Topographical Studies of the Nicotinic Acetylcholine Receptor

Thesis by
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To my parents

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Chapter 1

Exposure of the Acetylcholine Receptor to the Lipid Bilayer

ABSTRACT: All four subunits of the nicotinic acetylcholine receptor in membrane vesicles isolated from *Torpedo californica* have been labeled with the photoactivated hydrophobic probe, [^{3}H]adamantanediazirine, which selectively labels regions of integral membrane proteins in contact with the hydrocarbon core of the lipid bilayer. As all of the homologous subunits are exposed to the lipid bilayer, it is probable that they each interact with the surrounding membrane in a similar fashion.

Chapter 2

Identification of the Subunits of Acetylcholine Receptor that Interact with a Cholesterol Photoaffinity Probe

ABSTRACT: All four subunits of the acetylcholine receptor in membrane vesicles isolated from *Torpedo californica* have been labeled with [^{3}H]cholesteryl diazoacetate. As this probe incorporates into lipid bilayers analogously to cholesterol, this result indicates that acetylcholine receptor interacts with cholesterol. This investigation also demonstrates that this probe is a useful reagent for studying the interaction of cholesterol with membrane proteins.

Since the photogenerated carbene is situated near the lipid-water interface, this probe has potential as a topographic tool for mapping membrane protein structure. The labeling studies with both [^{3}H]adamantanediazirine and [^{3}H]cholesteryl diazoacetate support the concept that the acetylcholine receptor is a pseudosymmetric complex of homologous subunits, all of which interact with and span the membrane.

Chapter 3

Synthesis of Fluorine-Containing Agonists for the Acetylcholine Receptor

ABSTRACT: The syntheses of the fluorine-containing agonists for the *Torpedo californica* nicotinic acetylcholine receptor, fluoroacetylcholine bromide and p-fluorophenyltrimethylammonium iodide, are described. It is demonstrated that both are agonists using a cation flux assay with acetylcholine receptor enriched membrane vesicles. The potential for their use in ligand binding studies using ^{19}F NMR spectroscopy is discussed.

Chapter 4

Affinity Cleavage of the Acetylcholine Receptor: Evidence for a Low-Affinity Agonist Binding Site

ABSTRACT: The affinity cleavage reagent, p-thiocyanophenyltrimethylammonium iodide, specifically cleaves a peptide bond of the nicotinic acetylcholine receptor in membrane vesicles isolated from *Torpedo californica*. It is demonstrated that this reagent is an agonist using a cation flux assay. The cleavage is blocked by stoichiometric quantities of α-bungarotoxin. The yield of the cleavage reaction is reduced with addition of the agonist, phenyltrimethylammonium iodide. This affinity cleavage reaction provides evidence for a low-affinity binding site for agonists on the 60K subunit.

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Abbreviations

ACh, acetylcholine.

AChR, acetylcholine receptor.

α-BgTx, α-bungarotoxin.

BrACh, bromoacetylcholine.

BSA, bovine serum albumin.

CARB, carbamylcholine chloride.

DAP, bis(3-aminopyridinium)-1,10-decane diiodide.

DAPA, bis(3-azidopyridinium)-1,10-decane perchlorate.

ESR, electron spin resonance.

FAcCh, fluoroacetylcholine bromide.

FT-IR, fourier transform infrared.

HEPES, N-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonic acid.

IANBD, 4-[[(iodoacetoxy)ethyl]methylamino]-7-nitro-2,1,3,-benzoxadiazole.

IR, infrared.

MBTA, [4-(N-maleimido)benzyl]trimethylammonium diiodide.

NaDodSO_4, sodium dodecyl sulfate.

NBD, 7-nitro-2,1,3-benzoxadiazole.

NMR, nuclear magnetic resonance.

NTCB, 2-nitro-5-thiocyanobenzoic acid.

PFT, p-fluorophenyltrimethylammonium iodide.

PTA, phenyltrimethylammonium iodide.

TEA, triethylamine.

THF, tetrahydrofuran.

TLC, thin-layer chromatography.

TPMA, p-thiocyanophenyltrimethylammonium iodide.

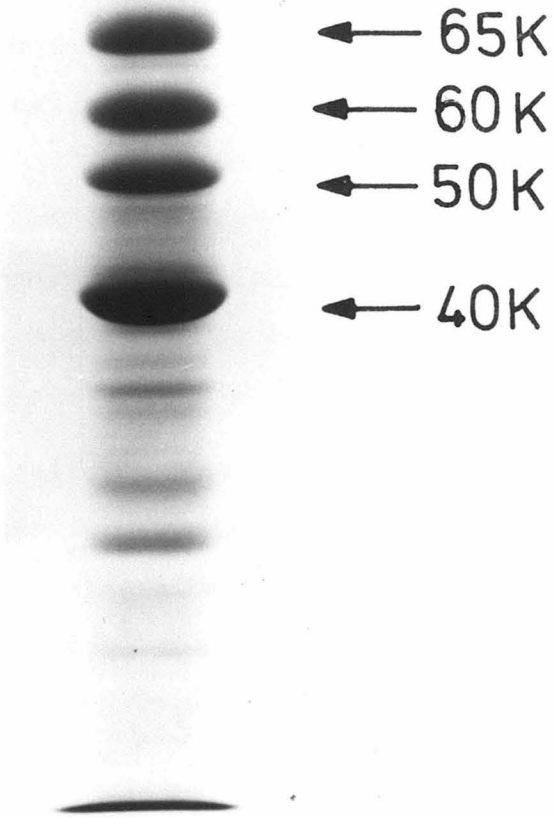
UV, ultraviolet.

Introduction

The understanding of the molecular basis of a neurotransmitter receptor has been greatly facilitated by the isolation of relatively large quantities of the nicotinic acetylcholine receptor (AChR) from the electric organs of marine rays in the genus *Torpedo*. The electric organs are a modified muscle tissue which, in addition to being an effective predatory offense (Bray & Hixon, 1978), are a rich source of the AChR. In the past ten years, the purification and unequivocal identification of the components of the AChR has been demonstrated by reconstitution of functional receptor into membrane vesicles [for a recent review, see Conti-Tronconi & Raftery, 1982]. In the last five years, all of the AChR subunits have been cloned (cDNAs), sequenced, subunit mRNAs synthesized using *in vivo* (Mishina et al., 1984) and *in vitro* systems (White et al. 1985; Sakmann et al., 1985), and functionally expressed in *Xenopus* oocytes for both *Torpedo californica* and calf. Considerable understanding has been gained on the molecular basis of AChR function [for recent reviews, see Conti-Tronconi & Raftery, 1982; Raftery et al., 1983; Adams, 1981; Taylor et al., 1983; Changeux et al., 1984]. Outstanding structural questions include the primary structural locations of the ion channel, possible multiple agonist binding sites, and regulatory sites. The complementary approaches of site directed mutagenesis, immunochemical mapping, and chemical labeling experiments should soon answer these structural questions. Correlation of structural data with kinetic data will lead to an understanding of the mechanisms involved in the transduction of a signal mediated by agonist concentration into a membrane depolarization. The possible regulation of AChR function by other mechanisms, such as phosphorylation which may regulate the rate of agonist induced desensitization of the receptor (Huganir et al., 1986), also remains an important area of research.

The *T. californica* AChR is a pentameric complex of four homologous subunits with apparent M_r 40K (α), 50K (β), 60K (γ), and 65K (δ) as determined by Laemmli gels (Figure 1) in a stoichiometry of 2:1:1:1, respectively (Raftery et al., 1980). The exposure of the receptor to both the extracellular and cytoplasmic sides of the membrane has been demonstrated by labeling (Hartig & Raftery,

FIGURE 1: Resolution of the AChR subunits on a $Na^{+}DodSO_4$-polyacrylamide gel using the buffer system of Laemmli (1970).


65K
60K
50K
40K

1977), proteolytic, and immunological studies(Karlin et al., 1978; Strader et al., 1979; Tarrab-Hazdai et al., 1978). Definitive evidence for the transmembrane nature of all four subunits of the AChR resulted from tryptic proteolysis on both the intracellular and extracellular sides of sealed vesicles (Strader & Raftery, 1980; Conti-Tronconi et al., 1982a). Although the exposure of AChR to the lipid bilayer had been investigated using photoactivated probes which partition predominantly into the lipid bilayer(Šator et al., 1979; Gonzalez-Ros et al., 1979; Tarrab-Hazdai et al., 1980), the results were contradictory. In this thesis, the exposure of all four subunits of the AChR to the hydrocarbon core of the lipid bilayer has been conclusively demonstrated using [^{3}H]adamantanediazirine (Middlemas & Raftery, 1983). This result agrees with the sequence data of Noda et al. (1982, 1983a, 1983b) which shows that all four subunits contain homologous putative membrane spanning regions. In this thesis, it has also been demonstrated that all four subunits interact with the cholesterol photoaffinity reagent, [^{3}H]cholesteryl diazoacetate. As the reactive group of this probe is situated near the the aqueous-lipid interface, this result also suggests that the subunits interact with the membrane in a symmetric fashion.

Noda et al. (1983b) proposed four membrane-spanning regions for each subunit based on the conserved sequence of hydrophobic regions in the subunits. It has been generally assumed that since the subunits are homologous, the basic folding of each subunit will be similar. Models with four transmembrane regions were also proposed by Claudio et al. (1983) and Devillers-Thiéry et al. (1983). Conversely, Guy (1983) and Finer-Moore & Stroud (1984) have proposed a model based on the sequence data with the four hydrophobic transmembrane segments and an additional amphipathic segment, which was proposed to compose part of the ion channel. As the amino terminal region of each subunit is likely to be extracellular, each model makes a different prediction for the location of the carboxyl terminal. Ratnam & Lindstrom (1986), using antibodies raised against the carboxyl terminal region, have concluded the carboxyl terminal region is intracellular. The result is consistent with the model proposing five transmembrane segments, one

of which is amphipathic. However, Ratnam et al. (1986) now have additional immunochemical data which suggest the two putative membrane-spanning segments most proximal to the carboxyl terminus are intracellular domains. One of these regions was proposed to be the amphipathic helix contributing to the ion channel. As these results confirm that the transmembrane topology of a membrane protein is difficult to predict based on hydropathy profiles derived from primary amino acid sequence data, these results reaffirm the need for experimental mapping of the lipid and aqueous exposed domains of membrane proteins to generate better understanding of how the primary structure of a membrane protein codes for the tertiary stucture.

The affinity labels that react with the AChR after reduction of a disulfide bond, BrACh (Damle et al., 1978; Moore and Raftery, 1979) and MBTA (Damle and Karlin, 1978), have demonstrated the existence of binding sites on the 40K subunits for agonists and antagonists. These binding sites undergo an agonist-induced increase in the binding affinity of the site which is correlated with physiological desensitization. Noda et al. (1982; 1983a,b) postulated the disulfide bond was composed of cysteine residues 128 and 142, as these residues are conserved in all four subunits and are located in the amino terminal region of the subunits. The expression of functional AChR in *Xenopus* oocytes by injection of mRNA synthesized in an in vitro phage SP6 RNA polymerase transcription system from the cDNAs has allowed site-directed mutagenesis studies and subunit hybrid receptor studies (AChR composed of subunits from different species) (Mishina et al., 1985; White et al., 1985). After changes of cysteine to serine for each of these four cysteine residues independently, it was postulated that cysteine residues 128 and 142 compose a disulfide bond and cysteine residues 192 and 193 compose a disulfide bond. This was based on the finding that the α-bungarotoxin binding activity was virtually eliminated and the amount of AChR expressed was substantially decreased in the mutants with either cysteine residue 128 or 142 changed to serine. On the other hand, the binding of α-bungarotoxin was nearly normal in the mutants with the change of cysteine residue 192 or 193. As they also found

that the changes of either cysteine residues 192 or 193 results in a complete loss of acetylcholine sensitivity, it was suggested that cysteine residues 192 and 193 form part of the agonist binding site. It was noted that cysteine residues 192 and 193 are both unique to the 40K subunit, whereas 128 and 142 are conserved in all four subunits. Kao et al. (1984) found that the affinity reagent, MBTA, labels cysteine residue 192 and possibly 193. It has now been demonstrated that cysteine residues 192 and 193 compose a disulfide bond near the agonist binding site which is labeled by MBTA (Kao & Karlin, 1986).

As the AChR is a pentamer of homologous subunits, the existence of multiple classes of binding sites for agonists is certainly a possibility. Evidence of other distinct binding sites, with a relatively low affinity, for agonists has been acquired (Dunn and Raftery, 1982; Conti-Tronconi et al., 1982b; Dunn et al., 1983). The existence of these sites was discovered by monitoring fluorescence changes of a covalently bound probe. Stopped-flow kinetic studies of this agonist-induced conformational change reveal its time dependence is similar to channel opening. As agonist binding to these sites is unaffected by desensitization or covalent labeling with BrACh, these sites are not those on the 40k subunit which can be labeled by the affinity techniques. It was also noted that the half-maximal response in electrophysiological experiments is also in a relatively low-affinity range (Adams, 1981). It has been postulated that the low affinity sites correspond to the physiologically relevant sites for channel opening and that desensitization and channel opening are parallel processes (Dunn & Raftery, 1982; Raftery et al., 1983). In this thesis, studies with an affinity cleavage reagent provide evidence for the existence of a low affinity binding site on the 60K subunit.

The expression of a hybrid AChR by White et al. (1985) containing the α, β, and γ subunits from *T. californica* and the δ subunit from calf in a *Xenopus* oocyte expression system resulted in functional receptors that have a 3–4-fold greater response to acetylcholine than does the *T. californica* receptor. Sakmann et al. (1985) in a similar experiment demonstrated the gating behavior of the a hybrid containing the α, β, and γ subunits from *T. californica* and the δ subunit from

calf is different, but that the channel conductance is similar, using single-channel current measurements. Mishina et al. (1986) found that the conductance and gating properties are different between fetal and adult calf AChR. This difference is dependent on the fetal ϵ subunit being replaced by the adult γ subunit. It is possible that the γ and δ subunits have functional roles in gating of the ion channel.

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Chapter 1

Exposure of the Acetylcholine Receptor to the Lipid Bilayer

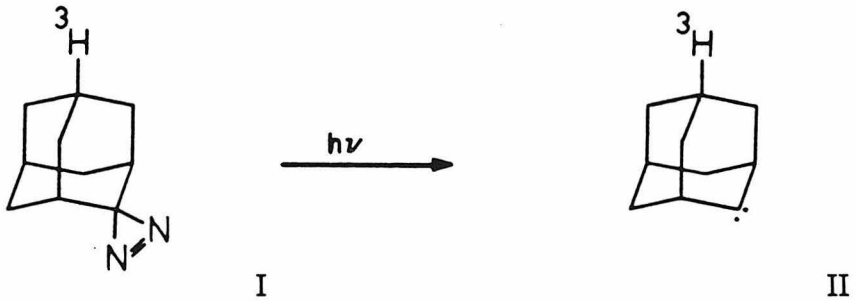
INTRODUCTION

The nicotinic AChR isolated from the electric organ of *T. californica* is a pentameric complex of four homologous subunits with apparent molecular weights of 40K, 50K, 60K, and 65K in a stoichiometry of 2:1:1:1 (Raftery et al., 1980). As the AChR is an ion channel, studies directed at the transmembrane nature of the subunits are crucial to our understanding of communication through the nicotinic synapse. A unique opportunity to investigate the transmembrane nature of an ion channel is afforded by the availability of membrane fragments greatly enriched in AChR prepared from Torpedo electric organ [for a recent review, see Conti-Tronconi & Raftery, 1982].

Several experimental approaches have shown that *T. californica* AChR is a transmembrane protein. Extracellular exposure of AChR was demonstrated using anti-AChR antibodies conjugated with ferritin visualized by electron microscopy (Karlin et al., 1978). In other electron microscopic studies, antigenic determinants were revealed on both sides of the electroplax membrane in *T. californica* (Tarrab-Hazdais et al., 1978; Strader et al., 1979). Exposure of the 40K, 50K, and 60K subunits to the aqueous phase was shown by labeling with ^{125}I using lactoperoxidase (Hartig and Raftery, 1977). Although trypsin added to the outside of *T. marmorata* vesicles resulted in no degradation, after sonication, the three subunits present in the preparation were degraded. This suggests intracellular exposure of the subunits (Wennogle and Changeux, 1980). Definitive evidence for the transmembrane nature of all four subunits of *T. californica* AChR was obtained by proteolysis with trypsin (Strader and Raftery, 1980). Trypsin added both externally and internally to sealed vesicles, 95% of which had their synaptic surface facing the external medium, resulted in proteolysis of all four subunits. Although both external and internal exposure to trypsin cause significant subunit degradation, neither alter agonist induced flux properties (Conti-Tronconi et al., 1982). This lends credence to the conclusion that proteolysis occurs at tryptic cleavage sites exposed on both sides of the membrane rather than an artifactual

exposure of cleavage sites caused by degradation and subsequent perturbation of the AChR-lipid complex.

Although it is evident that all of the subunits span the post-synaptic membrane, whether all four subunits are exposed to the lipid bilayer has not been established. Previous experiments using photoactivated probes which partition into the lipid bilayer yielded conflicting results. 5-[^{125}I]iodonapthyl–1–azide labeled exclusively the 40K subunit (Tarrab-Hazdai et al., 1980) while [^{3}H]pyrenesulfonyl azide labeled polypeptides of 48K and 55K (Šator et al., 1979; Gonzalez-Ros et al., 1979). In this chapter, we have investigated the exposure of AChR to the hydrocarbon core of the lipid bilayer using the photoactivated probe [^{3}H]adamantanediazirine (I). Photolysis of I generates [^{3}H]adamantylidene (II). [^{3}H]Adamantanediazirine,



I II

which generates a carbene upon photolysis, is probably a more effective hydrophobic probe than the alternative probes which generate nitrenes (Bayley & Knowles, 1980). This hydrophobic probe has previously been shown to efficiently label both unsaturated and saturated membrane lipids (Bayley & Knowles, 1978a,b) and has subsequently been used to selectively label regions of integral membrane proteins exposed to the lipid bilayer (Goldman et al., 1979; Bayle & Knowles, 1980; Farley et al., 1979; Kaufman & Strominger, 1979).

MATERIALS AND METHODS

Preparation of Membrane Fragments. Membrane fragments enriched in AChR were prepared from electric organs from *T. californica* as described by Elliott et al. (1980) and were further purified by alkali extraction, which removes peripheral

proteins (Neubig et al., 1979; Elliott et al., 1979). The membranes were finally suspended in *Torpedo* Ringer's solution adjusted to pH 7.4 (250 mM NaCl, 5 mM KCl, 20 mM Hepes, 0.02% NaN_3, and 2 mM $MgCl_2$). Protein concentrations were determined by the method of Lowry et al. (1951). The concentrations of α-BgTx sites were determined by the method of Schmidt and Raftery (1973) using [^{125}I]α-BgTx obtained from New England Nuclear and calibrated by the procedures of Blanchard et al. (1979). The specific activity of the preparations varied between 1.4 and 3.6 nmol of α-BgTx binding sites mg^{-1} of protein. All tritium samples were counted on a Beckman LS 233 counter following addition of 10 ml of Aquasol 2 (New England Nuclear).

Conversion of the primarily 13S dimeric form of AChR obtained in the membrane preparations to the 9S monomeric form was accomplished using a modification of the method of Conti-Tronconi et al. (1981). Reduction and alkylation were performed at 4°C in *Torpedo* Ringer's solution adjusted to pH 8.5. 0.1 Volume of dithiothreiotol freshly dissolved in the same buffer was added to membrane fragments suspended at a concentration of 2 mg protein ml^{-1}. The suspension was stirred for 1 hr, followed by the addition of 0.1 volume of 100 mM iodoacetamide freshly dissolved in Ringer's adjusted to pH 8.5. After stirring for 5 min, the suspension was diluted with Ringer's adjusted to pH 7.4 and centrifuged at 40,000 g for 45 min. After centrifugation, the membranes were resuspended in Ringer's solution (pH 7.4). The extent of the reaction was determined by sucrose gradient centrifugation of solubilized receptors trace labeled with [^{125}I]α-BgTx, which separates monomers from dimers. Typically, 99% of α-BgTx binding activity sediments in the 9S monomeric form after reduction and alkylation.

Synthesis of Adamantanediazirine and [^{3}H]Adamantanediazirine. [^{3}H]Adamantanediazirine (596 mCi $mmol^{-1}$) was synthesized and purified by the method of Bayley and Knowles (1980). [^{3}H]Adamantanone ethylene ketal needed for the synthesis was prepared by Amersham Corporation from bromoadamantanone ethylene ketal, which we provided. TLC was performed on silica gel 60 on plastic support developed with petroleum ether (b.p. 35–60°C): diethyl ether (3:1), which

were then sliced and counted. The UV-visible spectra were recorded on a Cary 118 spectrophotometer. The time course of photolysis for adamantanediazirine in AChR enriched membrane vesicles was monitored by the absorbance at 372 nm.

[^{3}H]Adamantanediazirine Binding to Membrane Fragments. [^{3}H]Adamantanediazirine (9mCi mmol^{-1}) in ethanol was added to membrane fragments in *Torpedo* Ringer's solution at room temperature under dim light. In each of 4 samples, the final concentrations of [^{3}H]adamantanediazirine, membrane protein, and ethanol were 25 μM, 1 mg ml^{-1}, and 1%, respectively. After 1 hr incubation in the dark, the radioactivity in a portion of each sample was determined. The samples were then centrifuged at 147,000 g for 45 min. The supernatant and the resuspended pellets were then counted to determine the radioactivity of each. No correction was made for trapped volume of free solution in the pellet. The radioactivity in the pellet was interpreted as the amount of ligand bound to membrane fragments. Counting efficiency was accounted for using [^{3}H]H_2O as an internal standard. This centrifugation method was also used to determine the fraction of [^{3}H]adamantanediazirine bound to the membrane vesicles at various concentrations of AChR enriched vesicles.

Labeling of Membrane Fragments. All photolabeling experiments were carried out in *Torpedo* Ringer's solution with constant stirring at room temperature. [^{3}H]Adamantanediazirine (596 mCi mmol^{-1}) in ethanol was added to membrane fragments in a quartz cuvette under dim light. The final concentrations of [^{3}H]adamantanediazirine, membrane protein, and ethanol were 33 μM, 1 mg ml^{-1}, and 1%, respectively. The sample was incubated in the dark for 30 min and then irradiated for 30 min using a UVSL-25 lamp (Ultraviolet Products) set on the long wavelength setting. After photolysis, the membrane fragments were diluted 28 fold with 1% BSA in *Torpedo* Ringer's solution. Following a 15-min incubation, a portion was removed to determine the radioactivity and the sample was then centrifuged for 15 min at 252,000 g. The BSA wash of the pellet was repeated, followed by 2 washes in buffer without BSA. Portions of both the supernatant and pellet were taken for determination of the radioactivity. The radioactivity remain-

ing in the pellet was considered to represent the covalent attachment of radiolabel to membrane fragments.

Variations in labeling conditions. Both monomeric and dimeric forms of the AChR in membrane fragments were photolabeled as described above with and without reduced gluatathione in the buffer. All other experiments were carried out on membrane preparations that were not reduced and alkylated.

Membrane fragments desensitized with 10 μM carbamylcholine chloride and 4 mM $CaCl_2$ were photolabeled as already described. That the membranes were desensitized was shown by the associated inhibition of the time course of α-BgTx-receptor complex formation using the procedure of Lee et al. (1977). A control lacking carbamylcholine was simultaneously labeled. Membrane fragments saturated by addition of a 2 fold excess of α-BgTx were photolabeled as previously described. Determination of the concentration of α-BgTx sites verified that the sites were saturated. A control lacking α-BgTx was photolabeled simultaneously.

Prelabeling was carried out by the addition of 0.007 volumes adamantanediazirine in ethanol under dim light to membrane fragments (1mg ml^{-1}) resulting in a concentration of 100 μM. The sample was incubated in the dark with stirring for 60 min, after which it was irradiated for 30 min with stirrring. Then 0.003 volumes of [^{3}H]adamantanediazirine in ethanol were added under dim light resulting in a concentration of 18 μM. After 30 min of incubation in the dark with stirring, the sample was irradiated for 30 min with stirring. The BSA extraction was carried out as already described. A control with ethanol added in the first step, followed by addition of [^{3}H]adamantanediazirine, was simultaneously labeled.

Gel Electrophoresis. $NaDodSO_4$-polyacrylamide gel electrophoresis was performed using the system of Laemmli (1970) with 8.75% (wt/vol) acrylamide cylindrical gels (0.5 x 10 cm). After electrophoresis, gels were stained with 0.5% Coomassie Blue R-250 in 10% acetic acid/25% propanol/65% water and destained in the same solvent without the dye. Gels were then sliced with a Hoeffer Scientific Instruments gel slicer. Each slice was sealed in a glass scintillation vial with 0.5 ml H_2O_2 and incubated at 60°C for 5 hr followed by determination of radioactivity.

RESULTS

[^{3}H]Adamantanediazirine was synthesized according to Bayley & Knowles (1980). All of the radioactivity in the purified [^{3}H]adamantanediazirine preparation comigrated with the cold diazirine on TLC (Figure 1). The UV-visible spectrum of the tritiated diazirine was identical to adamantanediazirine (Figure 2). The time course of photolysis for adamantanediazirine in AChR enriched membrane vesicles is shown in Figure 3.

The distribution of [^{3}H]adamantanediazirine between the aqueous phase and AChR enriched membranes was determined by incubation of the hydrophobic probe with membrane fragments, which were then pelleted by centrifugation. The partition coefficient for the diazirine between the aqueous buffer and the membrane fragments defined as (mol ligand bound/mg protein)/(mol free ligand/μl external solution) is 1320. Assuming a density for the hydrocarbon core of 0.75 g/ml and using published values for the protein-lipid ratio in AChR enriched membrane fragments (Schiebler & Hucho, 1978; Popot et al. 1978), the ratio of diazirine in the hydrocarbon core to that in the surrounding aqueous phase is $\sim$ 2000. If the binding of adamantanediazirine is a simple partitioning, then the partitioning of the probe between the aqueous phase and the membranes can be described by

$$\frac{b}{(b+f)} = \frac{Pc}{(1000+Pc)}$$

where b is the bound reagent per unit volume of suspension, f is the free reagent per unit volume of suspension, c is the concentration of protein, and P is the partition coefficient. The fraction of [^{3}H]adamantanediazirine bound at various membrane vesicle concentrations is consistent with a simple partitioning (Figure 4). These results indicate [^{3}H]adamantanediazirine partitions into the lipid bilayer where it can interact with regions of membrane proteins in contact with the lipids (Figure 5).

Irradiation of AChR enriched membrane fragments equilibrated with [^{3}H]adamantanediazirine resulted in labeling of all four subunits (Figure 6). In addition,

FIGURE 1: TLC of [^{3}H]adamantanediazirine on silica gel 60 developed with petroleum ether: diethyl ether (3:1).

