximately constant.

A typical [^{3}H]H_{12}-HTX binding experiment is shown in Figure 35. In this experiment the concentration of α-BuTx sites with respect to total protein after treatment decreased to 43% of the starting value. The ratio of [^{3}H]H_{12}-HTX sites to α-BuTx sites underwent an increase of 1.8 fold. Therefore the number of [^{3}H]H_{12}-HTX sites in the membranes remained approximately constant. However, the K_D changed: it increased two fold from 0.6 μM to 1.2 μM. The AcChR was changed in such a way that [^{3}H]H_{12}-HTX did not bind as tightly, though the total extent of binding was not greatly affected.

It was suggested above that H_{12}-HTX might bind in a ratio of one H_{12}-HTX per AcChR dimer. Reduction and alkylation of these membranes converted receptor dimers quantitatively to monomers, demonstrated by sucrose density gradient centrifugation of 2% cholate extracts (not shown). However in the membrane dimers may be held together by noncovalent interactions as well as by disulfide bonds. If H_{12}-HTX binds exclusively to dimers, then this is very likely to be the case.

It was previously shown that treatment of AcChR enriched membranes with trimethyloxonium tetrafluoroborate resulted in a decrease in cholinergic ligand binding while α-BuTx binding was very little affected (Chao et al., 1975). The number of Carb and AcCh sites decreased, but the K_Ds remained essentially unaltered. Binding of bifunctional ligands such as decamethonium or its fluorescent analog bis-(3-amino pyridinium)1,10-decane was most strongly affected and was, in fact, almost totally obliterated. It was concluded that the site

FIGURE 35: [^{3}H]H_{12}-HTX Binding to AcChR Enriched Membranes Before and After Reduction and Alkylation: (●) Control (○) membranes reduced with dithiothreitol and alkylated with iodoacetamide as described in Part 7 of the Experimental Section. In both cases the concentration of [^{125}I]α-BuTx binding sites was 1.5 μM.


BOUND/FREE
0.4
0.3
0.2
0.1
BOUND (μM)
0.1
0.2
0.3
0.4
0.5

of specific modification was adjacent to the acetylcholine binding site, most likely at a carboxyl group which interacts with the second quaternary nitrogen group of bifunctional ligands.

Reed and Raftery (1976) showed that the tetrodotoxin binding component (voltage sensitive sodium channel) in eel electroplax was inactivated by treatment with trimethyloxonium tetrafluoroborate. Inactivation could be blocked by preincubation with tetrodotoxin or saxitoxin. This result and the pH dependence of tetrodotoxin binding led to the conclusion that trimethyloxonium methylated a carboxyl group in the tetrodotoxin binding site.

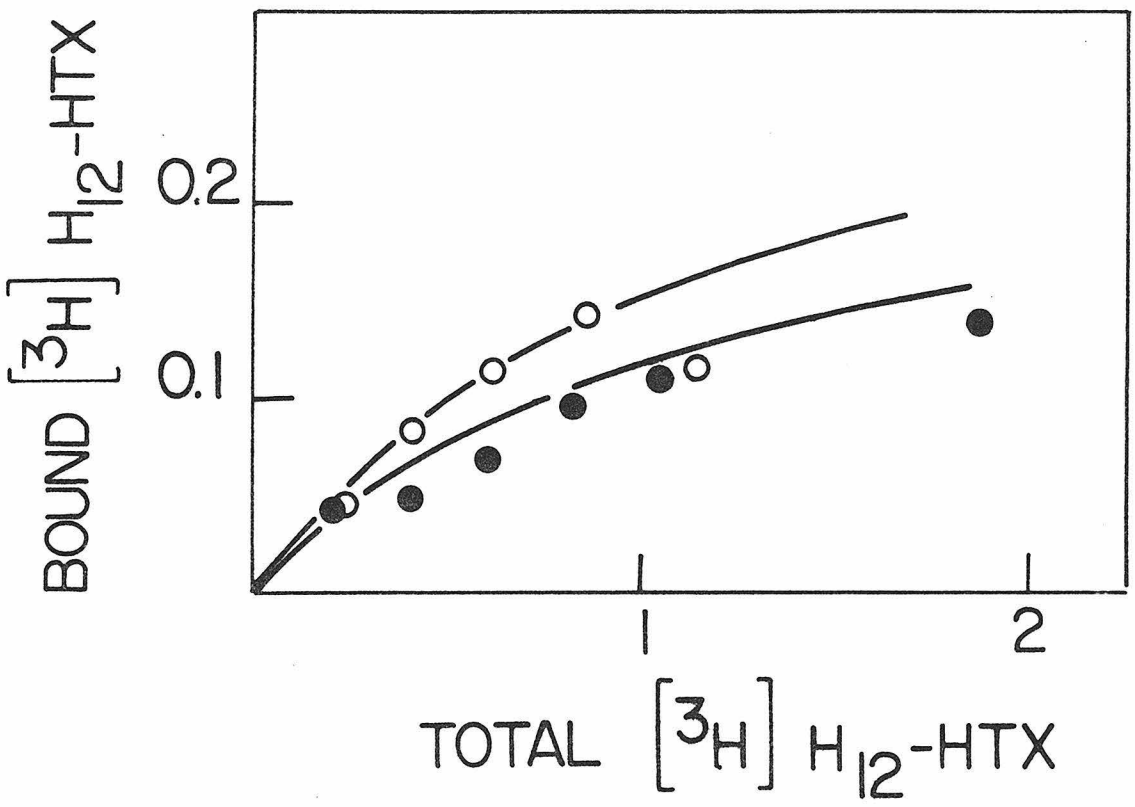
At physiological pH H_{12}-HTX carries a positive charge and so it is likely that the H_{12}-HTX binding site contains an anionic function. However, if this group is a carboxyl it has a low selectivity for the trimethyloxonium ion or it is not readily accessible to this ion. Treatment with 6 mg/ml trimethyloxonium tetrafluoroborate resulted in loss of 50% of α-BuTx binding activity and 64% of $[^3H]H_{12}$-HTX binding activity (Figure 36). Thus this ion does not modify the H_{12}-HTX site with high affinity or selectivity.