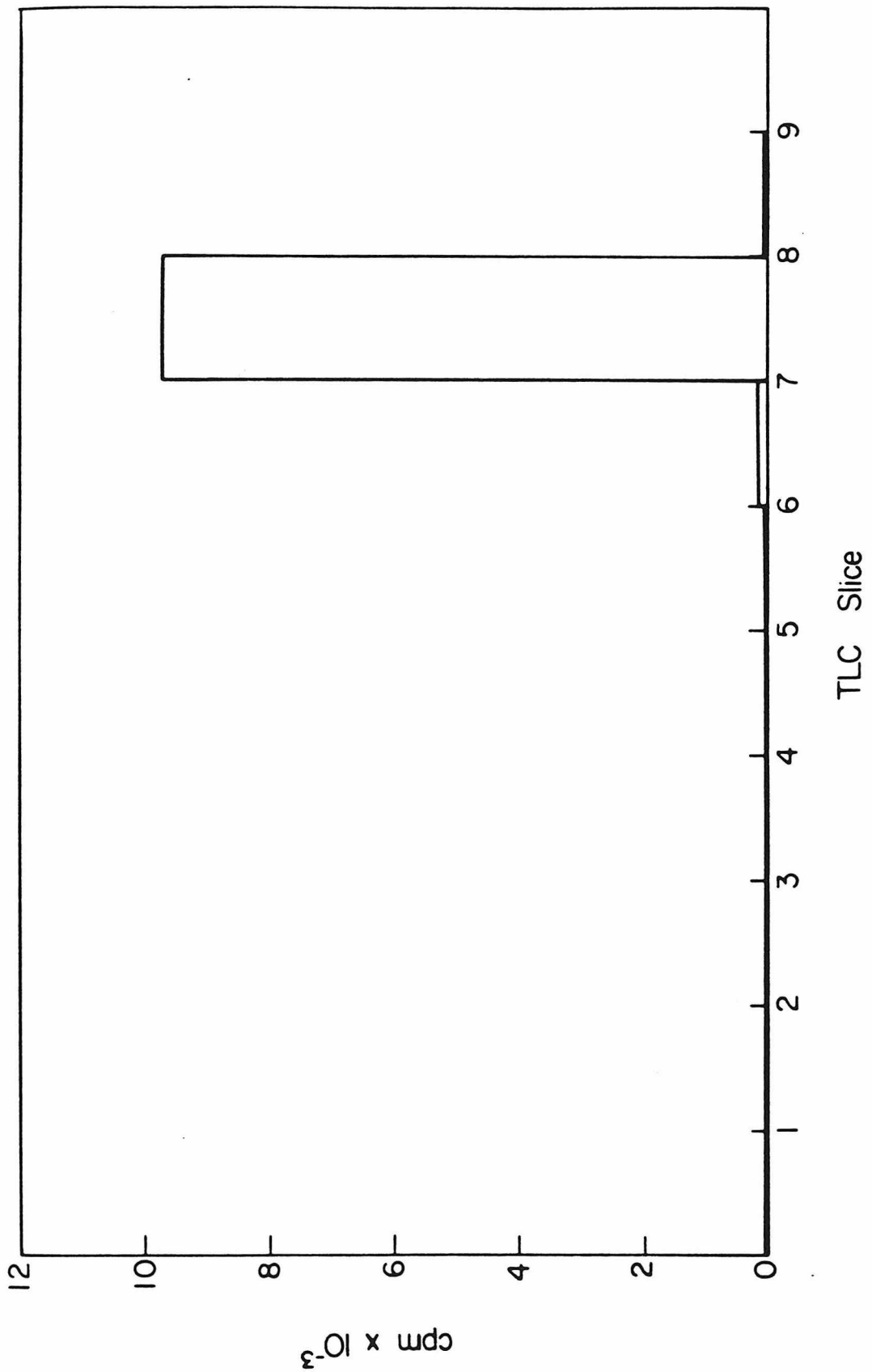

cpm x 10^{-3}
0
2
4
6
8
10
12
1
2
3
4
5
6
7
8
9
TLC Slice

FIGURE 2: The UV-visible spectrum of adamantanediazirine.

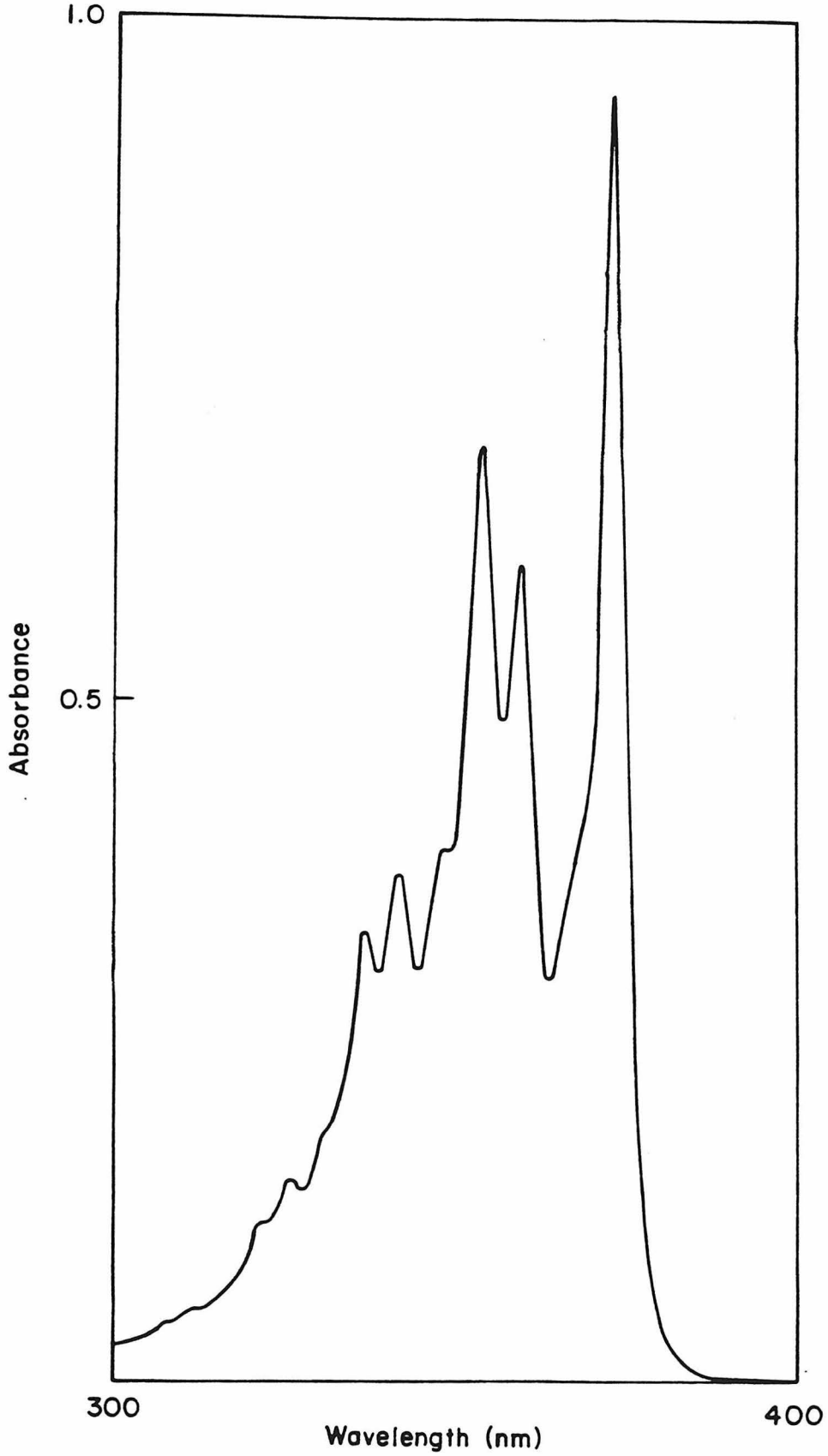

1.0
0.5
Absorbance
300
400
Wavelength (nm)

FIGURE 3: The time course of photolysis of adamantanediazirine in AChR enriched membrane vesicles.

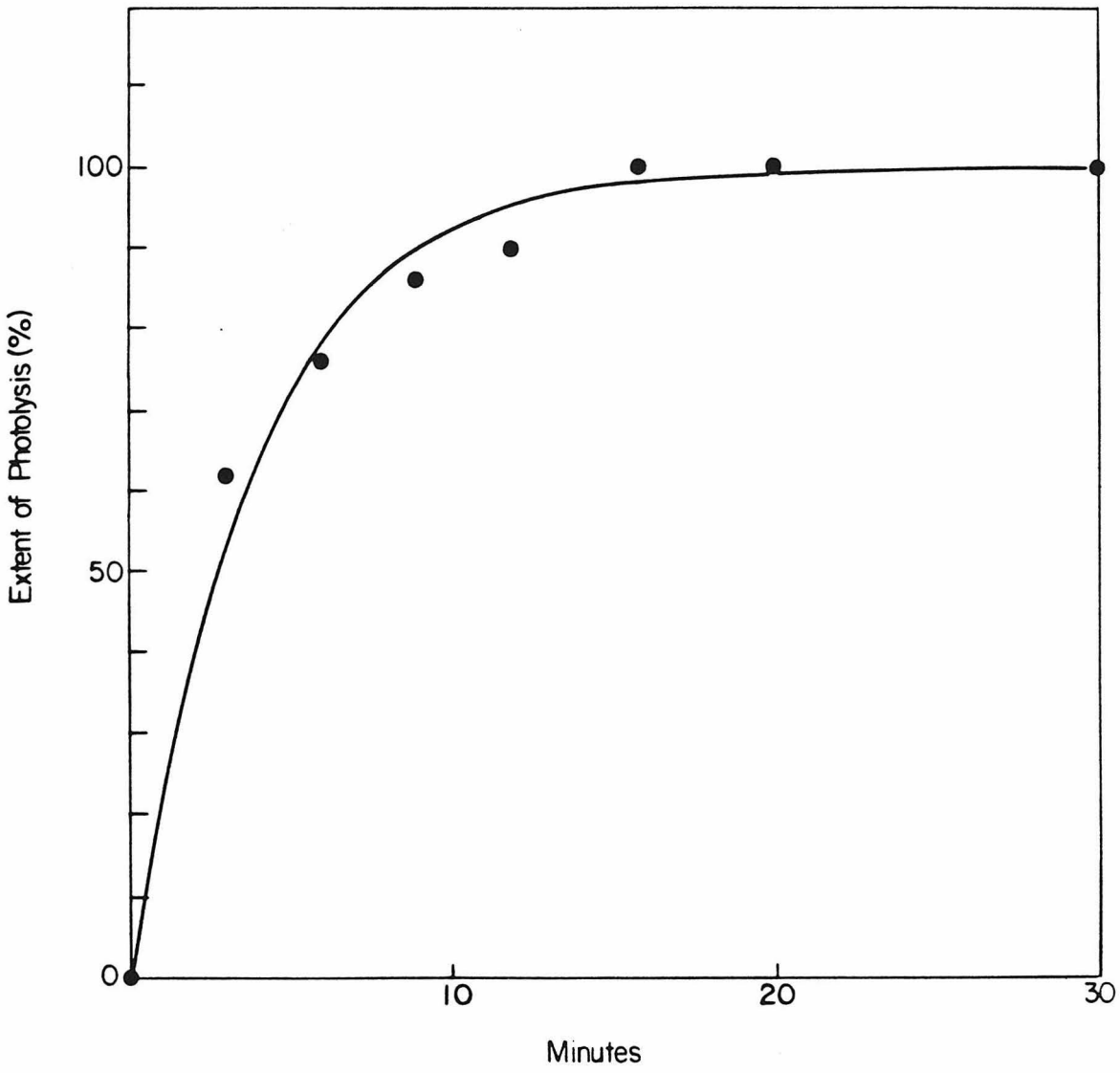

Extent of Photolysis (%)
100
50
0
10
20
30
Minutes

FIGURE 4: The fraction of [^{3}H]adamantanediazirine bound at various membrane vesicle concentrations is plotted. The data were fit to the equation described in the text for a simple partitioning of a hydrophobic reagent between an aqueous phase and membranes to generate the curve shown.

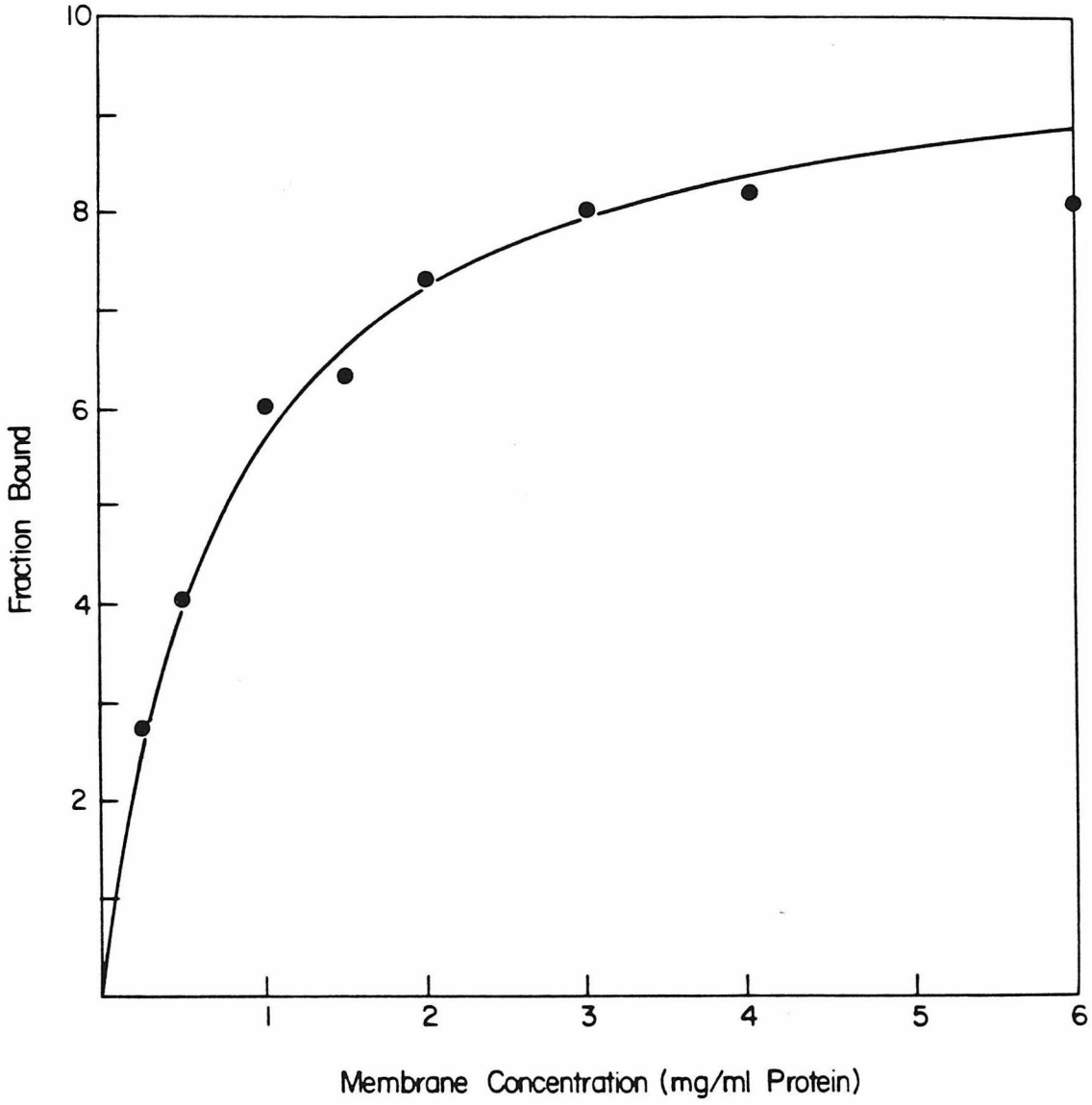

10
8
6
4
2
Fraction Bound
1
2
3
4
5
6
Membrane Concentration (mg/ml Protein)

FIGURE 5: A model depicting the partitioning of [^{3}H]adamantanediazirine into the hydrocarbon core of the lipid bilayer.

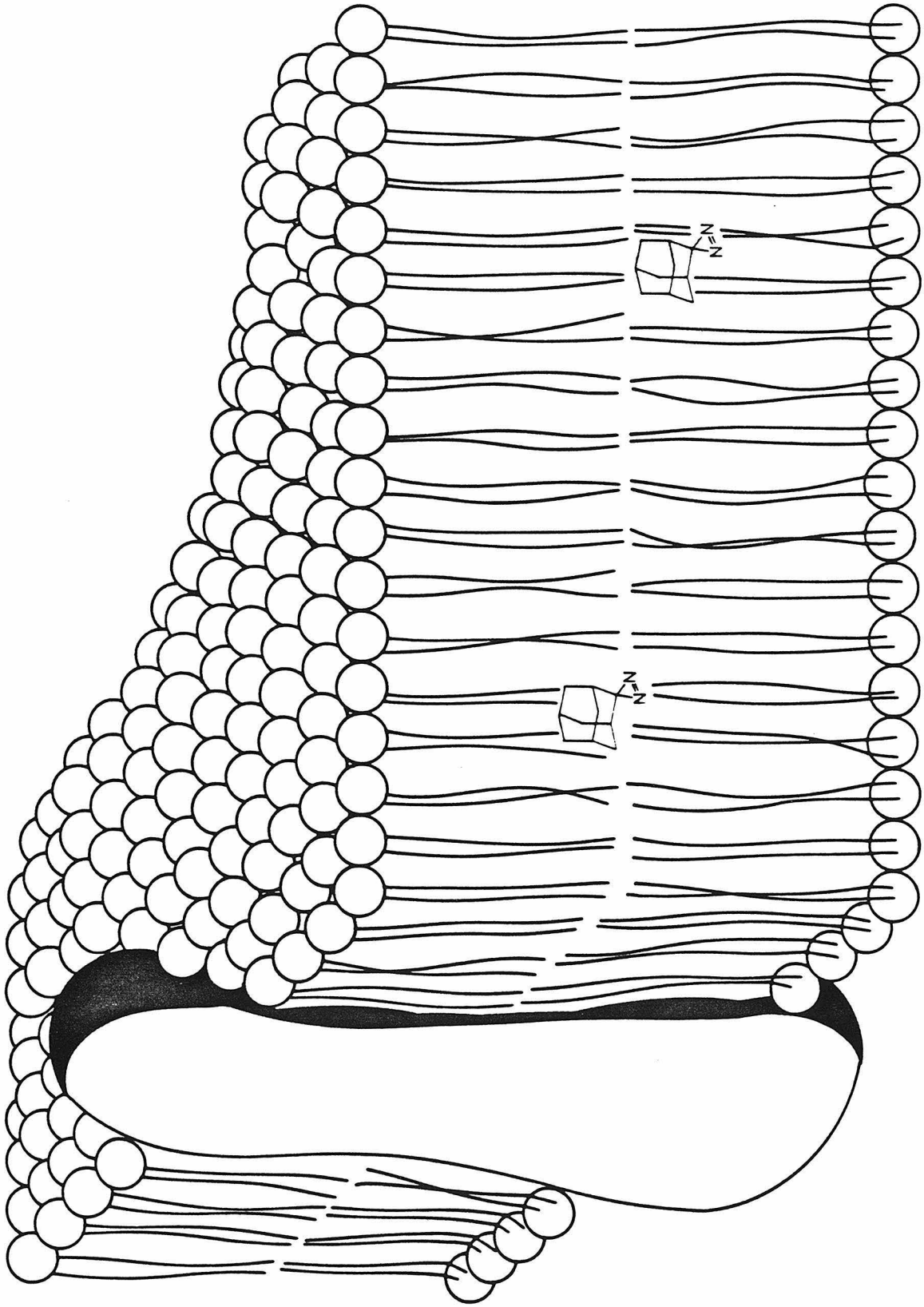
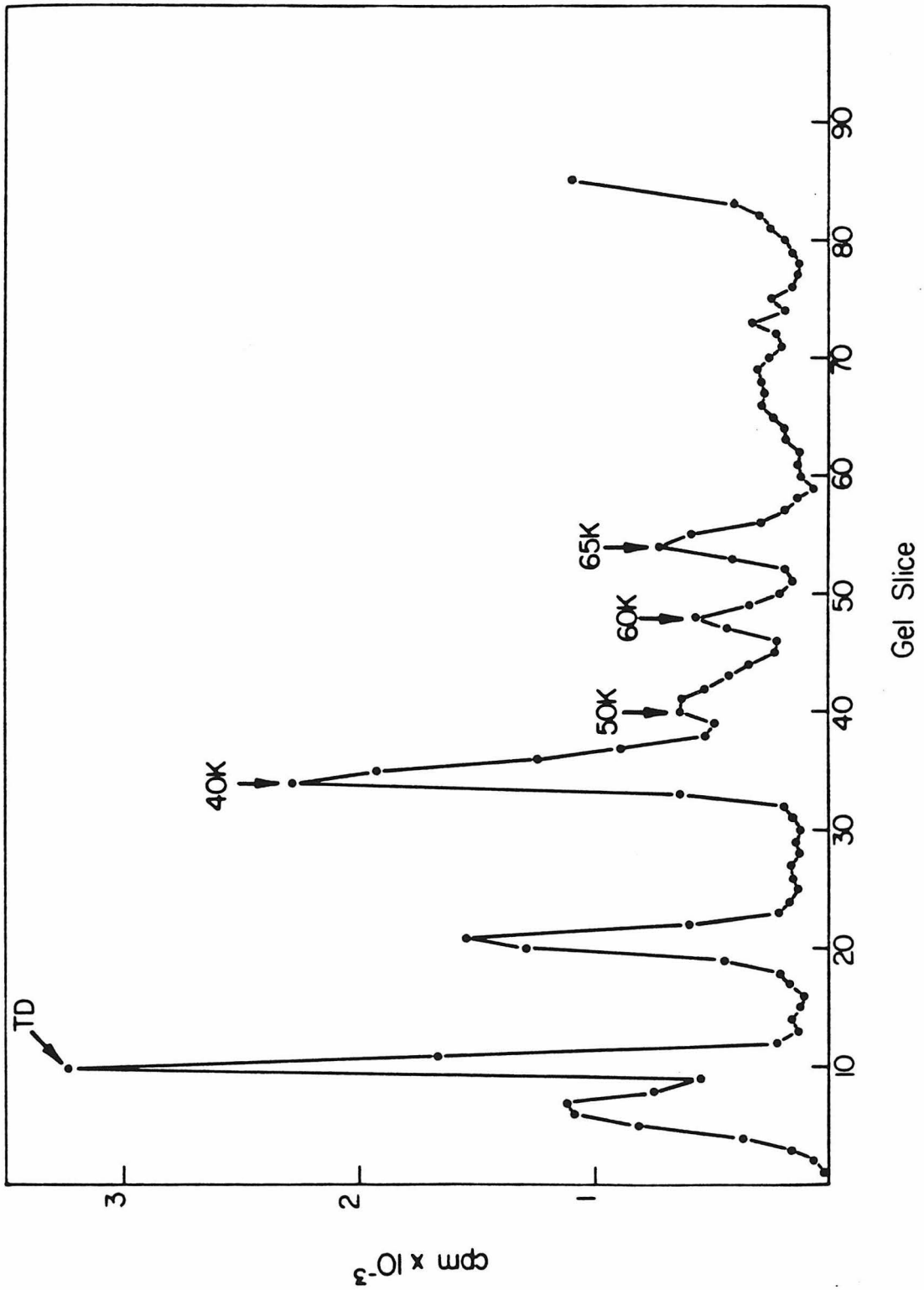

Z
Z
Z
Z

FIGURE 6: Polyacrylamide gel electrophoresis scan of AChR enriched membrane vesicles labeled with [^{3}H]adamantanediazirine (TD = tracking dye).


TD
40K
50K
60K
65K
cpm x 10⁻³
Gel Slice

there was a peak of radioactivity corresponding to a polypeptide of M_r 30K, which corresponds to unidentified proteins that are always contained in the AChR enriched vesicles preparation. There was also incorporation of radiolabel into a 43K and 90K dalton protein in experiments on membrane preparations which were not alkali extracted to remove these proteins (Figure 7). The 90K dalton protein may be a subunit of the Na^+-K^+ ATPase, which is an integral membrane protein. However, the proteins with M_r of 43K are thought to be peripheral membrane proteins. As [^{3}H]adamantanediazirine labels one or more of the 43K dalton proteins, it is likely that they are associated with the lipid bilayer. Alternatively, membrane associated proteins bound by integral membrane proteins, but that do not interact with the lipid bilayer, would presumably not be labeled by [^{3}H]adamantanediazirine. The large peak of radioactivity which comigrates with the dye front corresponds to radiolabeled lipids. In all of the experiments, between 30 and 40% of the radiolabel was incorporated covalently into membrane fragments upon photolysis (Figure 8). Of that, about 50% was incorporated into protein components with the remainder covalently attached to membrane lipids.

The ratio of label incorporation into the subunits is about 2:1:1:1, although the 40K is labeled more than expected by the stoichiometry of the subunits. The greater reactivity of the 40K subunit could be due to either its amino acid composition or a greater surface exposure. Dynamic interactions with other receptors or membrane proteins could also affect the time-averaged surface exposure of a particular subunit to the lipid bilayer.

The effect of agonist, which causes a conformational change which has been correlated with physiological desensitization (Weber et al., 1975; Weiland et al., 1976; Lee et al., 1977), on the photoincorporation of radiolabel into the subunits was investigated. Desensitization of the AChR by inclusion of 10 μM carbamylcholine in the buffer did not alter the incorporation of radiolabel into the subunits. Likewise, saturation of AChR with α-BgTx did not affect the radiolabel distribution among the subunits. Photolabeling of both the 9S monomer and the 13S dimer in membrane fragments also resulted in indistinguishable distributions of

FIGURE 7: Polyacrylamide gel electrophoresis scan of AChR enriched membrane vesicles which were not alkali extracted (extraction removes the 43K and 90K dalton proteins) labeled with [^{3}H]adamantanediazirine (TD = tracking dye).

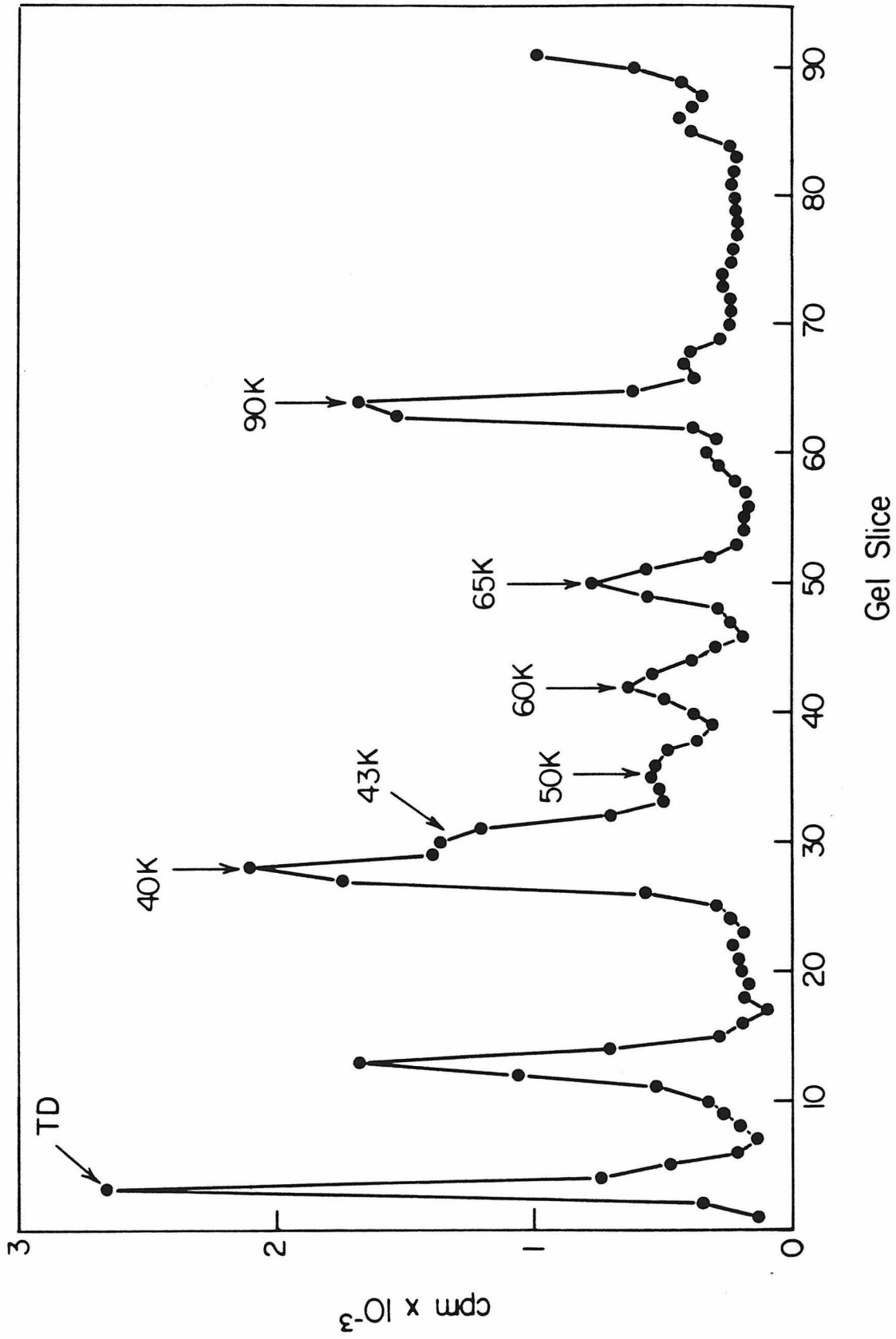
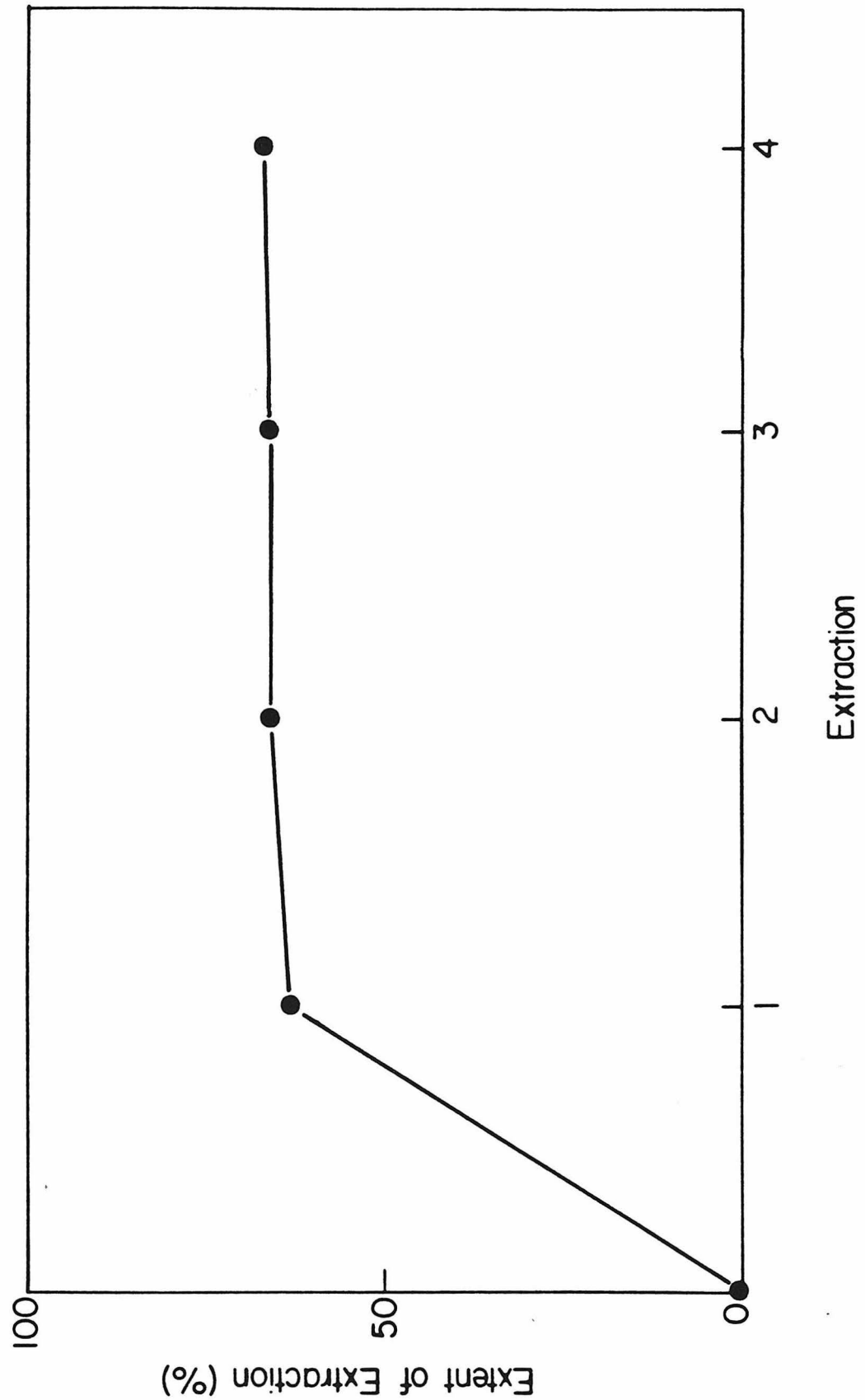

TD
40K
43K
50K
60K
65K
90K
cpm x 10-3
Gel Slice

FIGURE 8: Extraction of non-covalently bound [^{3}H]adamantanediazirine from AChR enriched vesicles after photolysis. Extractions 1 and 2 were with 1% BSA in *Torpedo* Ringer's solution and 3 and 4 were with *Torpedo* Ringer's solution.


Extent of Extraction (%)
100
50
0
1
2
3
4
Extraction

radiolabel among the subunits.

The inclusion of 20 mM reduced glutathione in the buffer did not alter the ratio of photoincorporation of radiolabel among the subunits, although the total incorporation of radiolabel into the subunits decreased by about 10% (Figure 9). As glutathione is an aqueous soluble scavenger of carbenes, this experiment confirms that the labeling occurs in regions of AChR exposed to the lipid bilayer.

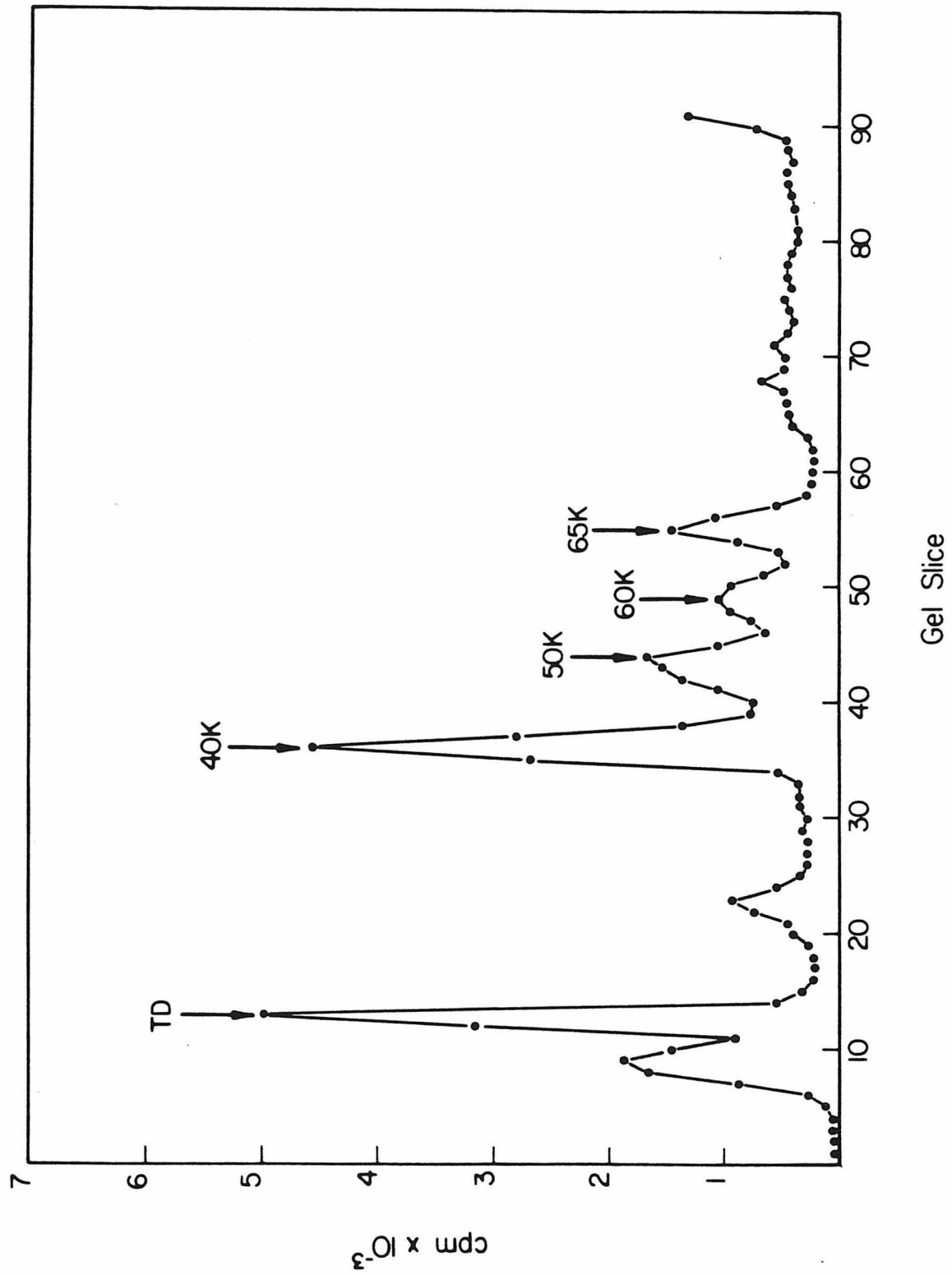
The possibility that photolabeling by [^{3}H]adamantanediazirine was occurring at high-affinity saturable binding sites was investigated. The membrane fragments were labeled with 100 μM adamantanediazirine and then labeled with 18 μM [^{3}H]adamantanediazirine. This prelabeling did not alter the the total covalent incorporation of radiolabel into the membrane fragments or the incorporation of radiolabel into the AChR subunits, which indicates that the diazirine interacts non-specifically with AChR after partitioning into the lipid bilayer.

DISCUSSION

The exposure of AChR subunits to the lipid bilayer has previously been investigated using hydrophobic photolabile probes, yielding conflicting results. Irradiation of 5-[^{125}I]iodonapthyl-1-azide equilibrated with AChR enriched membrane fragments isolated from *T. californica* exclusively labeled the 40K subunit (Tarrab-Hazdai et al., 1980). A similar reagent, [^{3}H]pyrenesulfonyl azide labeled polypeptides of 48K and 55K daltons in membranes (Šator et al., 1979; Gonzalez-Ros, 1979). This variability in labeling may have arisen from preferential reactivity of different nitrenes with particular subunits.

[^{3}H]Adamantanediazirine, which generates a carbene upon irradition, was proposed as an alternative to aryl nitrene probes by Bayley and Knowles (1978a,b). After finding that [^{3}H]adamantanediazirine labeled both unsaturated and saturated membrane lipids more efficiently than aryl nitrenes (Bayley & Knowles,1978a, 1978b), the probe was subsequently used to selectively label regions of integral membrane proteins in contact with the hydrocarbon core of the lipid bilayer (Gold-

FIGURE 9: Polyacrylamide gel electrophoresis scan of AChR enriched membrane vesicles labeled with [^{3}H]adamantanediazirine in the presence of 20mM reduced glutathione (TD = tracking dye).


cpm x 10^{-3}
7
6
5
4
3
2
1
TD
40K
50K
60K
65K
10
20
30
40
50
60
70
80
90
Gel Slice

man et al., 1979; Bayle & Knowles, 1980; Farley et al., 1979; Kaufman & Strominger, 1979). Our results also indicate that [^{3}H]adamantylidene may be a more effective hydrophobic probe than aryl nitrene probes.

AChR is a pentamer of homologous subunits (Raftery et al. 1980), all of which span the membrane (Strader & Raftery, 1980; Conti-Tronconi et al, 1982). Recently elucidated sequences of cDNAs for these subunits indicate that there are four hydrophobic regions conserved in each subunit (Noda et al., 1982, 1983a, 1983b; Sumikawa et al., 1982; Claudio et al., 1983; Devillers-Thiéry et al., 1983). We have demonstrated experimentally that all four subunits are exposed to the hydrocarbon core of the lipid bilayer.

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Noda, M., Takahashi, H., Tanabe,T., Toyosato, M., Kikyotani, S., Furutani, Y., Hirose, T., Takashima, H., Inayama, S., Miyata, T., & Numa, S. (1983) *Nature* **302**, 528–532.

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Chapter 2

Identification of the Subunits of Acetylcholine Receptor that Interact with a Cholesterol Photoaffinity Probe

INTRODUCTION

The nicotinic AChR is an integral membrane protein which transduces a signal mediated by ACh into a membrane depolarization. This depolarization is the result of a transient current caused by the opening of an ion channel [for recent review, see Adams (1981)]. A unique opportunity to correlate the molecular structure of a gated ion channel with its functional properties is afforded by the availability of membrane vesicles greatly enriched in AChR from *Torpedo* electric organ [for recent reviews, see Conti-Tronconi & Raftery (1982) and Raftery et al. (1983)].

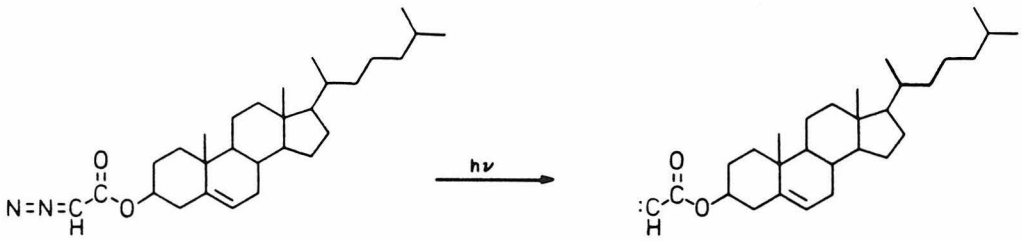
The AChR isolated from *T. californica* is a pentameric complex of homologous subunits with apparent M_r of 40K, 50K, 60K, and 65K in a stoichiometry of 2:1:1:1 (Raftery et al., 1980). Several experimental approaches have shown that *T. californica* AChR is a transmembrane protein. Extracellular exposure of AChR was demonstrated using anti-AChR antibodies conjugated with ferritin visualized by electron microscopy (Karlin et al., 1978). In other electron microscopic studies, antigenic determinants were revealed on both sides of the electroplax membrane (Strader et al., 1979, Tarrab-Hazdai et al., 1978). Exposure of the 40K, 50K, and 60K subunits to the aqueous phase was shown by labeling with ^{125}I using lactoperoxidase (Hartig & Raftery, 1977). Although trypsin added to the outside of *T. marmorata* vesicles resulted in no degradation, after sonication, the three subunits present in the preparation were degraded, suggesting their intracellular exposure (Wennogle & Changeux, 1980). Definitive evidence for the transmembrane nature of all four subunits of *T. californica* AChR was obtained by proteolysis with trypsin (Strader & Raftery, 1980). Trypsin added both externally and internally to sealed vesicles, 95% of which had their synaptic surface facing the external medium, resulted in proteolysis of all four subunits. Although both external and internal exposure to trypsin cause significant subunit degradation, neither alter agonist induced flux properties (Conti-Tronconi et al., 1982). This lends credence to the conclusion that proteolysis occurs at tryptic cleavage sites exposed on both

sides of the membrane rather than an artifactual exposure of cleavage sites caused by degradation and subsequent perturbation of the AChR-lipid complex.

The exposure of AChR to the lipid bilayer has been studied using photoactivated probes which partition predominantly into the lipid bilayer. 5-[^{125}I]iodonapthyl-1-azide labeled exclusively the 40K subunit (Tarrab-Hazdai et al., 1980) while [^{3}H]pyrenesulfonyl azide labeled polypeptides of 48K and 55K (Šator et al., 1979; Gonzalez-Ros et al., 1979). This difference in labeling pattern may be due to different reactivity of nitrenes. Using [^{3}H]adamantanediazirine, all four subunits of the AChR were labeled, demonstrating exposure of all four subunits to the hydrocarbon core of the lipid bilayer (Middlemas & Raftery, 1983). [^{3}H]Adamantanediazirine, which generates a carbene upon photolysis, is probably a more effective hydrophobic probe than the alternative probes which generate nitrenes (Bayley & Knowles, 1980). This result agrees with the sequence data of Noda et al. (1982, 1983a, 1983b) which show that all four subunits contain putative membrane spaning regions.

It is probable that AChR is a pseudosymmetric complex of homologous subunits, all of which interact with the lipid bilayer in a similar manner. The role of lipids in the function and structure of the AChR remains an important area of research. There is evidence that cholesterol is important to AChR function. In reconstitution experiments, inclusion of cholesterol was needed for protection of agonist-induced change in affinity of binding sites (desensitization)(Criado et al., 1982) and protection of agonist-induced $^{86}Rb^{+}$ flux (Dalziel et al., 1980). Using a spin labeled steroid probe, an immobilized component was revealed by ESR spectra which suggests AChR interacts with neutral lipids (Marsh & Barrantes, 1978).

Although cholesterol may be important to AChR function, it is not clear whether this is due to its effect on membrane properties or a specific interaction with AChR. In this communication, we have used the cholesterol photoaffinity probe, cholesteryl diazoacetate, to resolve whether or not cholesterol interacts with AChR. Cholesteryl diazoacetate (I) produces the reactive carbene (II) upon



I II

photolysis. Cholesteryl diazoacetate incorporates into lipid bilayers analogously to cholesterol and is therefore a direct tool to study the interaction of cholesterol with both lipids and membrane proteins. It is immobilized in bilayers like cholesterol and upon irradiation incorporates into the choline head group of phosphatidylcholine (Keilbaugh & Thornton 1983a,b). This result supports the notion that the carbene is generated near the lipid-water interface.

MATERIALS AND METHODS

^{1}H NMR spectra were recorded on a Varian Associates EM 390 (90 MHz) spectrometer using tetramethylsilane as the internal reference. Chemical shifts are reported in ppm downfield from the reference. UV-visible spectra were recorded on a Cary model 118 spectrophotometer. Fourier transform infrared (FT-IR) spectra were recorded on a Mattson Instruments Sirius 100 FT-IR equipped with Starlab minicomputer data station and high-resolution graphics terminals under a positive nitrogen purge.

Preparation of Membrane Fragments. Membrane fragments enriched in AChR were prepared from electric organs from *T. californica* as described by Elliot et al. (1980) and were further purified by alkali extraction (Neubig et al., 1979; Elliott et al., 1979). The membranes were finally suspended in Ca^{2+}-free *Torpedo* Ringer's solution adjusted to pH 7.4 (250 mM NaCl, 5 mM KCl, 20 mM HEPES, 0.02% NaN_3, and 2 mM $MgCl_2$). Protein concentration was determined by the method

of Lowry et al. (1951). The concentration of α-BgTx sites was determined by the method of Schmidt & Raftery (1973) using [^{125}I]α-BgTx obtained from New England Nuclear and calibrated by the procedures of Blanchard et al. (1979). The specific activities of the preparations varied between 2.0 and 3.7 nmol of α-BgTx binding sites mg^{-1} of protein. All tritium samples were counted on a Beckman LS 233 counter following addition of 10 ml Aquasol 2 (New England Nuclear).

Synthesis and Purification of [^{3}H]Cholesteryl Diazoacetate. Cholesteryl diazoacetate was synthesized and purified by the method of Keilbough & Thornton (1983a). A similar method was used to synthesize [^{3}H]cholesteryl diazoacetate from [7-^{3}H]cholesterol (12 Ci mmol^{-1}) purchased from ICN Pharmaceuticals, Inc. 2.8 μmol of cholesterol was added to 5.3 mCi of [7-^{3}H]cholesterol in benzene (1 mCi ml^{-1}). The solvent was removed under reduced pressure at 22 °C. The cholesterol was dissolved in 14 μl of CH_2Cl_2:THF (1:1) containing 5 μmol of redistilled TEA on ice. 5.2 μmol of glyoxylic acid chloride p-toluene sulfonyl hydrazone, prepared by the method of Blankley et al. (1969), was added in 17 μl of CH_2Cl_2 cooled to 0°C. After 30 min in the dark on ice, 5 μmol of TEA in 5 μl of CH_2Cl_2 was added. The solution was brought to 22°C. After 30 min, the solvent was removed under reduced pressure at 22 °C. The residue was extracted twice with 100 μl of toluene. The extracts were applied to a 0.5 x 4 cm silica gel 60 column (Merck) and eluted with toluene. Toluene was removed from the purified diazoacetate under reduced pressure. The diazoacetate was taken up in ethanol for determination of the specific activity and introduction into membranes.

The specific activity of [^{3}H]cholesteryl diazoacetate was determined by using UV absorbance at 247 nm (ϵ_{247}=17,000) and scintillation counting using [^{3}H]H_2O as an internal standard. Plastic-backed silica gel 60 TLC plates (Merck), which were eluted with toluene and then sliced and counted, were used to verify the purity of the tritiated diazoacetate. The unlabeled diazoacetate was visualized on TLC plates with I_2.

Photolysis of Cholesteryl Diazoacetate. Photolysis was carried out in a quartz cuvette with a UVSL-25 lamp (Ultraviolet Products) set on the short wavelength

setting. The rate of photolysis of cholesteryl diazoacetate in ethanol and cyclohexane was monitored by the absorbance at 247 nm. The rate of photolysis of the tritiated diazoacetate incorporated into vesicles enriched in AChR was monitored by taking time aliquots for TLC. When eluted with toluene, the diazoacetate migrated near the solvent front while the products of photolysis remained near the origin. The TLC plates were then sliced and counted.

Labeling of Membrane Vesicles. All photolabeling experiments were carried out in Ca^{2+}-free *Torpedo* Ringer's solution with constant stirring at room temperature. [^{3}H]Cholesteryl diazoacetate in ethanol was added to membrane vesicles in a quartz cuvette under dim light. The final concentrations of [^{3}H]cholesteryl diazoacetate, membrane protein, and ethanol were 12 μM, 1 mg ml^{-1}, and 1%, respectively. The sample was incubated in the dark with stirring for 30 min and then the cuvette was flushed with argon. The sample was irradiated for 10 min using a UVSL-25 lamp set on the short wavelength setting with constant stirring. The membranes were then pelleted by centrifuging for 15 min in an Eppendorf centrifuge, after which, the samples were taken up in buffer for gel electrophoresis.

Membrane vesicles desensitized with 10 μM carbamylcholine chloride and 4 mM $CaCl_2$ were photolabeled as described. Desensitization was shown by the associated inhibition of the time course of α-BgTx-receptor complex formation using the procedure of Lee et al. (1977).

Extraction of lipids from the protein was accomplished by adding 20 volumes of chloroform:methanol (2:1) to 1 volume of photolyzed membranes in a glass centrifugation tube. After vortexing, the samples were centrifuged at 12,000 *g* for 30 min. The supernatant was removed and the pellet was dissolved in buffer for gel electrophoresis. Aliquots of both the supernatant and pellet were taken for scintillation counting. Quenching was accounted for using [^{3}H]H_2O as an internal standard.

Gel Electrophoresis. NaDodSO_4-polyacrylamide gel electrophoresis was performed using the system of Laemmli (1970) with 10% acrylamide cylindrical gels (0.5x10 cm). After electrophoresis, gels were immediately sliced using a Hoeffer

Scientific Instruments gel slicer. Each slice was sealed in a glass scintillation vial with 0.5 ml of 30% H_2O_2 and incubated at 70°C for 5 hr followed by determination of radioactivity. Electrophoresis of unlabeled AChR preparations was performed simultaneously to determine the mobility of the subunits. These gels were stained with 0.5% Coomassie Blue R-250 in 10% acetic acid/25% propanol/65% H_2O and destained in the same solvent without the dye.

RESULTS

Cholesteryl diazoacetate was synthesized and purified by the method of Keilbough & Thornton (1983a). IR and NMR spectra and melting point agreed with the published values. The maximum absorbance in the UV spectrum is at 247 nm (Figure 1). The IR spectrum exhibits the characteristic absorption band at 2100 cm^{-1} of a diazo group (Figure 2). Using a similar approach, [^{3}H]cholesteryl diazoacetate with a specific activity of 1.64 Ci/mmol was synthesized and purified (11% yield). The mobility on TLC, the UV spectrum, and the rate of photolysis of the tritiated compound were identical to that of cholesteryl diazoacetate. In both labeled and unlabeled cholesteryl diazoacetate, greater than 99% of the UV absorbance at 247 nm disappears upon photolysis (Figure 3).

In order to ensure complete photolysis, the rate of photolysis was determined under various conditions (Figure 4). The half lives of photolysis in ethanol and cyclohexane are 47 and 76 seconds respectively and in membrane vesicles containing 1 mg ml^{-1} protein, the half life of photolysis was 49 seconds. It is interesting to note that the rate of photolysis is similar in both organic solvent and in unilamellar vesicles, although much longer irradiation times are required for photolysis in multilamellar vesicles (Keilbough & Thornton, 1983b). It is evident that exposure to UV light under these conditions will result in virtually complete photolysis of the diazo group in 10 min.

After incorporation of [^{3}H]cholesteryl diazoacetate into AChR enriched membrane vesicles, irradiation resulted in covalent labeling of all four subunits (Figure

FIGURE 1: The UV spectrum of cholesteryl diazoacetate in ethanol.