The effects of various inorganic cations on the rate of α-BuTx binding to AcChR have been determined (Schmidt and Raftery, 1974). Inhibition constants of about 5 mM were measured for Na^+, K^+, Li^+, Rb^+, Cs^+, NH_4^+, and Tris $\cdot$ H^+. The divalent cations Ca^{2+} and Mg^{2+} slowed the toxin on-rate more effectively with inhibition constants of about 0.2 mM.

Divalent cations were shown to increase the affinity of AcChR enriched membranes for $[^3H]H_{12}$-HTX (Figure 7 above). The K_D

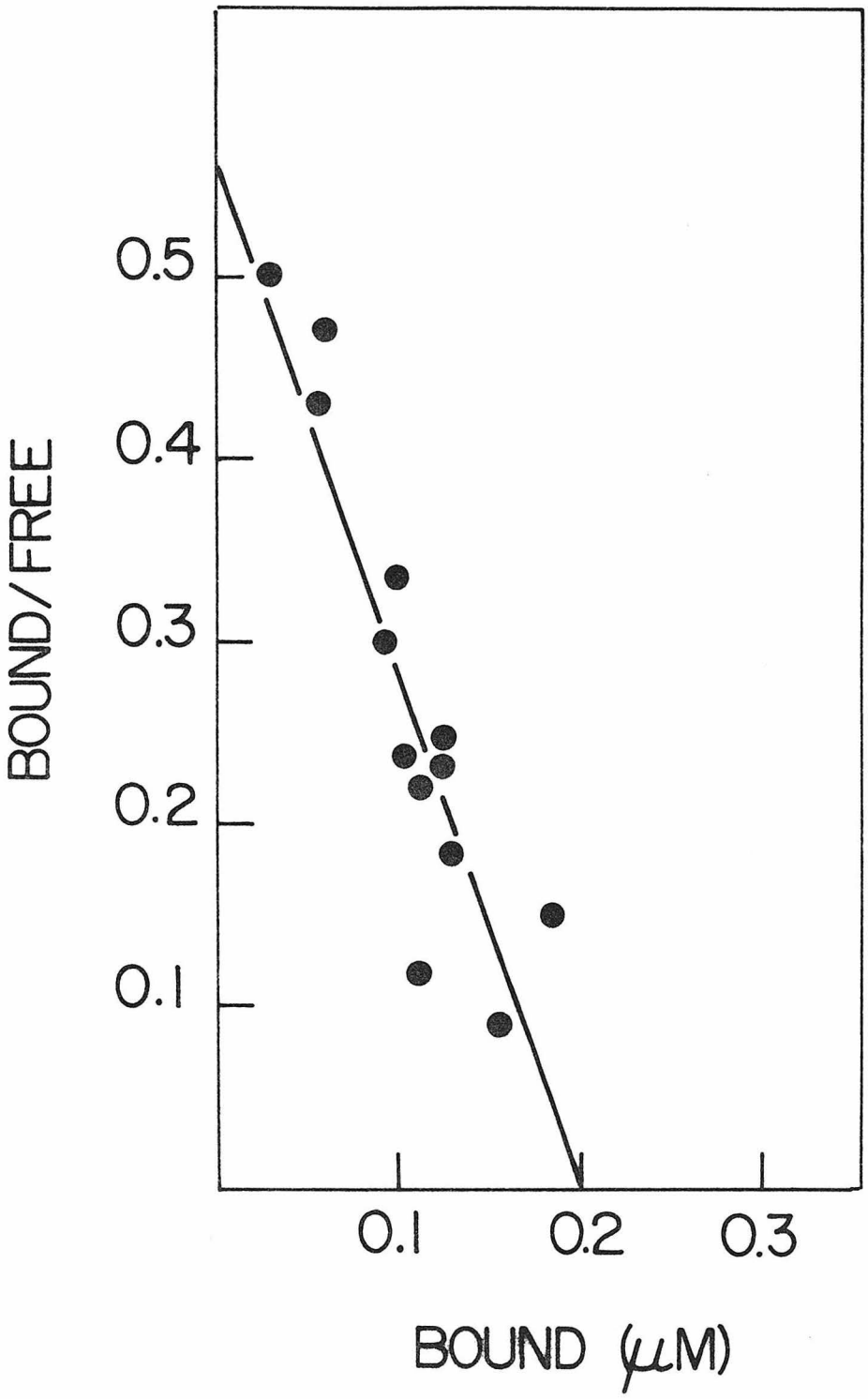
FIGURE 36: [^{3}H]H_{12}-HTX Binding to AcChR Enriched Membranes Before and After Treatment with $(CH_3)_3O^+BF_4^-$:

(○) Control membranes; (●) membranes after treatment with $(CH_3)_3O^+BF_4^-$ as described in Part 7 of the Experimental Section. In both cases the concentration of [^{125}I]α-BuTx binding sites was 0.86 μM.


BOUND [3H] H12-HTX
0.2
0.1
1
2
TOTAL [3H] H12-HTX

increased from 0.5 μM to 0.9 μM when the buffer was changed from Torpedo Ringers to 250 mM NaCl, 5 mM KCl, 20 mM Hepes (pH 7.4), 2 mM EDTA. This change was probably due to effects on the conformation of the AcChR; Ca^{2+} has been shown to have a large effect on the process of conversion from the low affinity to the high affinity state for agonists (Lee et al., 1977). The K_D (0.4 μM) for [^{3}H]H_{12}-HTX binding to AcChR enriched membranes in a low Na^+ buffer (10 mM Tris · HCl, 10 mM NaCl) was not greatly different from that determined in Torpedo Ringers (0.5 μM) (Figure 37). Therefore the effect of Na^+ on [^{3}H]H_{12}-HTX binding is not large.

FIGURE 37: Scatchard Plot of [^{3}H]H_{12}-HTX Binding to AcChR Enriched Membranes in Low Salt Buffer: The buffer was 10 mM Tris · HCl (pH 7.4), 10 mM NaCl and the concentration of [^{125}I]α-BuTx sites was 0.66 μM.


BOUND/FREE
0.5
0.4
0.3
0.2
0.1
0.1
0.2
0.3
BOUND (μM)

V. CONCLUSIONS

The stoichiometry of H_{12}-HTX binding relative to α-BuTx binding reported above suggests that one H_{12}-HTX binds per AcChR dimer. This may imply that there is one ionic conductance "channel" or one mechanism for regulation of channel opening per receptor dimer. There is evidence from membrane vesicle studies that dimers actively flux $^{22}Na^{+}$ (Miller et al., 1978).

It is considered unlikely in the Torpedo electroplax system that H_{12}-HTX acts to block conductance primarily by affecting the agonist induced conversion of AcChR to the high affinity ("desensitized") state. Some local anesthetics showed much larger differences in their affinity for the low affinity and the high affinity states for agonists; they had correspondingly greater effects on in vitro "desensitization."

The dependence on detergent concentration of H_{12}-HTX binding to cholate extracts of membranes was very interesting. Since binding was recovered only at cholate concentrations near the critical micelle concentration, some degree of reaggregation may be necessary for an active H_{12}-HTX site.

The H_{12}-HTX binding component has been positively identified with the AcChR, ending speculation that the binding site might be located on the 43,000 M_r polypeptide found in Torpedo membranes. It is hoped that in the future photoaffinity labeling studies using an azido derivative of [^{3}H]H_{12}-HTX will show which receptor polypeptide(s) contain the H_{12}-HTX binding site.

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