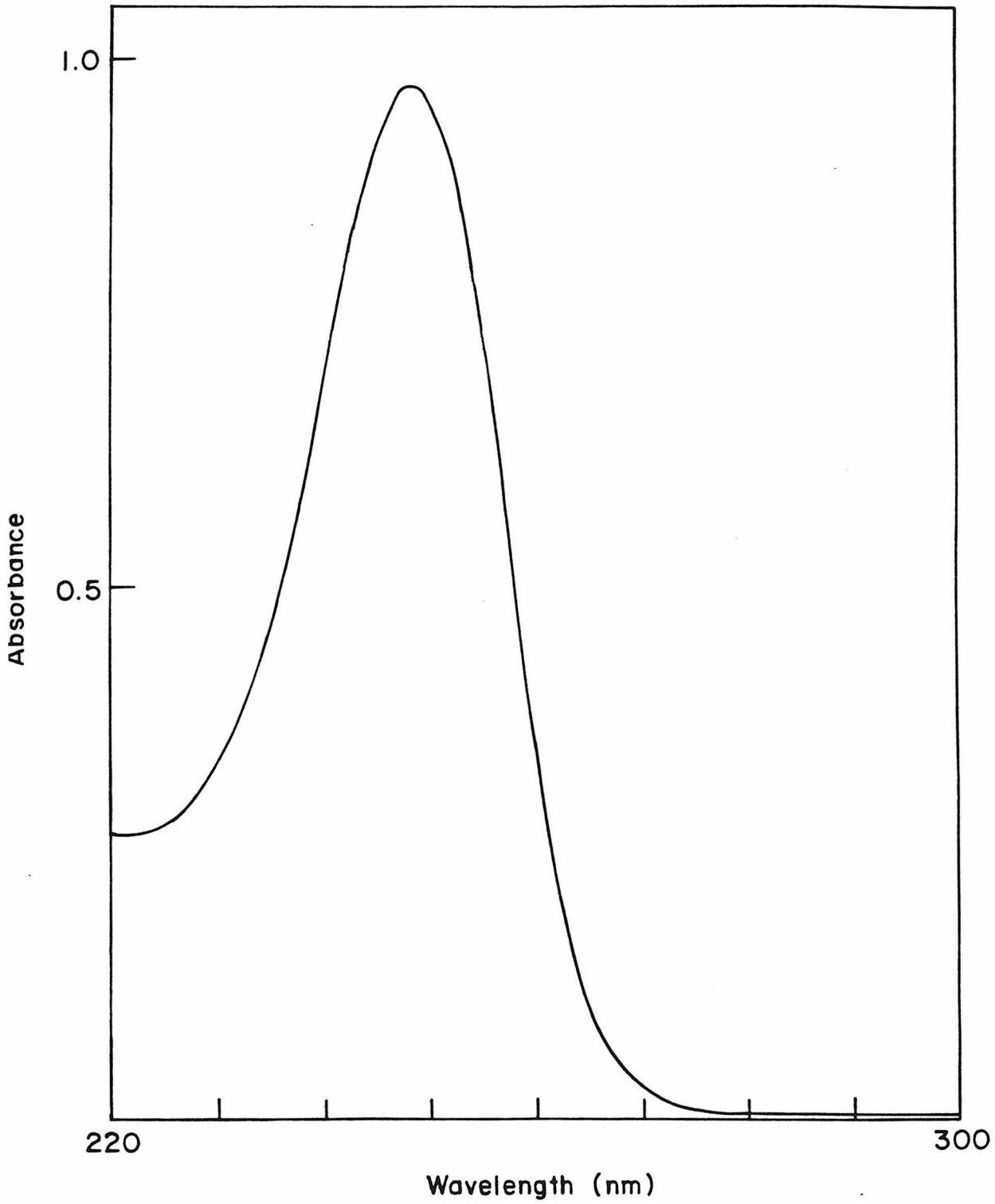

1.0
0.5
Absorbance
220
300
Wavelength (nm)

FIGURE 2: The IR spectrum of cholesteryl diazoacetate in CCl_4.

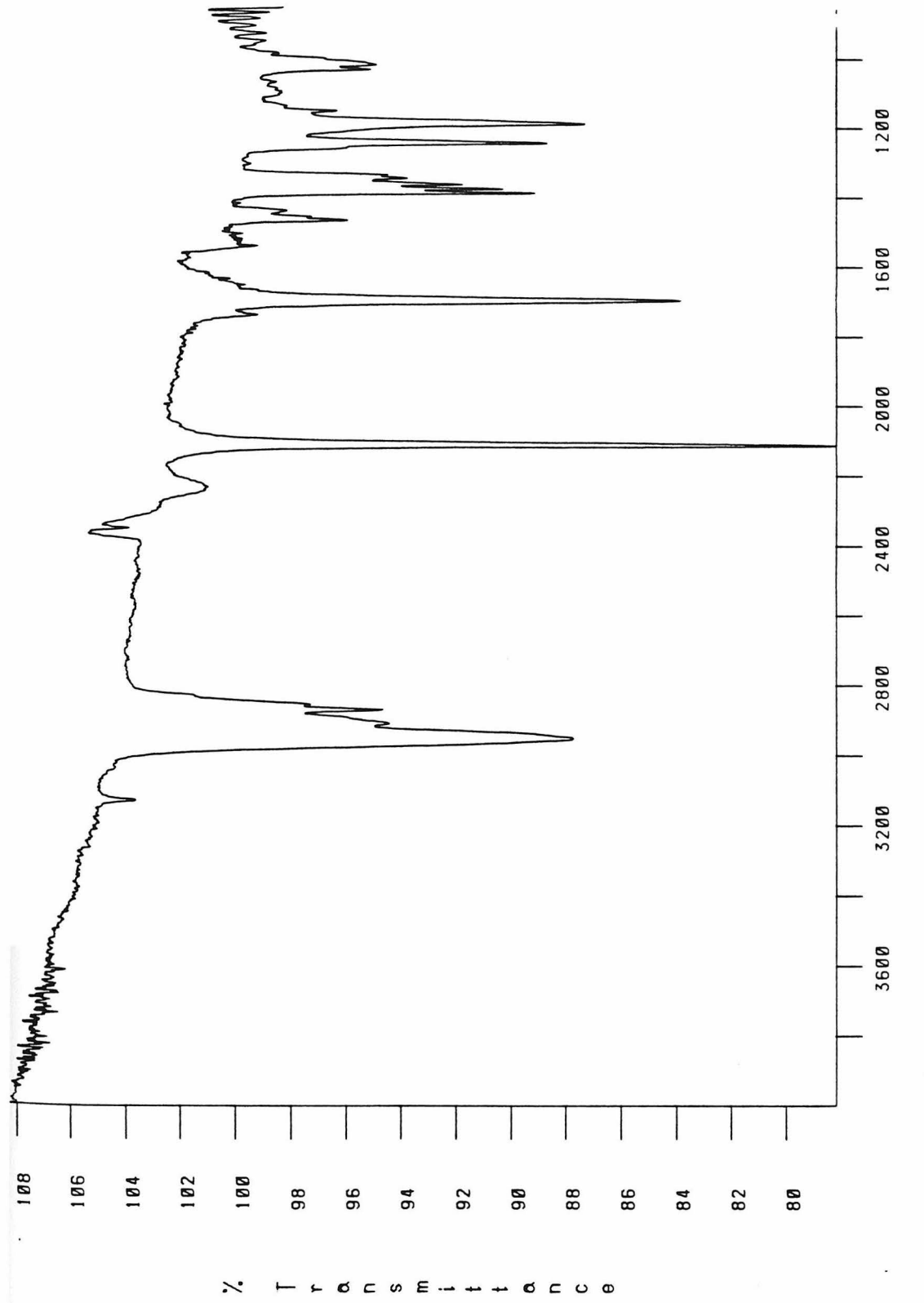

% Transmittance
108
106
104
102
100
98
96
94
92
90
88
86
84
82
80
3600
3200
2800
2400
2000
1600
1200
Wavenumbers

FIGURE 3: The UV spectra of cholesteryl diazoacetate before (a) and after (b) photolysis in ethanol.

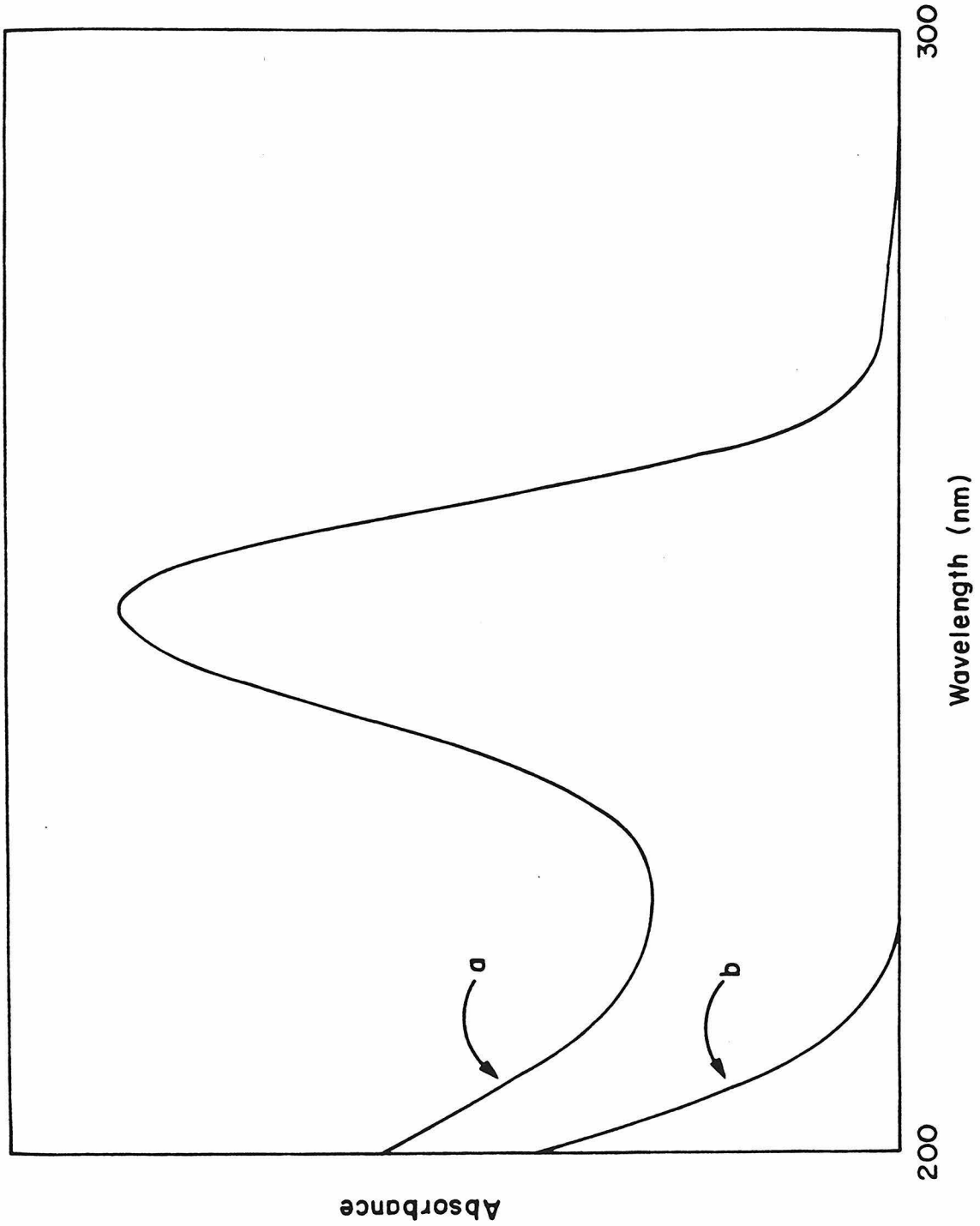
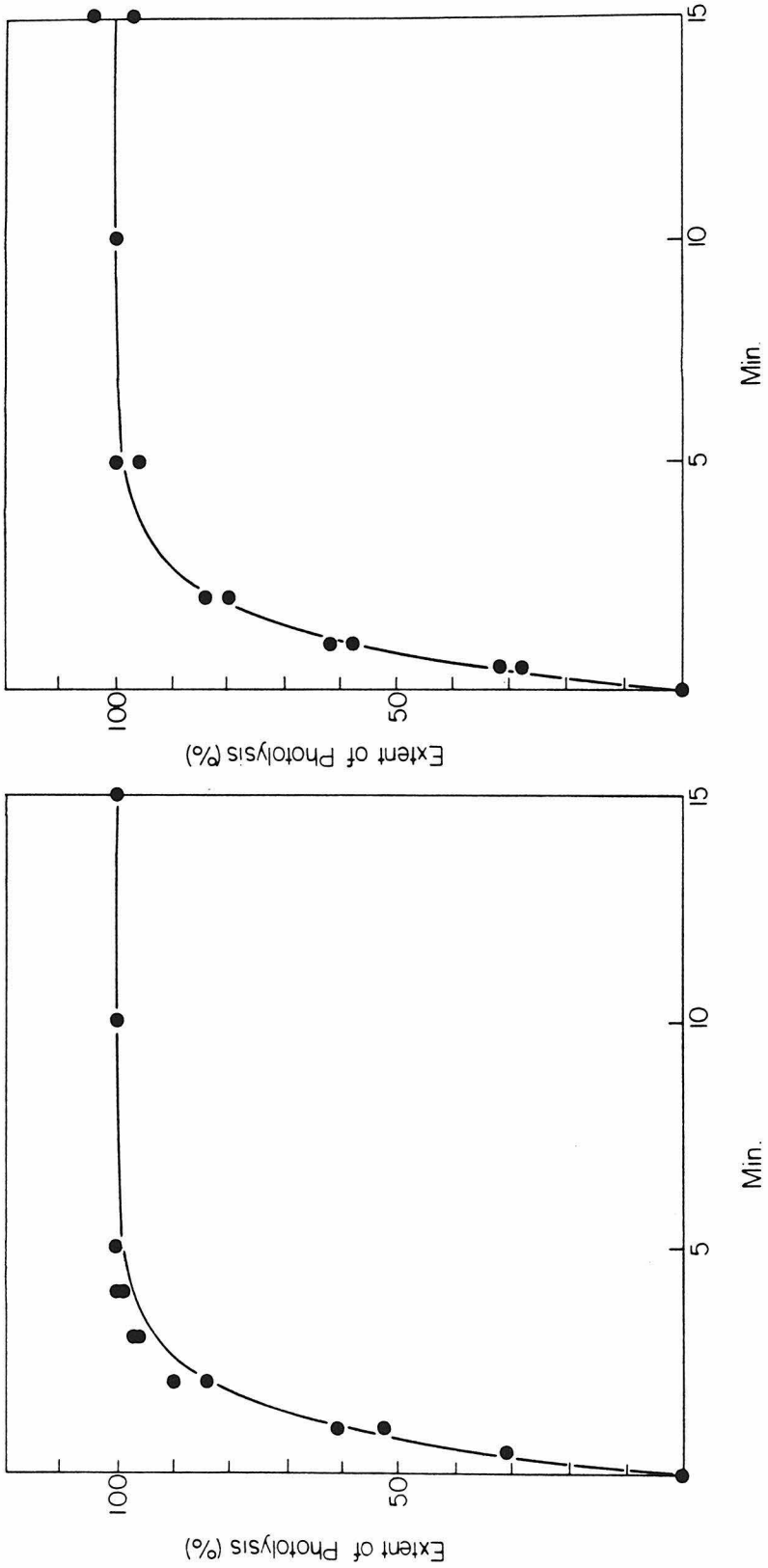

Absorbance
200
300
Wavelength (nm)
a
b

FIGURE 4: The time course of photolysis of cholesteryl diazoacetate in ethanol (left) and [^{3}H]cholesteryl diazoacetate in membrane vesicles (right).


Extent of Photolysis (%)
100
50
5
10
15
Min.
Extent of Photolysis (%)
100
50
5
10
15
Min.

5). The ratio of label migrating at the dye front, which corresponds to both labeled lipids and cholesterol, to label incorporated into protein is 75. Since much of the carbene will react with water to form cholesterol esters and cholesterol, the ratio above does not indicate the ratio of carbene insertion into lipids over proteins. The high background radioactivity is presumably caused by tritiated cholesterol esters and cholesterol (which migrate with the dye front presumably in detergent micelles) and therefore a chloroform:methanol extraction of both labeled lipids and cholesterol from the protein prior to electrophoresis was used to reduce this background. Although the radioactive lipids were not quantitatively extracted, about 70% of the radioactivity was extracted with the chloroform:methanol. The stoichiometry of covalent incorporation into the 40K, 50K, 60K, and 65K subunits, respectively, was about 4:1:1:2, i.e., a molar ratio of 2:1:1:2 (Figure 6). As all four subunits should be precipitated during the extraction in a quantitative manner, this ratio is likely to be correct.

Since there is evidence that cholesterol may be important in desensitization or at least essential for protection of desensitization properties of receptor during reconstitution experiments, the effect of agonist on the interaction of [^{3}H]cholesteryl diazoacetate with AChR was investigated. The probe was incorporated into AChR enriched vesicles, which were desensitized by exposure to 10 μM carbamylcholine as verified by inhibition of [^{125}I]α-BgTx binding. Irradiation of both desensitized and control AChR enriched membrane vesicles resulted in identical distributions of label among the four subunits.

In the above experiments, [^{3}H]cholesteryl diazoacetate was introduced into membranes in small portions of ethanol. An alternative method of introducing the probe was used to investigate the possibility of the label distribution being affected by the ethanol. The probe was introduced into the cuvette and ethanol was removed with a gentle stream of nitrogen. Membrane suspension was then introduced and stirred at 22°C for 4 hours. Although complete uptake of the probe from the walls of the cuvette was not possible, all four subunits were labeled after irradiation. This indicates that ethanol does not affect the labeling of AChR with

FIGURE 5: $NaDodSO_4$-polyacrylamide gel electrophoresis scan of AChR labeled with [^{3}H]cholesteryl diazoacetate in membrane vesicles.

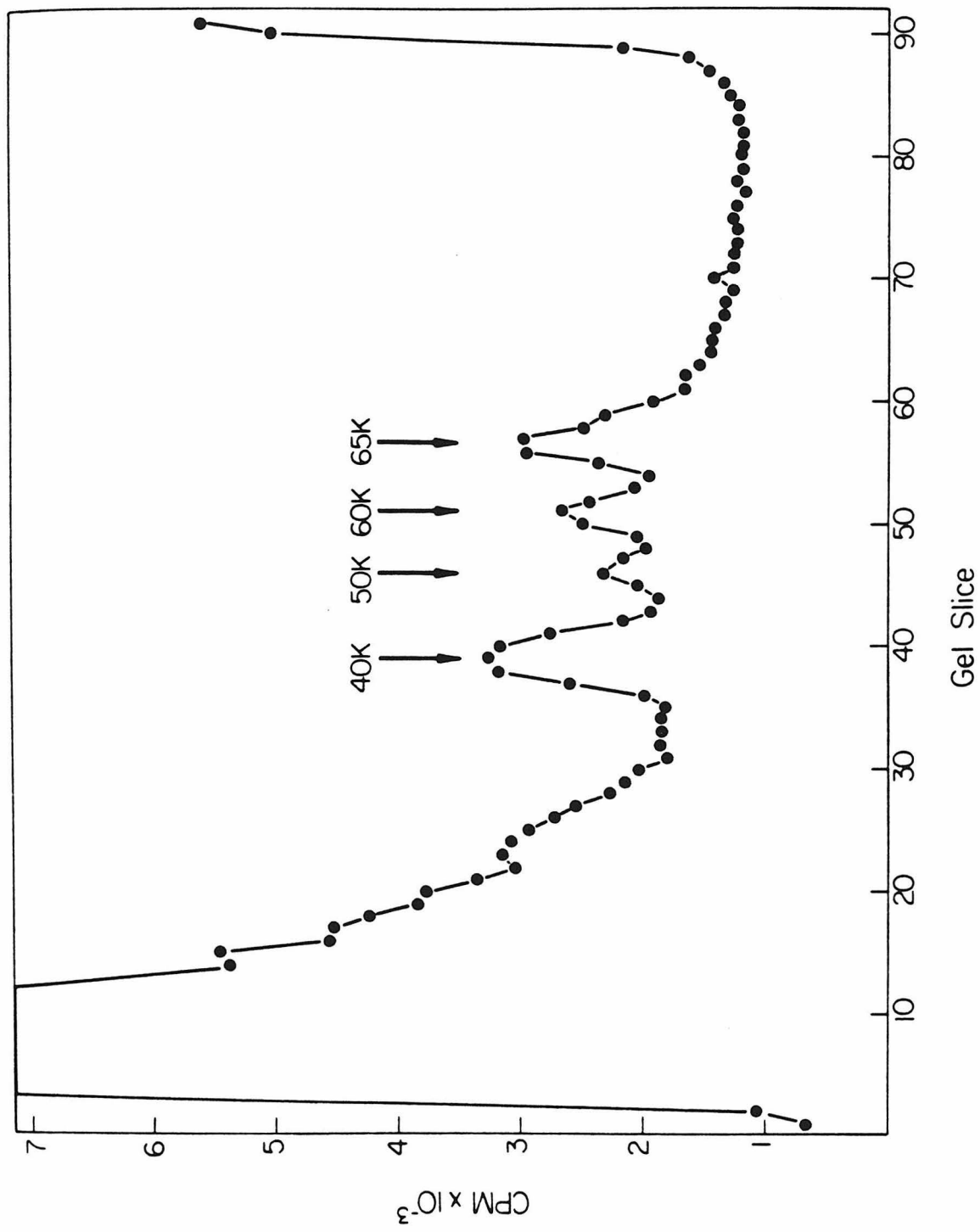
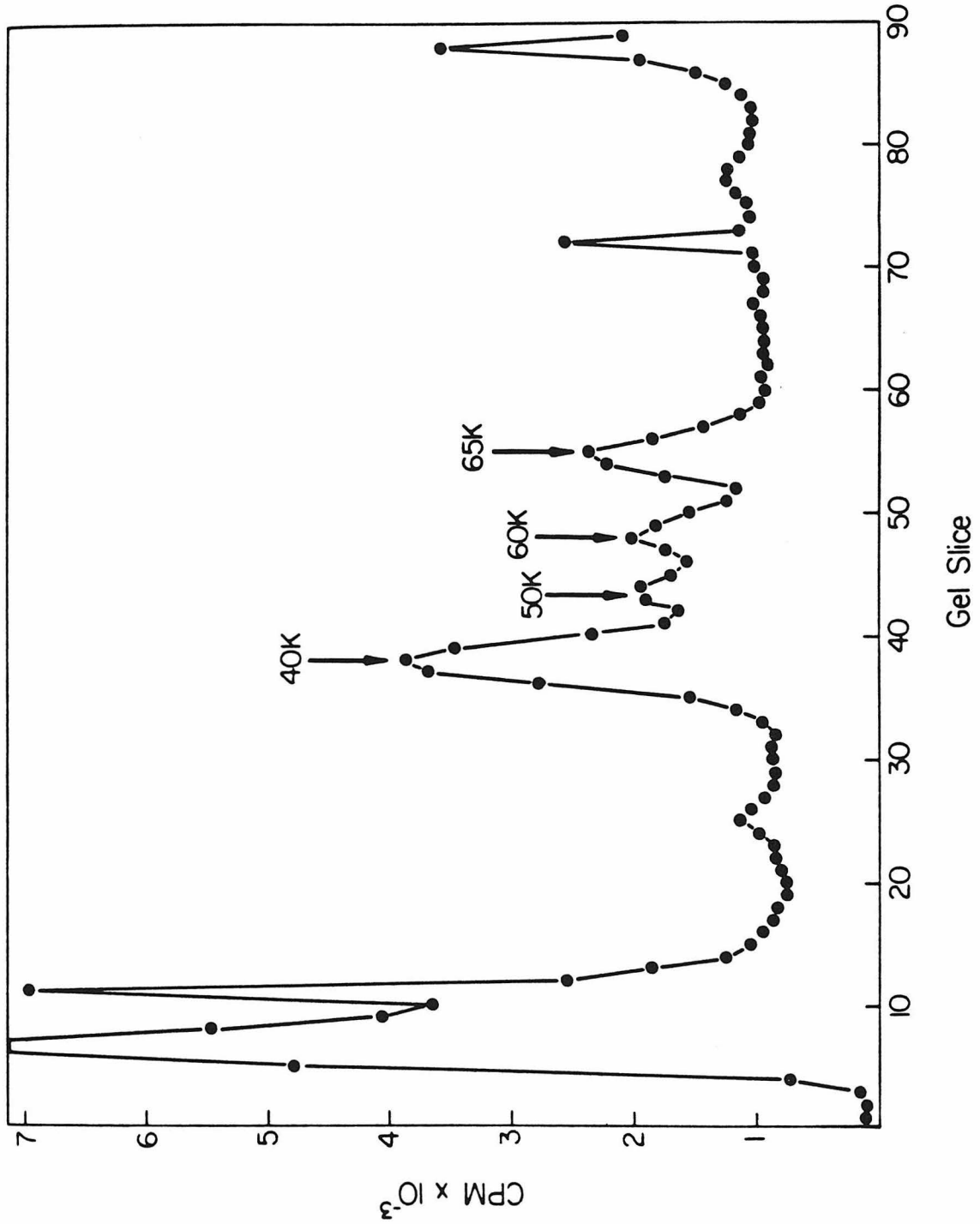

CPM x 10⁻³
Gel Slice
40K
50K
60K
65K

FIGURE 6: $NaDodSO_4$-polyacrylamide gel electrophoresis scan of AChR labeled with [^{3}H]cholesteryl diazoacetate in membrane vesicles after extraction of lipids with chloroform:methanol.


CPM x 10^{-3}
Gel Slice
40K
50K
60K
65K

[^{3}H]cholesteryl diazoacetate.

DISCUSSION

ESR experiments with a labeled steroid probe indicated an interaction of neutral lipids with AChR (Marsh & Barrantes, 1978). Using an androstanol spin labeled probe, the electron spin resonance spectra revealed an immobilized component along with the fluid component which is found in aqueous dispersions of extracted lipids. A similar result was obtained using stearic acid probes. AChR has been shown to incorporate more readily into cholesterol monolayers than other lipid monolayers, suggesting that it has an affinity for cholesterol (Popot et al., 1978). The photolabeling results presented in this communication using [^{3}H]cholesteryl diazoacetate demonstrate definitively that cholesterol interacts with AChR.

Investigations of the distribution of cholesterol in membranes is possible using saponin, filipin and digitonin, which form complexes with cholesterol that can be identified by electron microscopy after freeze-fracture (Elias et al., 1978,1979). Several groups have found little or no evidence for the presence of cholesterol in AChR rich regions of membranes using these probes. However, in one study, evidence for the presence of cholesterol in areas of membrane rich in AChR clusters was found. In both non-innervated and innervated *Xenopus* embryonic muscle cells, there was virtually no indication of cholesterol complexes in areas rich in aggregates of AChR particles (Bridgman & Nakajima, 1981). In *Rana* cutaneous pectoris and sartorious muscles, filipin-sterol complexes were absent from regions occupied with AChR aggregates (Nakajima & Bridgman, 1981) and there was also an almost complete absence of filipin-sterol complexes in AChR rich regions of the electroplax membrane of *T. marmorata* (Perrelet et al., 1982). However, in rat myotubes, areas of membrane rich in AChR clusters reacted extensively with filipin, which indicates the presence of cholesterol (Pumplin & Bloch, 1983). Although in the same study, membrane rich in AChR clusters in chick myotubes

did not react with saponin or filipin (Pumplin & Bloch, 1983).

The role which cholesterol plays in AChR function has been investigated. Inclusion of cholesterol during reconstitution was found to be essential to protect agonist-induced affinity change of cholinergic binding sites (Criado et al., 1982). In another study it was reported that cholesterol was necessary for the maintenance of agonist induced $^{86}Rb^{+}$ flux (Dalziel et al., 1980). Zabrecky & Raftery (1985) found the binding affinity of AChR for carbamylcholine increased as the cholesterol content of membrane vesicles was reduced. The cholesterol content was reduced by fusion of AChR enriched vesicles with phospholipid vesicles of defined composition. Although, it is apparent that cholesterol is important to AChR function, it is not clear whether this is because of its effect on membrane properties or a specific interaction with AChR.

A recent study, using photoreactive phospholipid probes, found that all four subunits of both *T. californica* and *T. marmorata* AChR were labeled (Giraudat et al., 1985). The study clearly demonstrated that all four subunits interact with charged lipids. Our results, which indicate neutral lipids interact with the AChR, are consistent with these findings.

Cholesteryl diazoacetate is a useful reagent for the study of membrane proteins. It is readily photolyzed and exhibits a low level of Wolff rearrangement (Keilbough & Thornton, 1983a). Reasonable yields of incorporation into lipids and membrane proteins can be obtained. Since the photo-generated carbene is situated near the lipid-water interface (Keilbough and Thornton, 1983b), this probe has potential as a topographic tool to map membrane protein structure. Using a photo-activated lipid probe, which also generates a carbene near the lipid-water interface, regions of glycophorin A near the interface have been determined (Ross et al., 1982). As cholesteryl diazoacetate incorporates into membrane proteins and lipids, it is useful in the study of the interaction of cholesterol with both lipids and membrane proteins.

In this communication, we have demonstrated covalent labeling of AChR with [^{3}H]cholesteryl diazoacetate. Since this probe incorporates into lipid bilayers

in a fashion analogous to cholesterol, this result confirms that AChR interacts with cholesterol. It is interesting to note that the labeling of AChR with this probe is very similar to the labeling with [^{3}H]adamantanediazirine, a probe which labels proteins exposed to the hydrocarbon core of the lipid bilayer (Middlemas & Raftery, 1983). As both of these reagents label all of the subunits, it is probable that all of the subunits interact with the membrane in a related manner.

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Chapter 3

Synthesis of Fluorine-Containing Agonists for the Acetylcholine Receptor

INTRODUCTION

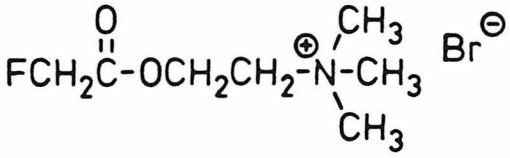
^{19}F nuclear magnetic resonance (NMR) spectroscopy has been used for the study of enzymes (Raftery et al., 1971). ^{19}F is well suited for this approach to enzymology, as it has a large chemical shift range (500 ppm) as well as very high sensitivity (83% of ^{1}H) [for a review of ^{19}F NMR spectroscopy, see Emsley et al. (1968)]. Another important aspect of fluorine is that it can be substituted for a proton in many biological substrates and ligands without significantly altering their biological properties.

Equilibrium binding studies of agonists to the AChR reveal the existence of two sites with relatively high affinity (equilibrium dissociation constants in the range of 1–100nM). Under equlibrium conditions, these binding sites have undergone an agonist induced increase in affinity which is correlated with physiological desensitization. Although various approaches on a faster time scale have allowed estimates of the resting dissociation constant of these sites, a discrepancy exists in the results [for a recent review, see Changeux et al., 1984]. Two conformational transitions have been observed, a slow one (about 0.01 sec^{-1}) and an intermediate one (between 2–50 sec^{-1}). The problem arises that channel activation occurs faster than these observed conformational transitions. Recently, evidence for a faster conformational change (400–600 sec^{-1}, i.e., $t_{\frac{1}{2}}$ 1.7–1.2 msec), one that is more closely correlated in time to channel opening, has been acquired (Dunn and Raftery, 1982). This conformational change is caused by agonist binding to sites which are distinct from the previously identified high-affinity binding sites (Conti-Tronconi et al., 1982; Dunn et al., 1983). The existence of these low-affinity sites, with equilibrium dissociation constants between 50μM to 1mM for various agonists, was discovered by monitoring fluorescence changes of the covalently bound probe, NBD. As agonist binding to these sites is unaffected by desensitization or covalent labeling with BrACh (an affinity reagent which specifically labels the high-affinity sites), these sites are distinct from the high-affinity sites.

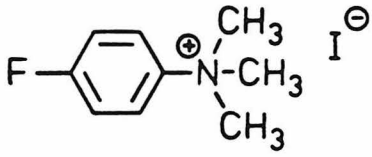
In order to use NMR spectroscopy to study exchange of a ligand at a binding

site, a high concentration of membrane-bound ligand binding sites must be obtained. In addition, a ligand must be available that is readily observed by using current NMR spectroscopy instrumentation and the ligand binding parameters must be suitable for study of the exchange between free and bound states. These conditions can be met with the AChR, as demonstrated by Miller et al. (1979) in a study of the binding of choline to the receptor.

The interaction of agonists with these low-affinity sites may be in a range amenable to study by ^{19}F NMR. In this chapter, the syntheses of fluoroacetylcholine (I) and p-fluorophenyltrimethylammoniumiodide (PFT) (II) are described.



I



II

It is also demonstrated that both of these compounds are agonists for the AChR.

MATERIALS AND METHODS

^1H NMR and ^{19}F NMR spectra were recorded on a Varian Associates XL-200 (200 MHz) spectrometer or a Bruker WM 500 (500 MHz) spectrometer using sodium 3-(trimethylsilyl)propane sulfonate as the internal reference for ^1H NMR spectra and trifluoroacetic acid as the internal reference for ^{19}F NMR spectra. All spectra were recorded in $[^2H]H_2O$ or H_2O. Chemical shifts are reported in ppm downfield or upfield (negative values) from the references.

Sodium fluoroacetate, bromoethanol, and iodomethane were purchased from Aldrich. Phthaloyl chloride was the generous gift of Dr. R.E. Ireland. Bromoethanol was redistilled before use. Benzene was dried and distilled over sodium. N,N-dimethyl-p-fluoroaniline was purchased from TCI. $^{22}Na^+$ was purchased from NEN.

Synthesis of Fluoroacetyl Chloride. All procedures should be done in a hood, as monofluoroacetyl compounds are toxic. Fluoroacetyl chloride was synthesized by the method of Pattison et al. (1956). 5 g Sodium fluoroacetate was added to 8.5 ml of phthaloyl chloride. After mixing thoroughly, the mixture was heated with vigorous stirring in a flask fitted for distillation. The distillate of boiling point 70–71° was collected and was used immediately. The yield was 3.2 g (66%).

Synthesis of Bromoethyl Fluoroacetate. Bromoethyl fluoroacetate was synthesized by the method of Blohm (1951). 2.2 g of fluoroacetyl chloride was added to 5.6 ml of bromoethanol in 11 ml of benzene. The solution was refluxed for 1 hr. The solution was fractionally distilled under reduced pressure. Bromoethyl fluoroacetate was collected between 115–116 ° at 75 mm. The yield was 2.5 g (59%).

Synthesis of Fluoroacetylcholine Bromide. Fluoroacetylcholine bromide was synthesized by the method of Blohm (1951). 1 ml of trimethylamine, which had been passed through a KOH tower and then condensed, was added to 2.5 g of bromoethyl fluoroacetate in 8.3 ml of benzene. After 48 hr at 22 °, the crystalline mass was collected and washed with two 20 ml portions of hot acetone. The remaining acetone was removed *in vacuo* over P_2O_5. The yield was 0.85 g (26%). The compound melted at 122–124 °, which is in agreement with the literature (Blohm, 1951). ^{1}H NMR: δ 3.21 (s, 9H); 3.79 (t, 2H); 4.71 (t, 2H); 5.06 (d, 2H, J = 46 Hz). ^{19}F NMR: δ -49.25 (t, J = 50 Hz).

Synthesis of p-Fluorophenyltrimethylammonium Iodide. 1 ml of iodomethane was added to 1 g of N,N-dimethyl-p-aniline in 4 ml of benzene. After 24 hr, the crystalline product was collected on a sintered glass funnel and washed with two 5 ml portions of benzene. The remaining benzene was removed *in vacuo* over P_2O_5. The yield was 1.23 g (61%). The m.p. of the white crystals was 226 °. ^{1}H NMR: δ 3.66 (s, 9H); 7.37 (m, 2H); 7.88 (m, 2H). ^{19}F NMR: δ -34.63 (s).

Preparation of AChR Enriched Membrane Fragments. Membrane fragments enriched in AChR were prepared from electric organs from *T. californica* as described by Elliot et al. (1980) and were further purified by alkali extraction (Neu-

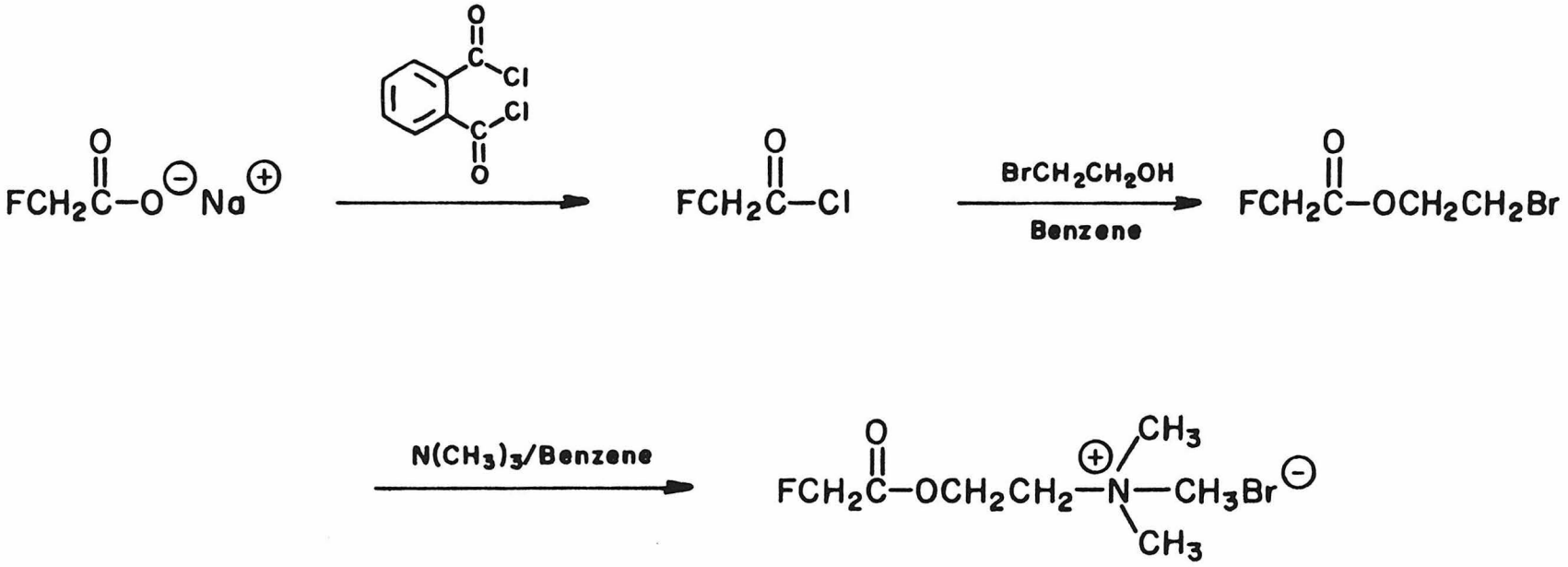
big et al., 1979; Elliott et al., 1979). The membranes were finally suspended in Ca^{2+}-free *Torpedo* Ringer's solution adjusted to pH 7.4 (250 mM NaCl, 5 mM KCl, 20 mM HEPES, 0.02% NaN_3, and 2 mM $MgCl_2$). Protein concentration was determined by the method of Lowry et al. (1951). The concentration of α-BgTx sites was determined by the method of Schmidt & Raftery (1973) using [^{125}I]α-BgTx obtained from New England Nuclear and calibrated by the procedures of Blanchard et al. (1979). The specific activities of the preparations varied between 2.0 and 3.7 nmol of α-BgTx binding sites mg^{-1} of protein.

$^{22}Na^+$ Flux Assay. Membrane vesicles were suspended in BSA solution (10 mg ml^{-1}) and incubated for 15–20 min at 22°. The membrane vesicles were then pelleted by centrifugation at 40,000 *g*. This treatment produces sealed membrane vesicles probably by removing free fatty acids which may result from the extraction at pH 11. The pellets were resuspended in 10 mM 2, 2′, 2″-nitriloethanol buffer adjusted to pH 7.4 containing 200mM NaCl. The membranes were pelleted by centrifugation and resuspended in the the same buffer containing 15μCi ml^{-1} of $^{22}Na^+$ and subjected to two cycles of freeze thaw in order to allow equilibration of $^{22}Na^+$ inside and outside the vesicles. These vesicles were assayed for carbamylcholine, fluoroacetylcholine and PFT induced $^{22}Na^+$ flux by the method of Wu & Raftery (1979).

RESULTS

The synthesis of fluoroacetylcholine bromide is shown in Figure 1. The direct distillation of fluoroacetyl chloride from the sodium salt and phthaloyl chloride developed by Pattison et al. (1956) is convenient for the synthesis of fluoroacetyl esters. Previously, production and purification of the acid from the sodium salt, followed by synthesis of the acid chloride was required. This convenient synthesis of fluoroacetyl esters should allow the routine introduction of fluorine labels into compounds of biological interest. Although the introduction of trifluoroacetyl groups is synthetically easy, the esters of trifluoroacetic acid may not be stable

FIGURE 1: The synthesis of fluoroacetylcholine bromide.



in aqueous conditions. Therefore, the introduction of the monofluoroacetyl group may be advantageous. The synthesis of PFT is shown in Figure 2.

Cation flux assays reveal both fluoroacetylcholine bromide and PFT are agonists for the AChR (Figures 3 and 4). Although Blohm (1951) observed the cholinergic symptoms of fluoroacetylcholine bromide were less than acetylcholine when administered intravenously to rabbits, the assay was probably monitoring parasympathetic effects involving the muscarinic AChR.

The ^{1}H NMR spectrum of fluoroacetylcholine bromide, which indicates the α protons are split by coupling to the fluorine, is shown in Figure 5. The ^{19}F NMR spectrum is shown in Figure 6, which also has splitting due to coupling of the α protons with fluorine. The ^{1}H and ^{19}F NMR spectra of PFT are shown in Figure 7 and 8. The fluorine chemical shifts of fluoroacetate and fluoroacetylcholine are different, indicating there would not be a problem in identifying a signal from fluoroacetylcholine as opposed to its product of hydrolysis (Figures 6 and 9).

The hydrolysis of a fluoroacetate ester could cause problems in NMR experiments, which can require extensive periods of time for data gathering. However, preliminary results indicate that less than 10% of fluoroacetylcholine bromide is hydrolyzed at neutral pH and room temperature in 12 hr. On the other hand, trifluoroacetylcholine iodide was hydrolyzed rapidly. The principal advantage of PFT is it cannot be hydrolyzed by acetylcholine esterase, thus caution does not have to be excercised to ensure all of the esterase is blocked by irreversible inhibitors. Both agonists may prove useful as acetylcholine analogs for the study of the nicotinic AChR using ^{19}F NMR spectroscopy. In addition, fluoroacetylcholine could be useful for the investigation of both the muscarinic AChR and acetylcholine esterase.

FIGURE 2: The synthesis of PFT.

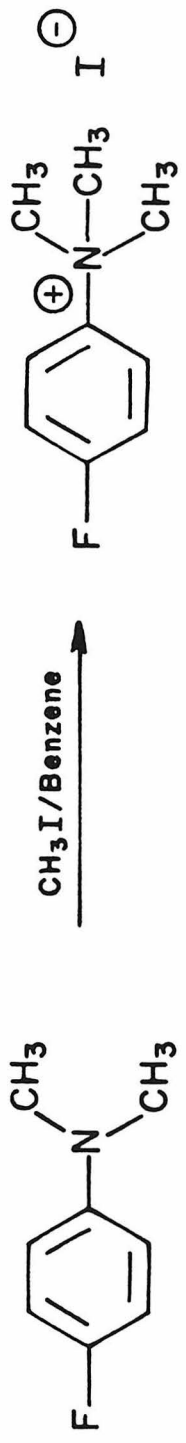

CH3
N
CH3
F
CH3I/Benzene
CH3
N
CH3
F
I

FIGURE 3: Fluoroacetylcholine bromide induced cation efflux. $^{22}Na^{+}$-loaded vesicles were assayed for $^{22}Na^{+}$ retained in vesicles as a function of time after mixing with agonist by the method of Wu & Raftery (1979). Control (0 μM agonist) (x), 100 μM carbamylcholine (•), 200 μM fluoroacetylcholine bromide (▲), and 0.2% Triton X–100 (■).

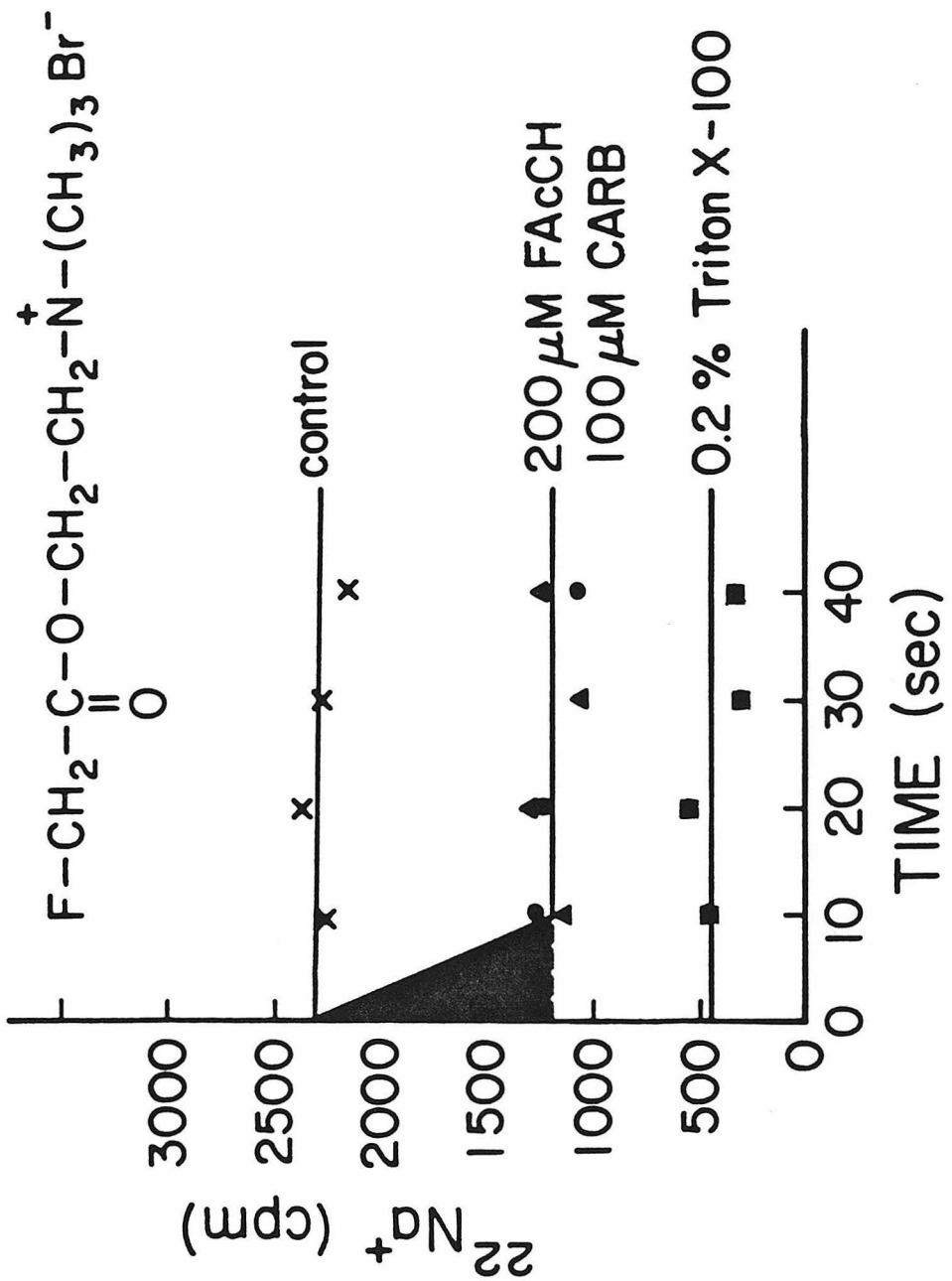

F-CH2-C-O-CH2-CH2-N+-(CH3)3 Br-
O
control
200 μM FAcCH
100 μM CARB
0.2 % Triton X-100
22Na+ (cpm)
3000
2500
2000
1500
1000
500
0
0 10 20 30 40
TIME (sec)

FIGURE 4: PFT induced cation efflux. $^{22}Na^{+}$-loaded vesicles were assayed for $^{22}Na^{+}$ retained in vesicles as a function of time after mixing with agonist by the method of Wu & Raftery (1979). Control (0 μM agonist) (x), 100 μM carbamylcholine (•), 200 μM PFT (▲), and 0.2% Triton X–100 (■).

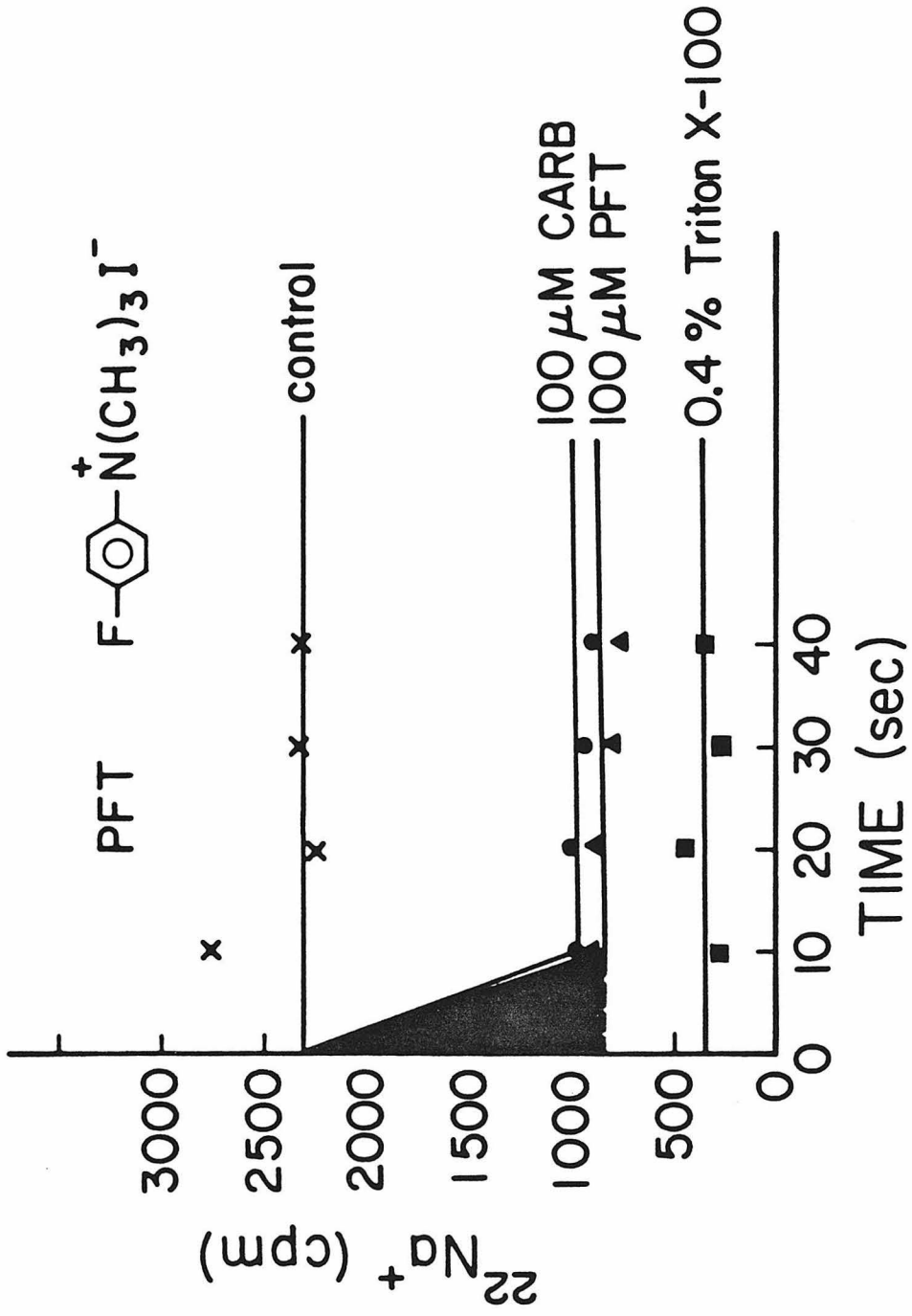

PFT
F
N(CH3)3 I-
control
100 μM CARB
100 μM PFT
0.4 % Triton X-100
22Na+ (cpm)
3000
2500
2000
1500
1000
500
0
0
10
20
30
40
TIME (sec)

FIGURE 5: The ^{1}H NMR spectrum of fluoroacetylcholine bromide in $[^{2}H]H_2O$.

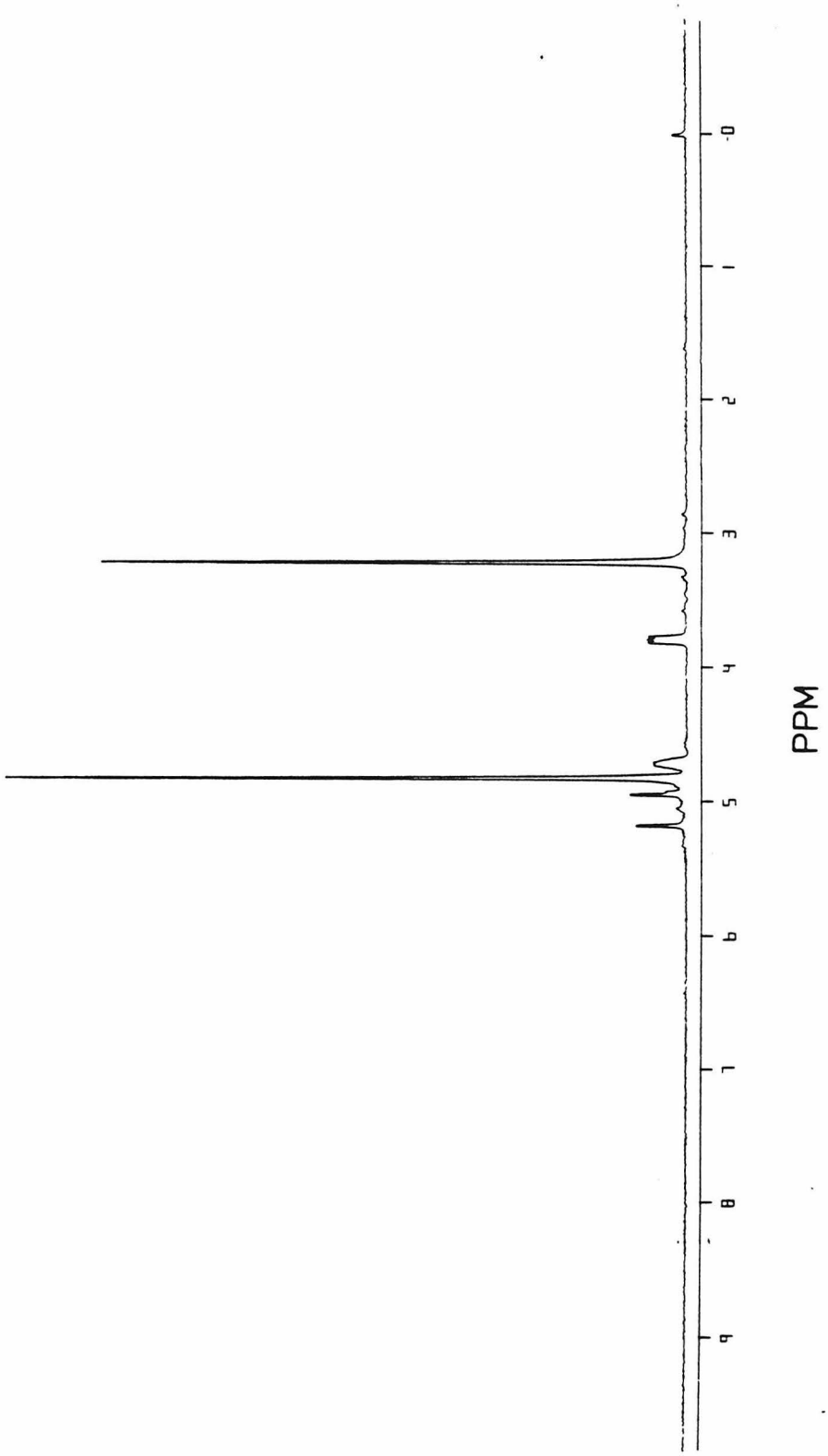

9
8
7
6
5
4
3
2
1
0
PPM

FIGURE 6: The ^{19}F NMR spectrum of fluoroacetylcholine bromide in H_2O.

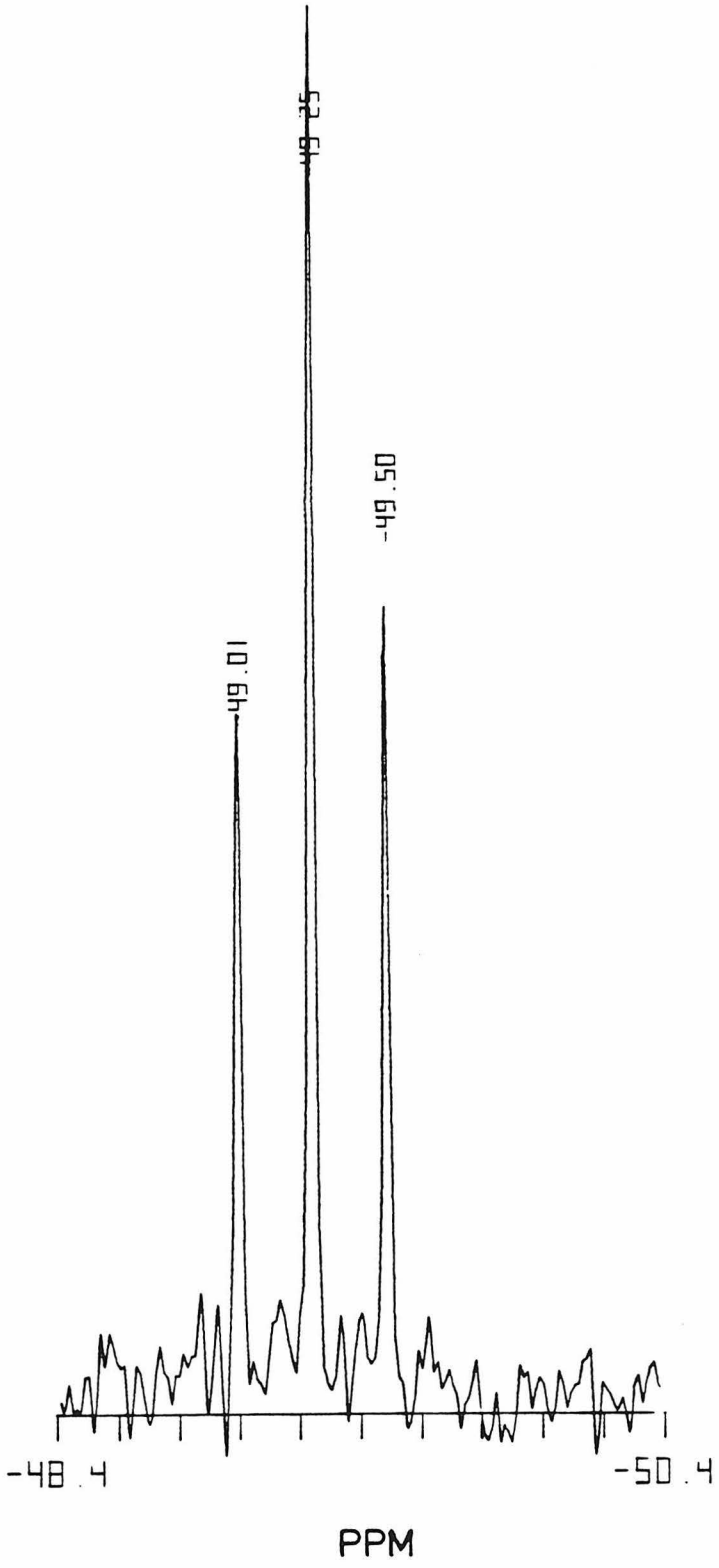

48.25
-49.50
-50.01
-48.4
-50.4
PPM

FIGURE 7: The ^{1}H NMR spectrum of PFT in $[^{2}H]H_2O$.

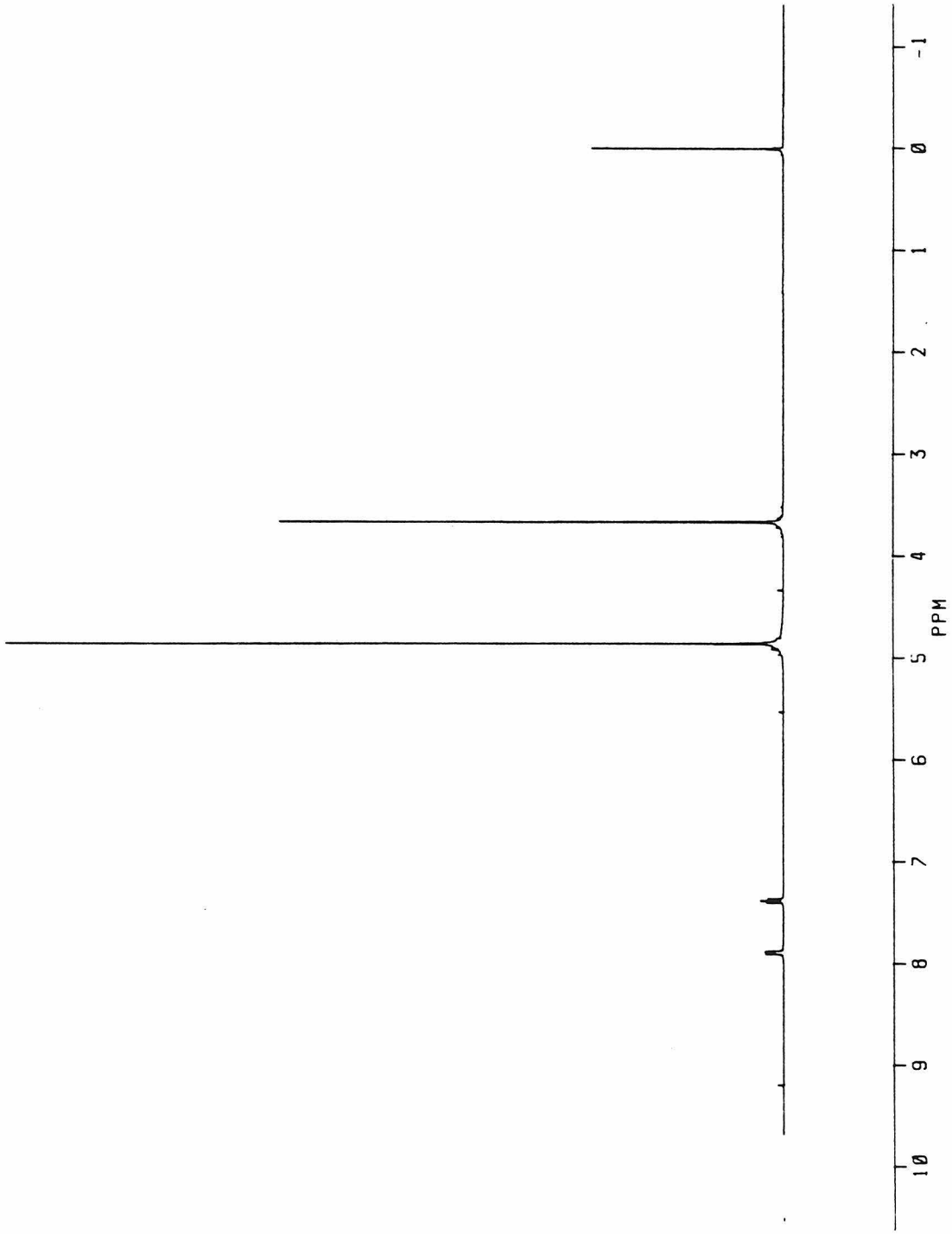

10
9
8
7
6
5
4
3
2
1
0
-1
PPM

FIGURE 8: The ^{19}F NMR spectrum of PFT in H_2O.

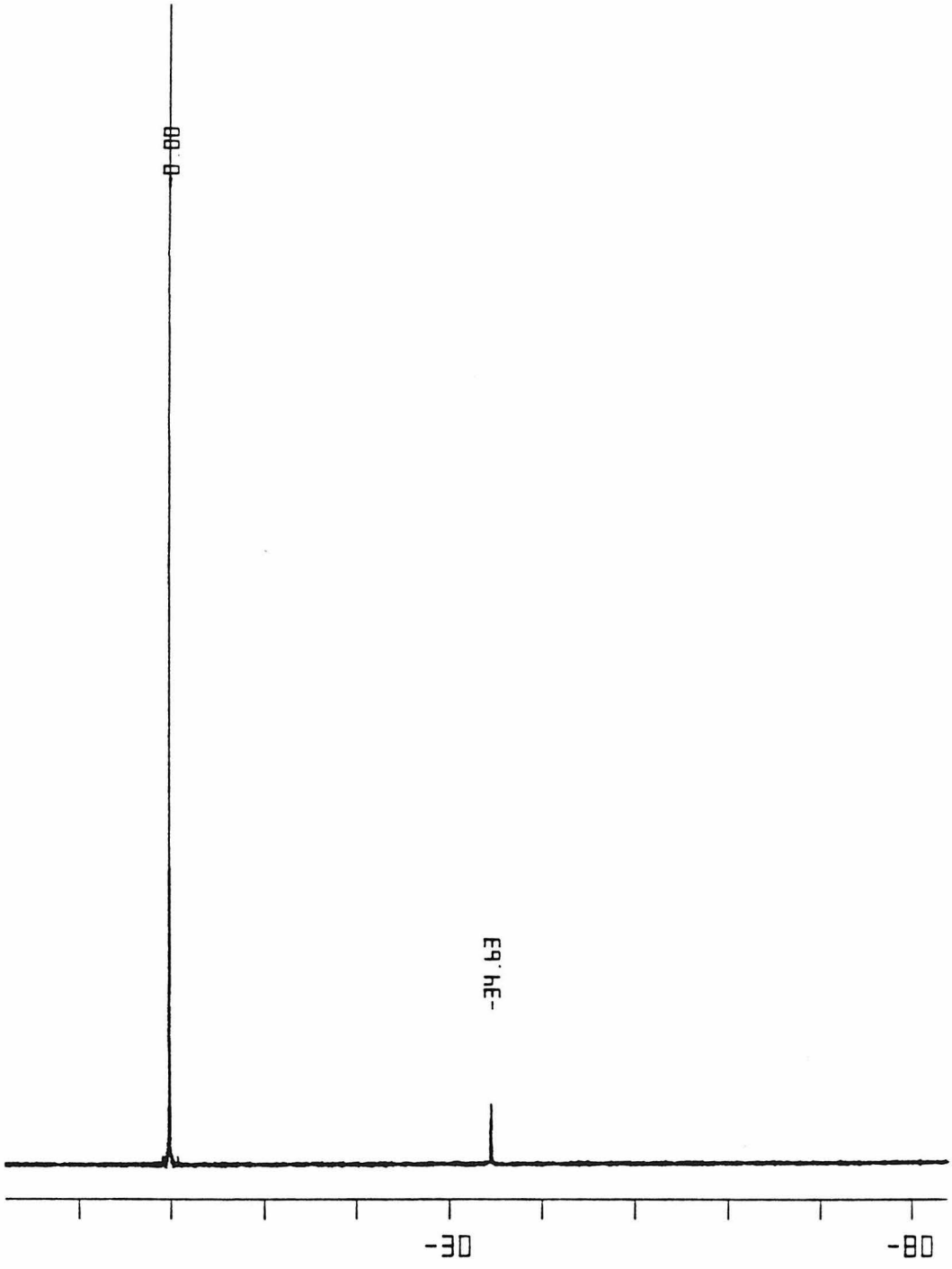
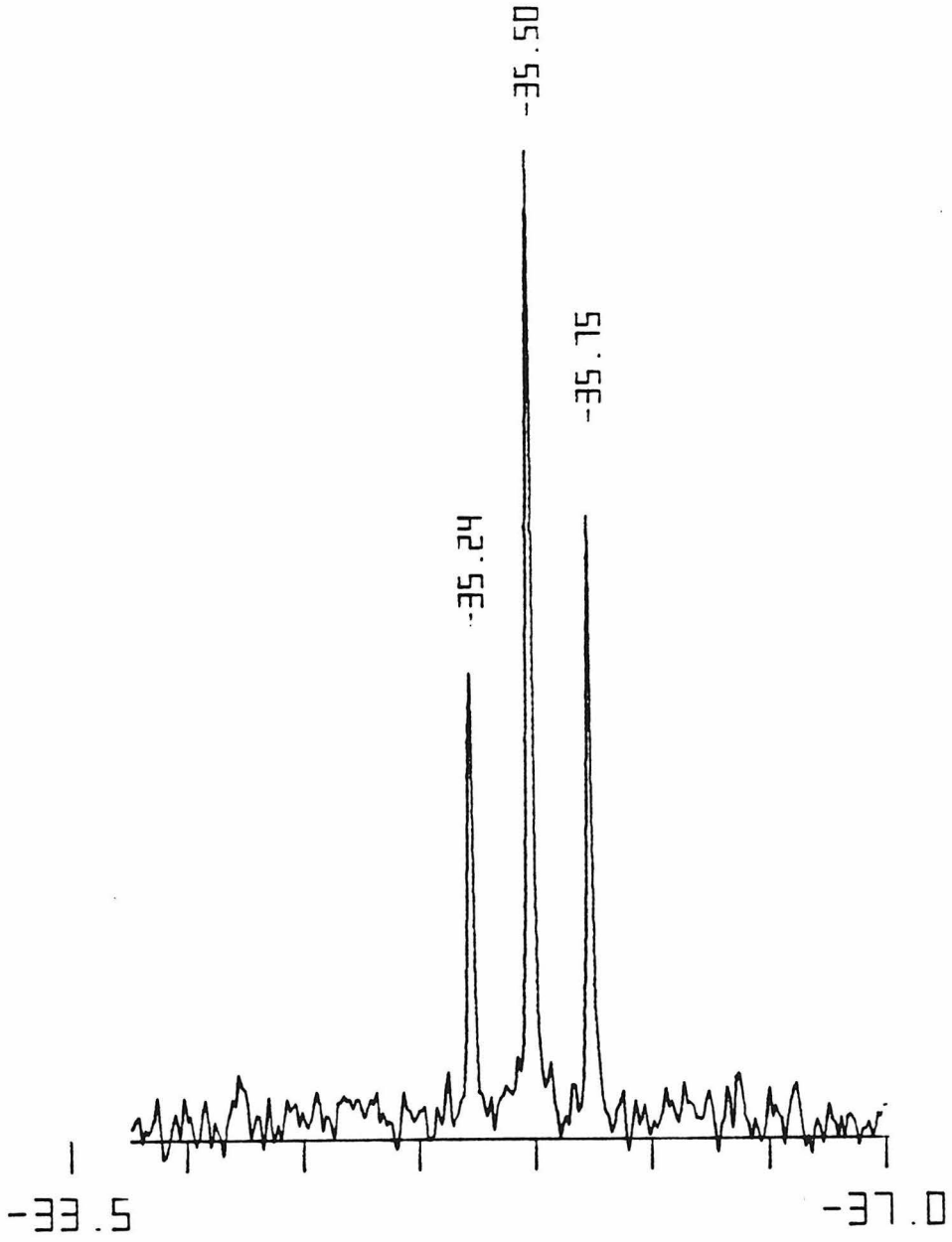

0.00
-34.63
-30
-80
PPM

FIGURE 9: The ^{19}F NMR spectrum of sodium fluoroacetate in H_2O.


-35.50
-35.75
-35.24
-33.5
-37.0
PPM

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Chapter 4

Affinity Cleavage of the Acetylcholine Receptor: Evidence for a Low-Affinity Agonist Binding Site

INTRODUCTION

The nicotinic AChR isolated from *Torpedo californica* is a multisubunit protein of homologous subunits with apparent M_r of 40K (α), 50K (β), 60K (γ), and 65K (δ) in a stoichiometry of 2:1:1:1 (Raftery et al., 1980). The receptor is a transmembrane complex of five subunits, all of which span the lipid bilayer (Strader & Raftery, 1980; Middlemas & Raftery, 1983), that forms an agonist gated ion channel [for recent reviews, see Conti-Tronconi & Raftery (1982); Raftery et al. (1983); Adams (1981)].

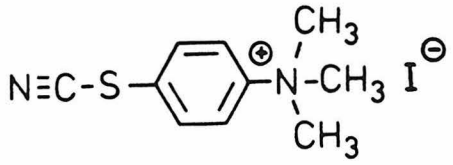
Binding sites for both agonists and antagonists have been revealed using the cholinergic affinity labels, BrACh (Damle et al., 1978; Moore and Raftery, 1979) and MBTA (Damle and Karlin, 1978), which specifically label the 40K subunit after reduction of a disulfide bond. These binding sites undergo an agonist-induced affinity change which is correlated with physiological desensitization. As this change increases the affinity of the receptor for agonists, we will refer to the sites on the 40K subunits as high-affinity sites. Recently, evidence of other distinct binding sites for agonists has been acquired (Dunn and Raftery, 1982; Conti-Tronconi et al., 1982; Dunn et al., 1983). The existence of these low-affinity sites was discovered by monitoring the change in fluorescence of a covalently bound probe, which is presumably caused by a conformational change of the AChR. This conformational change occurs on a similar timescale as ion channel opening. Since agonist binding to these sites is unaffected by desensitization or covalent labeling with BrACh, these sites are not those on the 40K subunit which can be labeled by the affinity techniques. An important area of research is determining the physiological roles of these multiple binding sites and primary structural locations.

An approach developed to determine the binding sites of molecules on DNA uses affinity cleavage reagents (Schultz et al., 1982; Taylor et al., 1984; Baker & Dervan, 1985). Such reagents require linking a DNA cleaving functionality to a molecule that binds DNA. After cleavage of the DNA, binding sites are determined by cleavage pattern analyses using Maxam-Gilbert sequencing gels. Conversely,

an approach allowing specific cleavage for any sequence uses oligodeoxynucleotides linked to a cleavage functionality (Dreyer & Dervan, 1985).

Affinity cleavage of neurotransmitter receptors could provide a complementary approach to affinity labeling in the determination of the primary structural locations of ligand binding sites. The molecular weights of the products of a protein cleavage reaction determined by polyacrylamide gel electrophoresis yield direct information about the possible sites of cleavage. Another advantage of an affinity cleavage strategy, in the case of membrane proteins, is the relative ease of purification of large polypeptides. Affinity labeling usually requires the generation of small hydrophobic peptides, which can be difficult to purify, for the determination of the primary structural location of labeling. In addition, the subsequent Edman degradations for amino acid sequence analysis often proceed in better yields with larger polypeptides.

In this communication, we report the synthesis of the affinity cleavage reagent, p-thiocyanophenyltrimethyl ammonium iodide (TPMA) (I) and its specific cleavage of a peptide bond near an agonist binding site in the AChR. This reagent,



I

which is an agonist for the AChR, cleaves at the amino peptide bond of cysteine residues. The mechanism of cleavage is expected to be similar to that of NTCB, a general reagent for cleavage of the amino peptide bond of cysteine residues in proteins (Vanaman & Stark, 1970; Degani et al., 1970).

MATERIALS AND METHODS

^{1}H NMR spectra were recorded on a Bruker WM 500 (500 MHz) spectrometer

using sodium 3-(trimethylsilyl)propane sulfonate as the internal reference for ^1H NMR spectra. Chemical shifts are reported in ppm downfield from the reference. UV-visible spectra were recorded on a Cary model 118 spectrophotometer. Fourier transform infrared (FT-IR) spectra were recorded on a Mattson Instruments Sirius 100 FT-IR equipped with Starlab minicomputer data station and high resolution graphics terminals under a positive nitrogen purge. Benzene was dried and distilled over sodium.

Preparation of Membrane Fragments. Membrane fragments enriched in AChR were prepared from electric organs from *T. californica* as described by Elliott et al. (1980) and were further purified by alkali extraction (Neubig et al., 1979; Elliott et al., 1979). The membranes were then suspended in 10 mM HEPES buffer containing 10 mM iodoacetamide, 35 mM $NaNO_3$, and 0.02% NaN_3 adjusted to pH 8.0. After 15 minutes at 22°, the membranes were diluted with 75 volumes of H_2O and centrifuged at 40,000 *g* for 50 minutes. The membrane pellets were finally suspended in 10 mM HEPES buffer containing 35 mM $NaNO_3$ and 0.02% NaN_3 adjusted to pH 7.4. Protein concentration was determined by the method of Lowry et al. (1951). The concentration of α-BgTx sites was determined by the method of Schmidt & Raftery (1973) using [^{125}I]α-BgTx obtained from New England Nuclear and calibrated by the procedures of Blanchard et al. (1979). The specific activities of the preparations varied between 2.0 and 3.7 nmol of α-BgTx binding sites mg^{-1} of protein. The concentration of AChR was taken to be half of the concentration of α-BgTx sites.

Synthesis of p-Thiocyanodimethylaniline. Synthesis of p-thiocyanodimethylaniline was accomplished by the method of Brewster & Schroeder (1943).

Synthesis of p-Thiocyanophenyltrimethyl Ammonium Iodide. 2.3 ml of iodomethane was added to 3 g of p-thiocyanodimethylaniline in 12 ml of dry benzene. The vessel was sealed. After 7 days at room temperature, the crystalline product was collected on a sintered glass funnel. After removal of the remaining solvent *in vacuo*, the product was recrystallized in absolute ethanol. Any remaining ethanol was removed *in vacuo*. The yield was 0.75 g (14%) of white crystals, m.p. 153–

154°. Anal. calcd. for $C_{10}H_{13}N_2S$: C, 37.50%; H, 4.06; N, 8.75. Found: C, 37.35%; H, 4.04; N, 8.56. ^1H NMR (C^2HCl_3): δ 3.70 (s, 9H), 7.99 (d, 2H, J=9.2 Hz), 7.89 (d, 2H, J=8.7 Hz). IR (KBr): 3453 (w), 3009 (w), 2155 (m), 1489 (s), 1468 (m), 1455 (w), 1120 (w), 1009 (m), 930 (m), 843 (m), and 825 (m) cm^{-1}. λ_{max} (10 mM sodium borate buffer, pH 9.2), 230 nm, ϵ=19,600.

Synthesis of 2-Nitro-5-thiocyanobenzoic Acid. NTCB was synthesized according to the method of Degani & Patchornik (1971).

$^{22}Na^+$ Flux Assay. Membrane vesicles were suspended in BSA solution (10 mg ml^{-1}) and incubated for 15–20 min at 22°. The membrane vesicles were then pelleted by centrifugation at 40,000 *g* for 50 minutes. This treatment produces sealed membrane vesicles, probably by removal of free fatty acids resulting from hydrolysis of lipids during the extraction at pH 11. The pellets were resuspended in 50 mM sodium cacodylate buffer containing 100 mM NaCl adjusted to pH 6.5. The membranes were pelleted by centrifugation and resuspended in the the same buffer containing 15 μCi ml^{-1} of $^{22}Na^+$ and subjected to two cycles of freeze thaw to allow equilibration of $^{22}Na^+$ inside and outside of the vesicles. These vesicles were assayed for carbamylcholine and TPMA induced $^{22}Na^+$ efflux by the method of Wu & Raftery (1979). The $^{22}Na^+$ retained in the vesicles was determined at 10, 20, 30, and 40 sec after the addition of agonist and averaged.

UV Spectra of TPMA and the Product of Reduction. The wavelength of maximum absorption of TPMA is 230 nm and the extinction coefficient is 19,600 in 10 mM sodium borate buffer, pH 9.2. After reduction of 50 μM TPMA with 80 μM cysteine in 10 mM sodium borate buffer, pH 9.2, the λ_{max} and the extinction coefficient of the product (presumably the thiolate anion) are 275 nm and 16,700, respectively.

Determination of the Reaction Rates of NTCB and TPMA with Sulfhydryl Reagents. NTCB or TPMA was reacted with equimolar concentrations of cysteine or β-mercaptoethanol in 10 mM sodium borate buffer, pH 9.2. The NTCB reactions were monitored by the increase in absorbance at 412 nm and the TPMA reactions were monitored by the increase in absorbance at 275 nm. The second-

order reaction constants were determined by plotting the reciprocal of the product concentration against time. The slopes of the plot, which are equivalent to the second-order rate constants, were determined by a linear least squares analysis.

Cleavage Reactions. All cleavage reactions were performed in 100 mM sodium borate buffer (pH 9.2) in a final volume of 1.0 ml at 37° for 20 hr. Preliminary experiments revealed optimal cleavage occurred in about 20 hr. Except for the membrane vesicle suspension, all reagents were dissolved in 100 mM sodium borate buffer (pH 9.2). The stock membrane vesicles suspension never exceeded 20% of the cleavage reaction final volume. Stock solutions of 10 and 100 mM TPMA and NTCB dissolved in 100 mM sodium borate buffer (pH 9.2) were stored at -80°. After cleavage under conditions described in the figure legends, the membranes were centrifuged for 15 min in an Eppendorf centrifuge. The pellets were then dissolved in 100 μl of gel electrophoresis sample buffer with dithiothreitol (7.5 mg ml^{-1}). 25 μl of these samples were loaded on Laemmli gels.

Cyanylation at pH 8.0 in the presence of PTA. The cyanylation reactions at pH 8.0 were performed in 10 mM Hepes buffer containing 35 mM $NaNO_3$ and 0.02% NaN_3 in a final volume of 1 ml at 37° for 14 hr with conditions described in the figure legends. Stock solutions of TPMA and PTA were dissolved in the pH 8.0 buffer and stored at -80°. After centrifugation in an Eppendorf centrifuge for 15 min, the pellets were then resuspended in 1 ml of 100 mM sodium borate (pH 9.2) and incubated at 37° for 20 hr. Preparation of the samples for gel electrophoresis was done as already described.

Multiple Additions of TPMA. The cleavage reaction was performed in 100 mM sodium borate (pH 9.2) with 250 μM TPMA and 1 μM AChR in a final volume of 1ml for 21 hr. Two subsequent additions of an identical amount of TPMA (from a 10 mM stock solution) were added at 7 and 14 hr. Preparation of the samples for gel electrophoresis was done as already described.

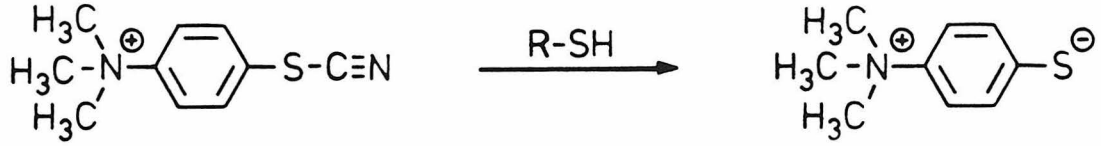
Gel Electrophoresis. $NaDodSO_4$-polyacrylamide gel electrophoresis was performed using the system of Laemmli (1970) with 12% polyacrylamide slab gels. These gels were stained with 0.5% Coomassie Blue R-250 in 10% acetic acid/25%

propanol/65% H_2O and destained in the same solvent without the dye. Gels were scanned using a LKB Ultroscan XL Laser Densitometer. Heights of the peaks were used to calculate a relative cleavage value. The smallest peak of a gel was set equal to 0 and the largest peak was set equal to 1.0.

RESULTS

The synthesis of TPMA is shown in Figure 1. The IR spectrum exhibits a sharp absorption band at 2155 cm^{-1} (Figure 2). A sharp and medium intensity band between 2170–2130 cm^{-1} is characteristic of an aryl thiocyanate, whereas an aryl isothiocyanate has a broad and strong band between 2105–2060 (Ben-Efraim, 1977). The ^1H NMR spectrum is shown in Figure 3.

Upon reaction with cysteine, a new absorbance peak appears (λ_{max} = 275 nm), which is presumably the absorbance peak of the thiolate (Figure 4). This reaction of TPMA (I) with sulfhydryl reagents presumably generates the thiolate ion (II). The second-order reactions of TPMA and NTCB with cysteine have



I II

rate constants of 21 mM^{-1} min^{-1} and 14 mM^{-1} min^{-1}, respectively (Figure 5). The reactions of TPMA and NTCB with β-mercaptoethanol have second-order rate constants of 23 mM^{-1} min^{-1} and 15 mM^{-1} min^{-1}, respectively (Figure 6). Although the thiolate leaving group of NTCB is resonance stabilized by the para nitro group, the thiolate leaving group of the TPMA reaction is probably stabilized by an electron inductive effect of the quarternary ammonium group. As reactivities of the two compounds with sulfhydryl reagents are almost equivalent (the rate constant for TPMA is about 1.5 fold greater than the rate constant for

FIGURE 1: Synthesis of TPMA.

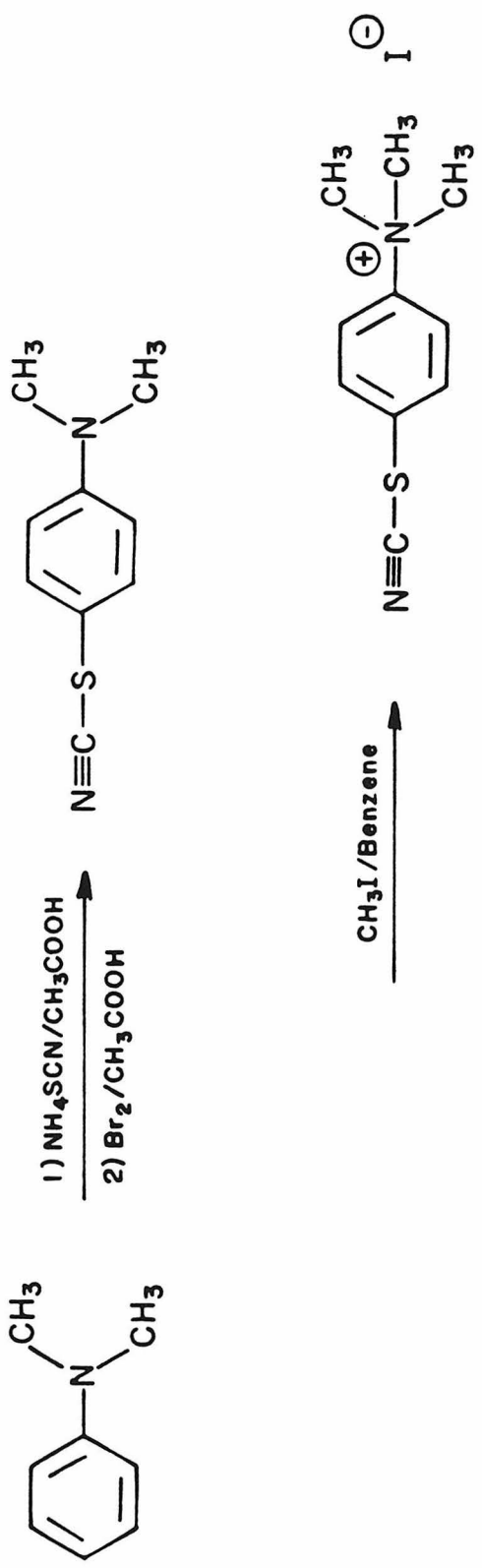

1) NH4SCN/CH3COOH
2) Br2/CH3COOH
CH3I/Benzene
I⊖

FIGURE 2: FT-IR spectrum of TPMA (KBr pellet).

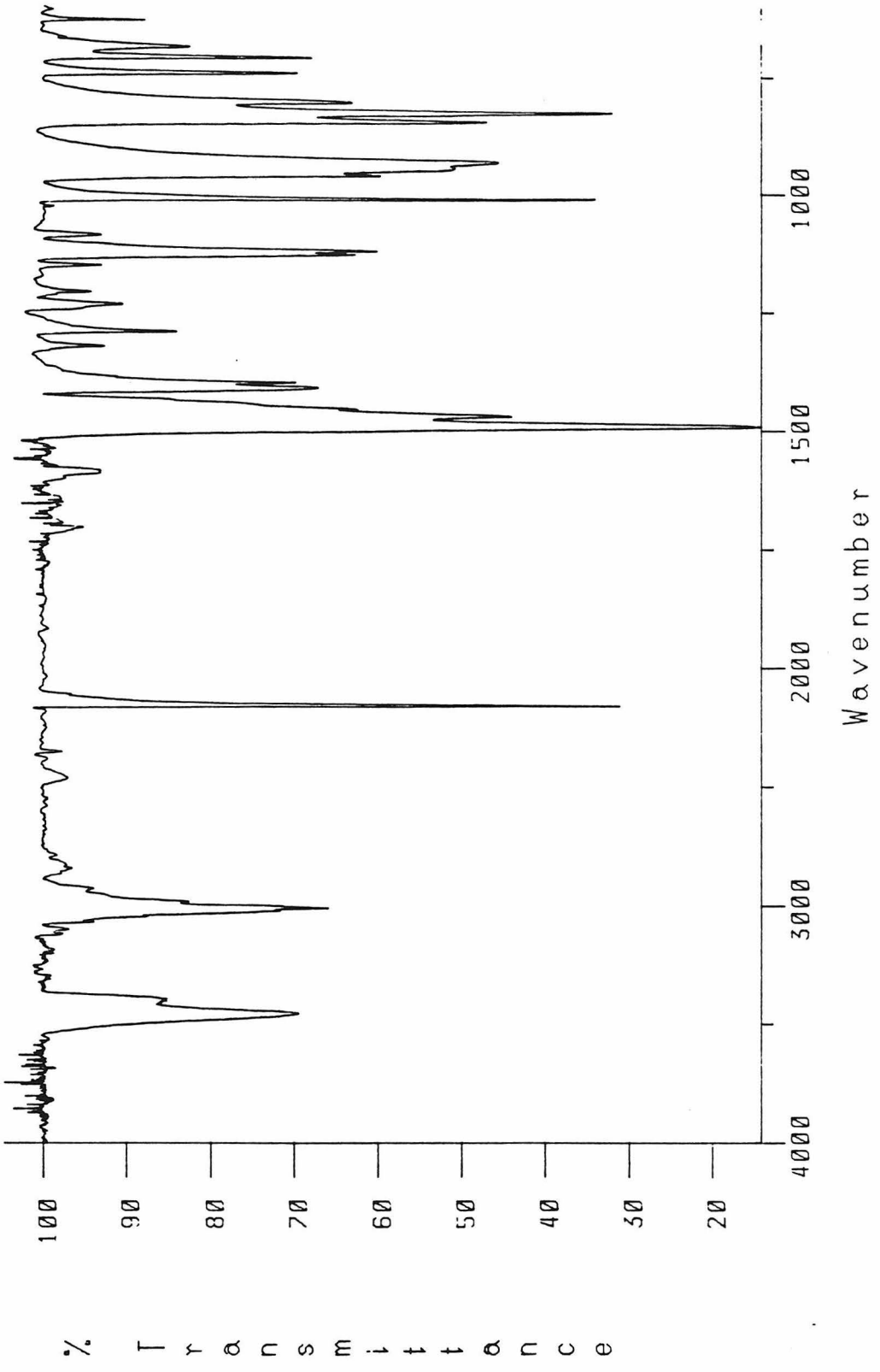

% Transmittance
100
90
80
70
60
50
40
30
20
4000
3000
2000
1500
1000
Wavenumber

FIGURE 3: ^{1}H NMR spectrum of TPMA in $[^{2}H]H_2O$.

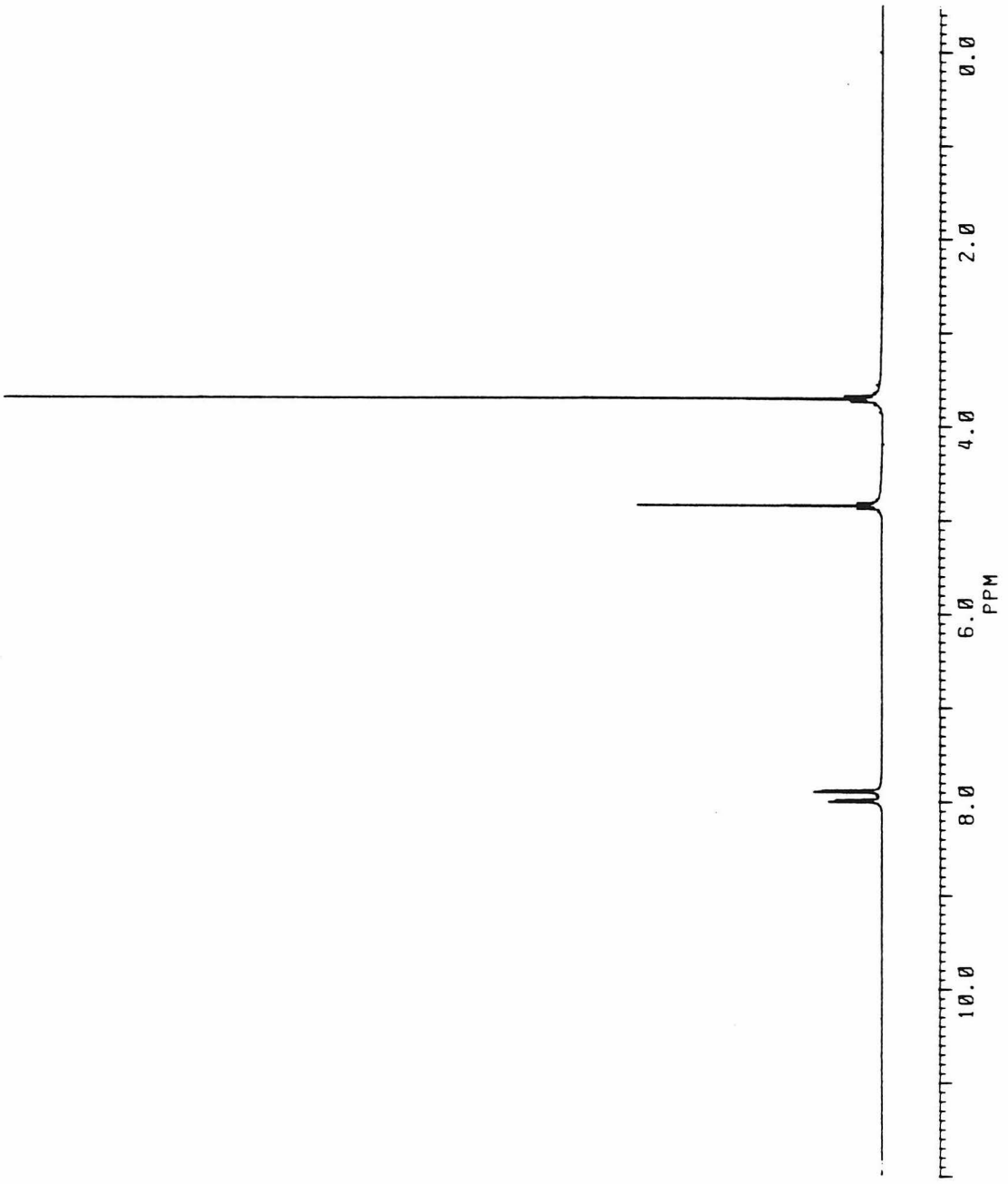

10.0
8.0
6.0
4.0
2.0
0.0
PPM

FIGURE 4: The UV spectrum of TPMA before (top) and after (bottom) reaction with cysteine as described in Materials and Methods.

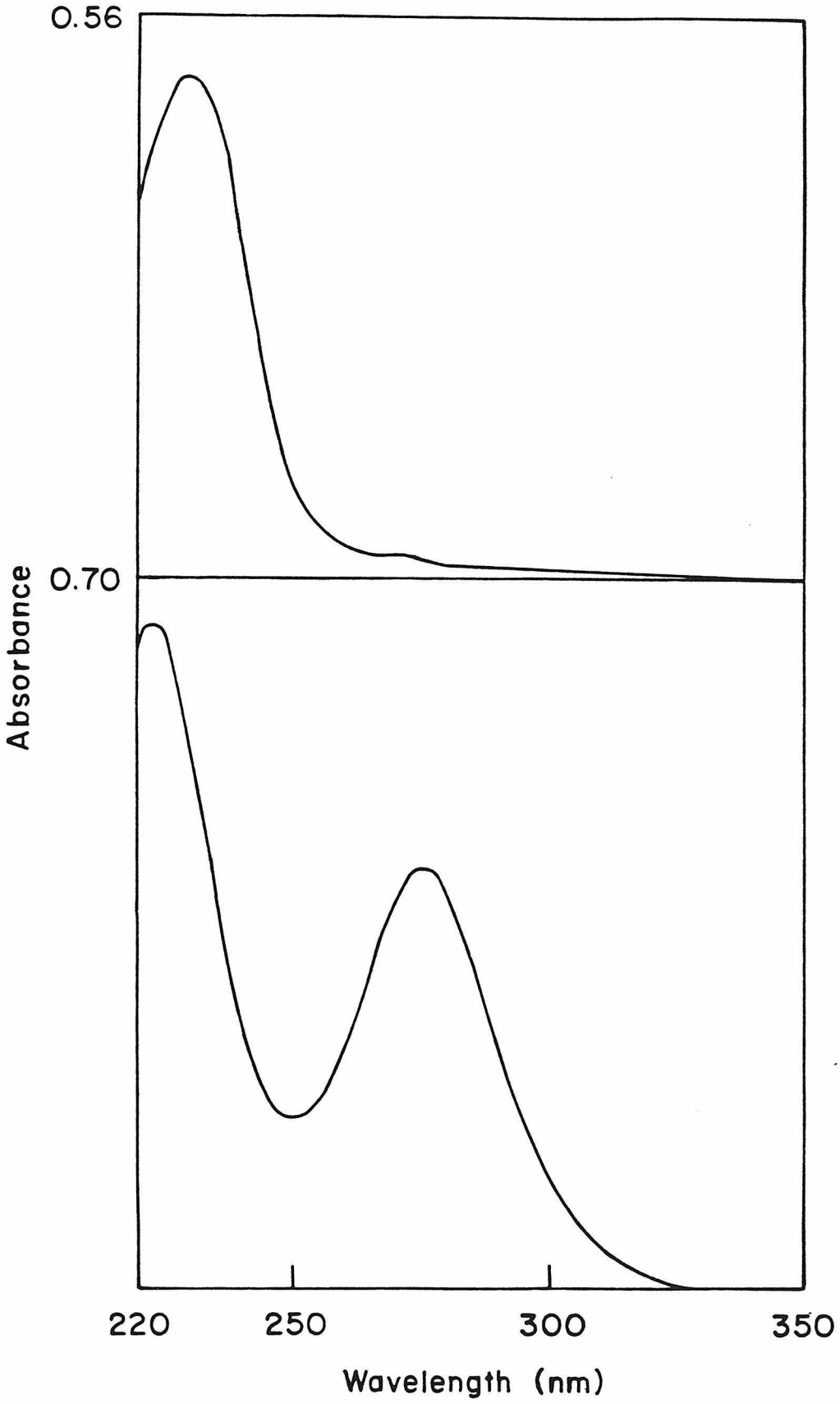

0.56
0.70
Absorbance
220
250
300
350
Wavelength (nm)

FIGURE 5: Reaction of equimolar concentrations (17 μM) of TPMA and cysteine (•) and NTCB and cysteine (■).

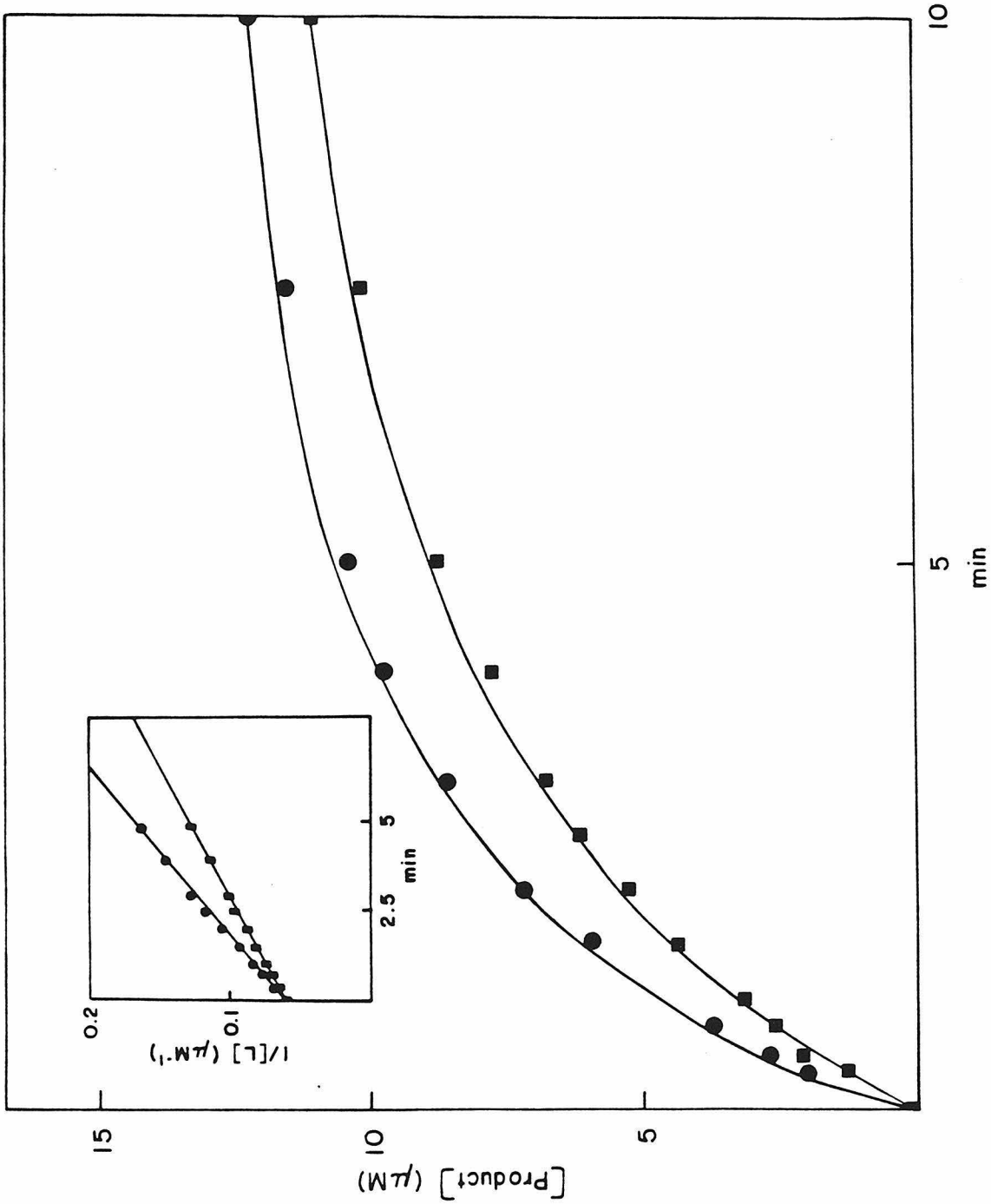
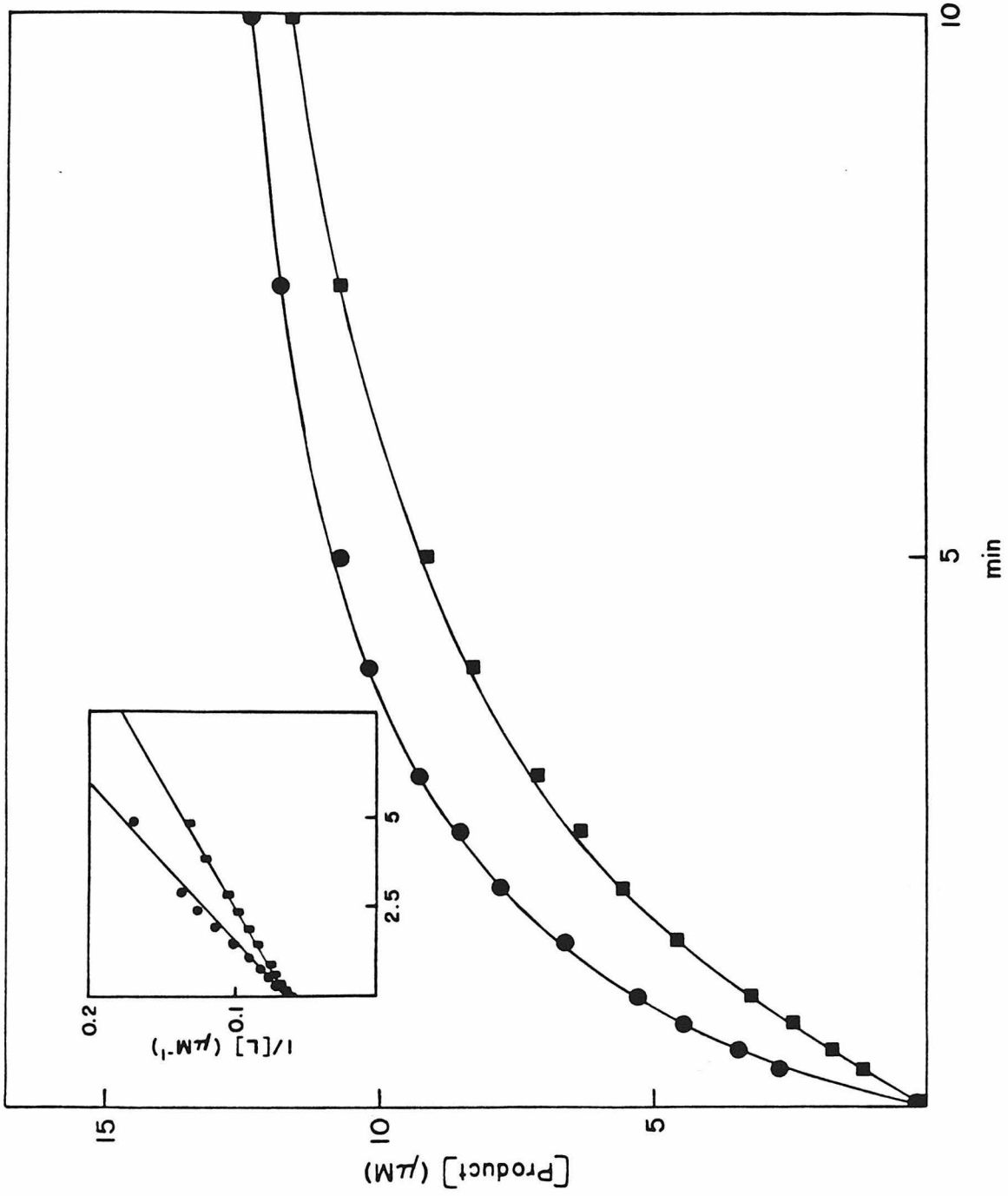

[Product] (μM)
15
10
5
5
min
10
1/[L] (μM⁻¹)
0.2
0.1
2.5
5
min

FIGURE 6: Reaction of equimolar concentrations (17 μM) of TPMA and β-mercaptoethanol (•) and NTCB and β-mercaptoethanol (■).


[Product] (μM)
15
10
5
5
10
min
1/[L] (μM⁻¹)
0.2
0.1
2.5
5

NTCB), TPMA should cyanylate cysteine residues as efficiently as NTCB.

TPMA induces cation flux in AChR enriched vesicles and this flux is antagonized by α-bungarotoxin (Figure 7). These flux experiments demonstrate that TPMA is an agonist for the AChR.

The reaction of TPMA with AChR yields two products with M_r of 26K and 22K (Figure 8). The concentration dependence of the cleavage was studied for both TPMA and NTCB, in order to contrast the affinity reagent (TPMA) to a non-affintiy reagent (NTCB) of similar chemical reactivity (Figures 9 and 10). The relative yields of cleavage product were determined by scanning the gels (Figure 11). The concentrations where 50% cleavage occurs for TPMA and NTCB are about 150 μM and 1.5 mM, respectively. As there is an order of magnitude difference in the concentration dependence of the cleavage reaction, it is likely TPMA binds specifically to and subsequently cleaves the AChR. The concentration dependence of the cleavage by TPMA is indicative of its binding to a site for agonists with relatively low affinity. Conversely, the equilibrium dissociation constant for the binding sites (high affinity) on the 40K subunits are in the range of 1 nM to 100 nM for agonists under equilibrium conditions.

The cleavage of AChR by TPMA is blocked by stoichiometric quantities of α-bungarotoxin with α-bungarotoxin binding sites (Figure 12). The formation of a product with M_r of about 68K is correlated with the blockage of cleavage. This is most likely to be a crosslinked product between the 8K dalton α-bungarotoxin and the 60k subunit. This result suggests the site of cleavage is contained in the 60K subunit. The yield of the 26K and 22K dalton products of the cleavage reaction are reduced by inclusion of the agonist, phenyltrimethylammonium iodide (PTA) (Figure 13). The cleavage reaction involves two consecutive steps, cyanylation of the protein followed by cleavage of the peptide bond. As cleavage is probably the rate limiting step and PTA may only compete with cyanylation, the competition experiment must be done in a time range before the cyanylation goes to completion. Difficulty arises in generating sufficient cleavage in a short enough time for blockage of the cyanylation reaction by agonist. In order to eliminate this

FIGURE 7: Agonist-induced flux of TPMA. $^{22}Na^{+}$-loaded vesicles were assayed for $^{22}Na^{+}$ retained in vesicles as a function of time after mixing with agonist by the method of Wu & Raftery (1979). 0 μM agonist (A), 100 μM TPMA (B), 1 mM TPMA (C), 100 μM carbamylcholine (D), 0.4% Triton X–100 (E), excess α-BgTx over α-BgTx sites and 1 mM TPMA (F), α-bungarotoxin and 100 μM carbamylcholine (G).

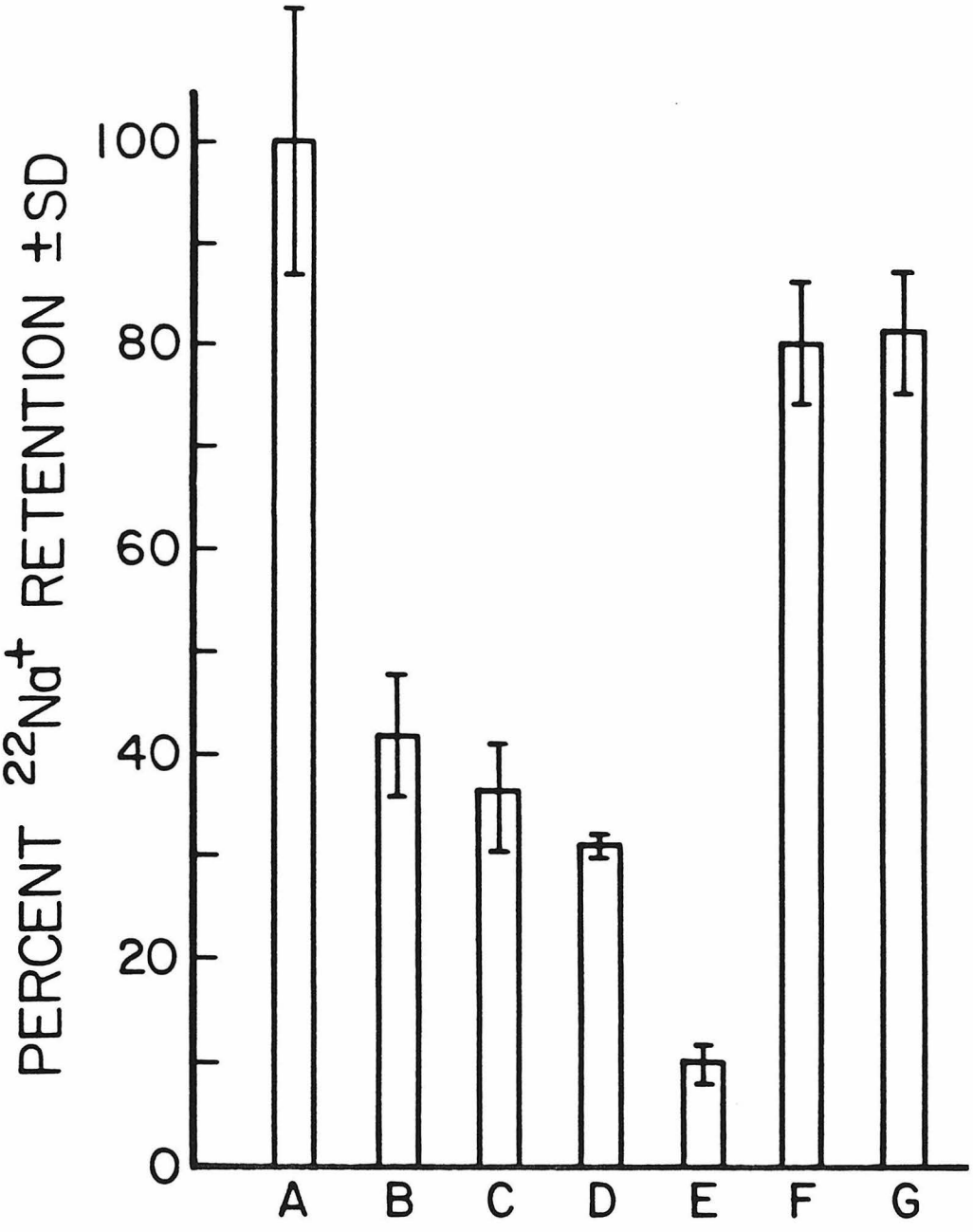

PERCENT $^{22}Na^{+}$ RETENTION ±SD
100
80
60
40
20
0
A
B
C
D
E
F
G

FIGURE 8: The reaction of TPMA with 1 μM AChR. Lane 1, 0 μM TPMA; lane 2, 250 μM TPMA.

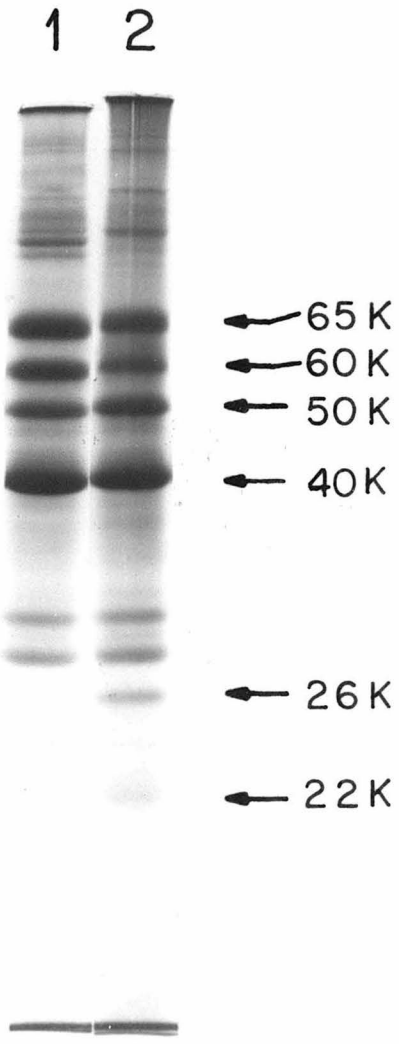

1
2
65K
60K
50K
40K
26K
22K

FIGURE 9: The TPMA concentration dependence of cleavage of 1 μM AChR. Lanes 1–12; 0, 10, 50, 100, 150, 200, 250, 300, 400, 600, 1000, and 2000 μM TPMA, respectively.

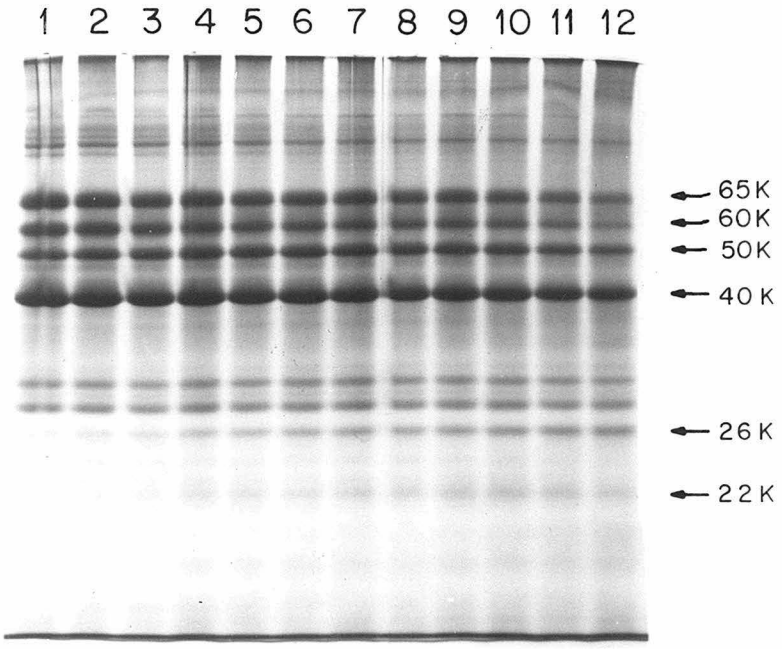

1 2 3 4 5 6 7 8 9 10 11 12
65K
60K
50K
40K
26K
22K

FIGURE 10: The NTCB concentration dependence of cleavage of 1 μM AChR. Lanes 1–12; 0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0, 3.0, 5.0, and 7.5 mM NTCB respectively.

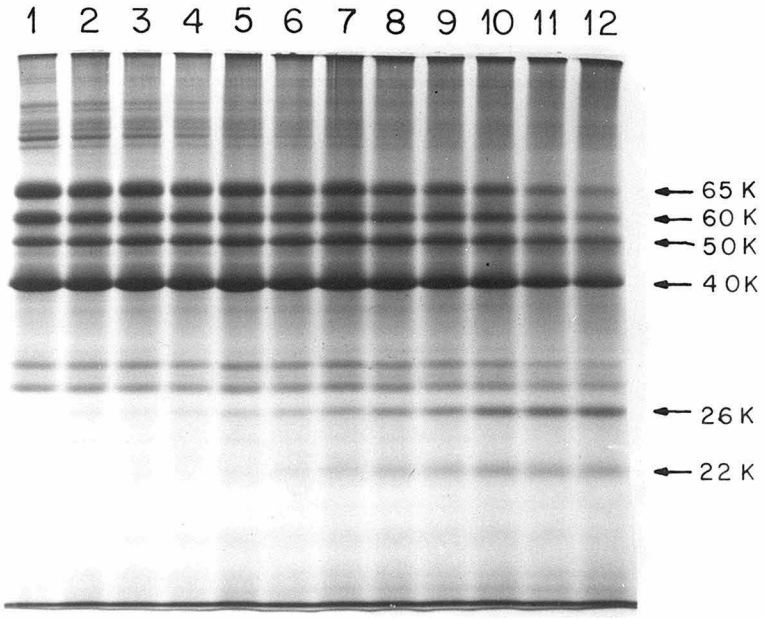

1 2 3 4 5 6 7 8 9 10 11 12
65 K
60 K
50 K
40 K
26 K
22 K

FIGURE 11: The relative yield of the 28K dalton product is plotted against the concentration of either TPMA (•) or NTCB (■). Data was gathered by scanning the gels shown in Figures 9 and 10.

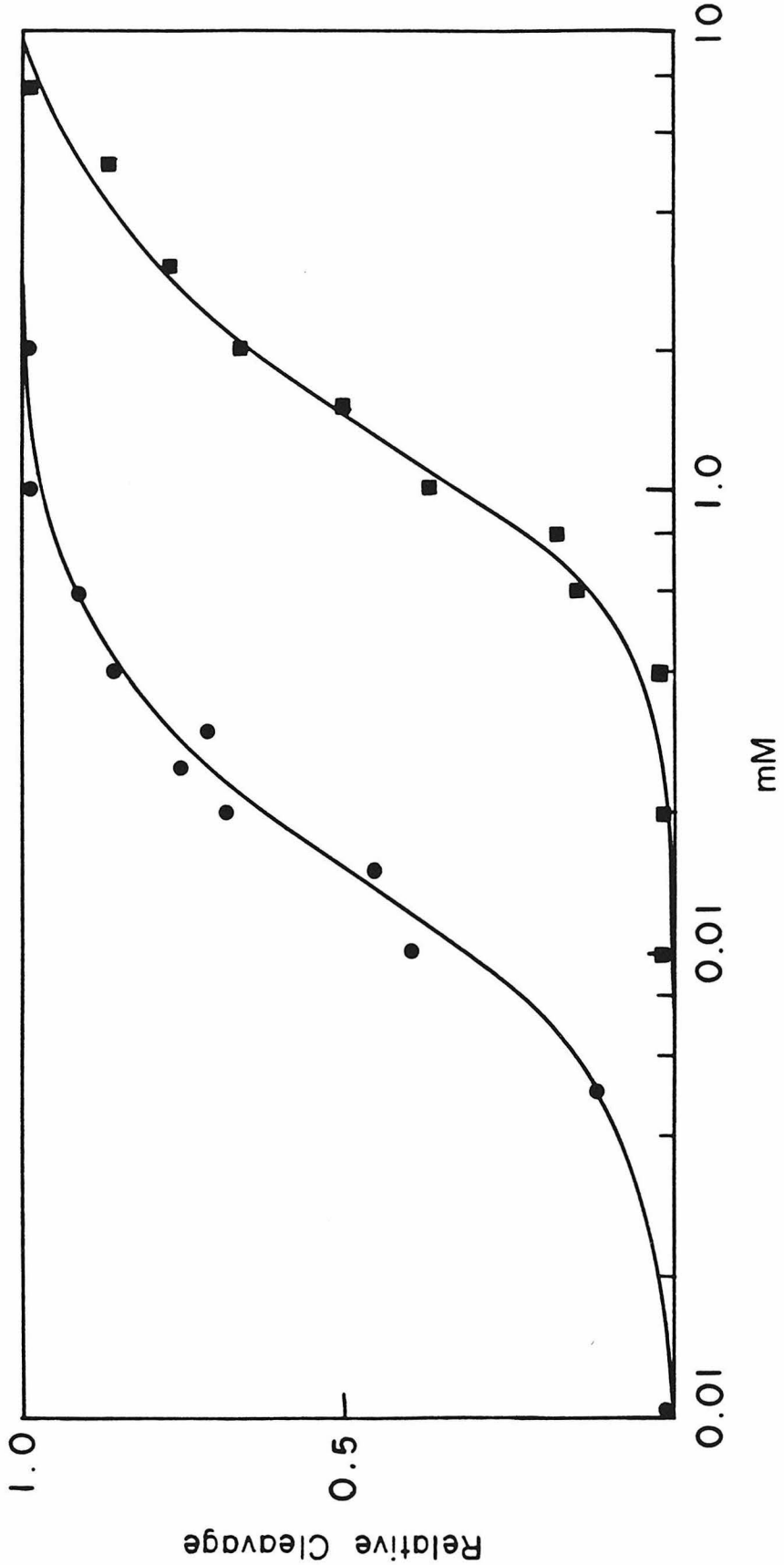

Relative Cleavage
1.0
0.5
0.01
0.01
1.0
10
mM

FIGURE 12: Blockage of the reaction of 250 μM TPMA and 0.5 μM AChR with stoichiometric quantities of α-bungarotoxin. Lanes 1–6; 0, 0.25, 0.50, 0.75, 1.0, and 2.0 μM α-bungarotoxin.

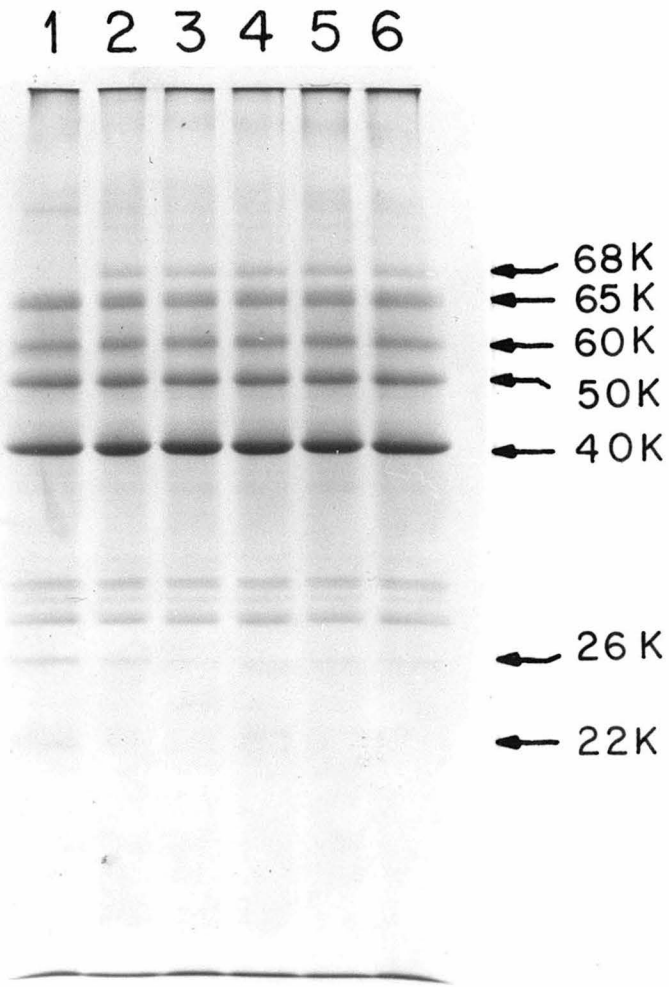
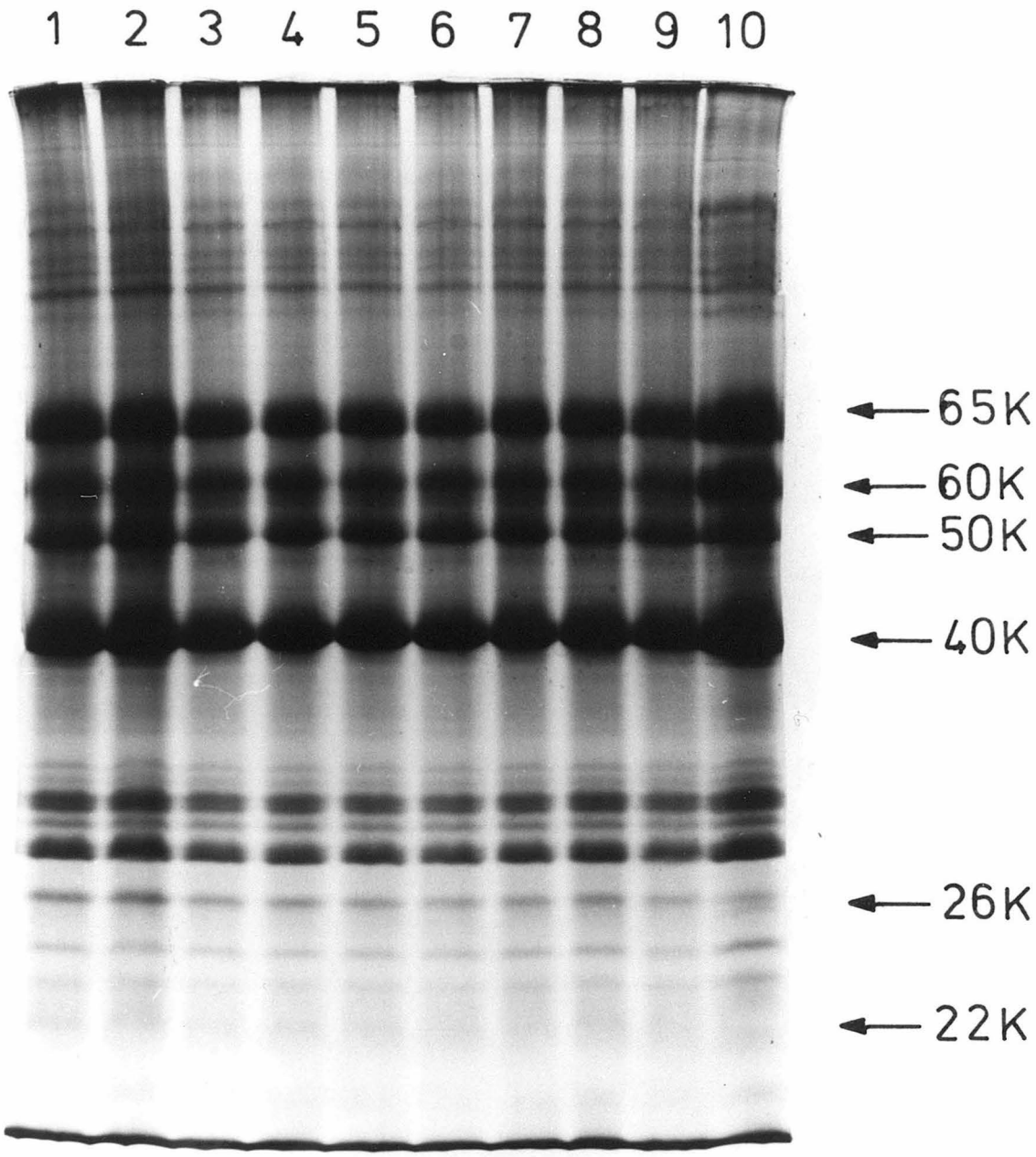

1 2 3 4 5 6
68K
65K
60K
50K
40K
26K
22K

FIGURE 13: Decrease in the yield of the cleavage reaction of 250 μM TPMA with 1.0 μM AChR by the addition of PTA. Lanes 1–9; 0, 0.5, 1.0, 2.0, 4.0, 5.0, 6.0, 7.0, and 8.0 mM PTA. Lane 10 is a control lacking TPMA and TPA, which indicates the background cleavage in the absence of TPMA.


1 2 3 4 5 6 7 8 9 10
65K
60K
50K
40K
26K
22K

problem, the cyanylation step was performed in the presence of agonist, followed by centrifugation to remove the TPMA and PTA. Then the pellets were taken up in the cleavage buffer to effect cleavage. Inclusion of PTA during the cyanylation reaction reduces the yield of the cleavage (Figure 14). Separation of the cleavage into two steps should prove useful in further investigations on the kinetics of the cyanylation. As α-bungarotoxin is a competitive antagonist and PTA is an agonist, it is probable the cleavage reaction is taking place at an agonist binding site.

The 60k subunit is degraded most rapidly and to a greater extent than the other subunits. Since TPMA is not stable over the entire time course of the reaction, multiple additions of TPMA were used to increase the yield of the reaction (Figure 15 and 16). As the 60K subunit is degraded extensively, this indicates the site of cleavage is most likely in the 60K subunit. It is probable that there is a low-affinity binding site for agonists on the 60K subunit.

DISCUSSION

An affinity cleavage reagent requires the linking of a peptide bond cleaving functionality with a ligand that binds to a specific site. As PTA is an agonist for the AChR, it is not surprising that TPMA is also an agonist. In addition, the stabilization of the thiolate leaving group by the positively charged quarternary ammonium group allows the reagent to efficiently cyanylate proteins.

A model depicting the interaction of the cationic TPMA with an anionic agonist binding site is shown in Figure 17. The likely candidates for the site of cleavage in the 60K subunit are cysteine residues 230, 252, and 301 based on the M_r of the cleavage products [residues are numbered according to Noda et al. (1983b)]. Cysteine residue 252 is unique to the 60K subunit, whereas cysteine residue 301 is conserved in the 60K and 65K subunits. Cysteine residue 230 is conserved in all four subunits. Preliminary experiments on the rate of cyanylation of the AChR receptor by TPMA indicate the reaction is significantly slower than

FIGURE 14: Decrease in the yield of the cleavage reaction of 500 μM TPMA with 1.0 μM AChR by the addition of PTA at pH 8.0 as described in Materials and Methods. Lanes 1–5; 0, 0.1, 1.0, 5.0, and 10.0 mM PTA.

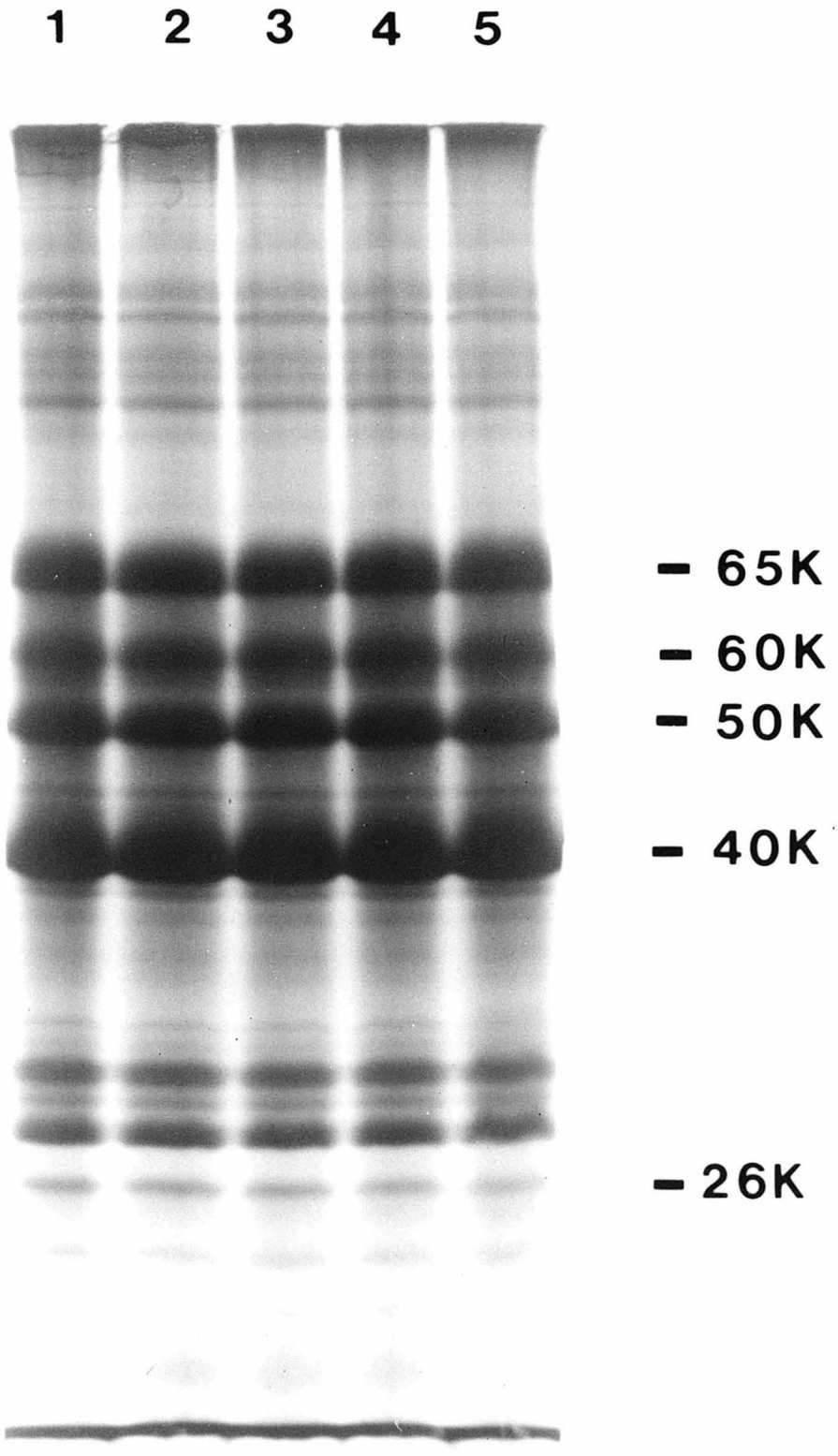

1
2
3
4
5
65K
60K
50K
40K
26K

FIGURE 15: Multiple additions of TPMA to increase the yield of the cleavage reaction. Conditions are described in Materials and Methods section. Lanes 1, No TPMA added; Lane 2, multiple additions of TPMA.

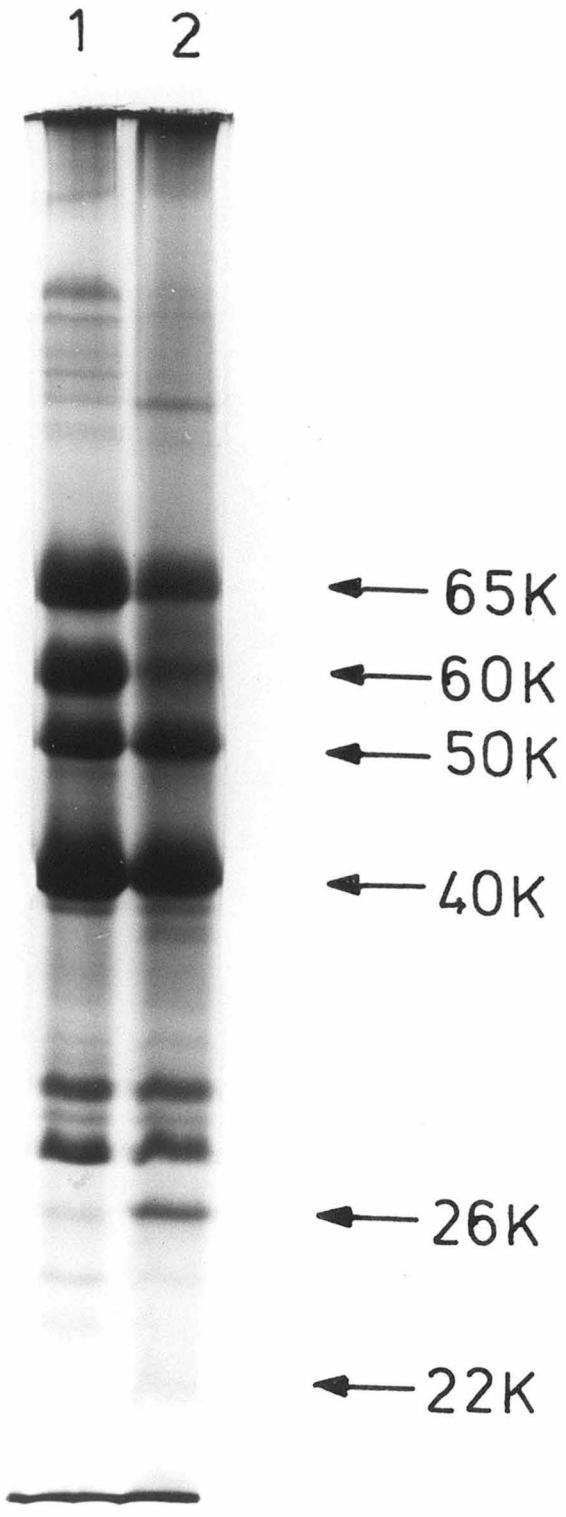

1
2
65K
60K
50K
40K
26K
22K

FIGURE 16: A gel scan of the reaction of AChR with multiple additions of TPMA. Conditions are described in Materials and Methods section. No TPMA added (a) and multiple additions of TPMA (b).

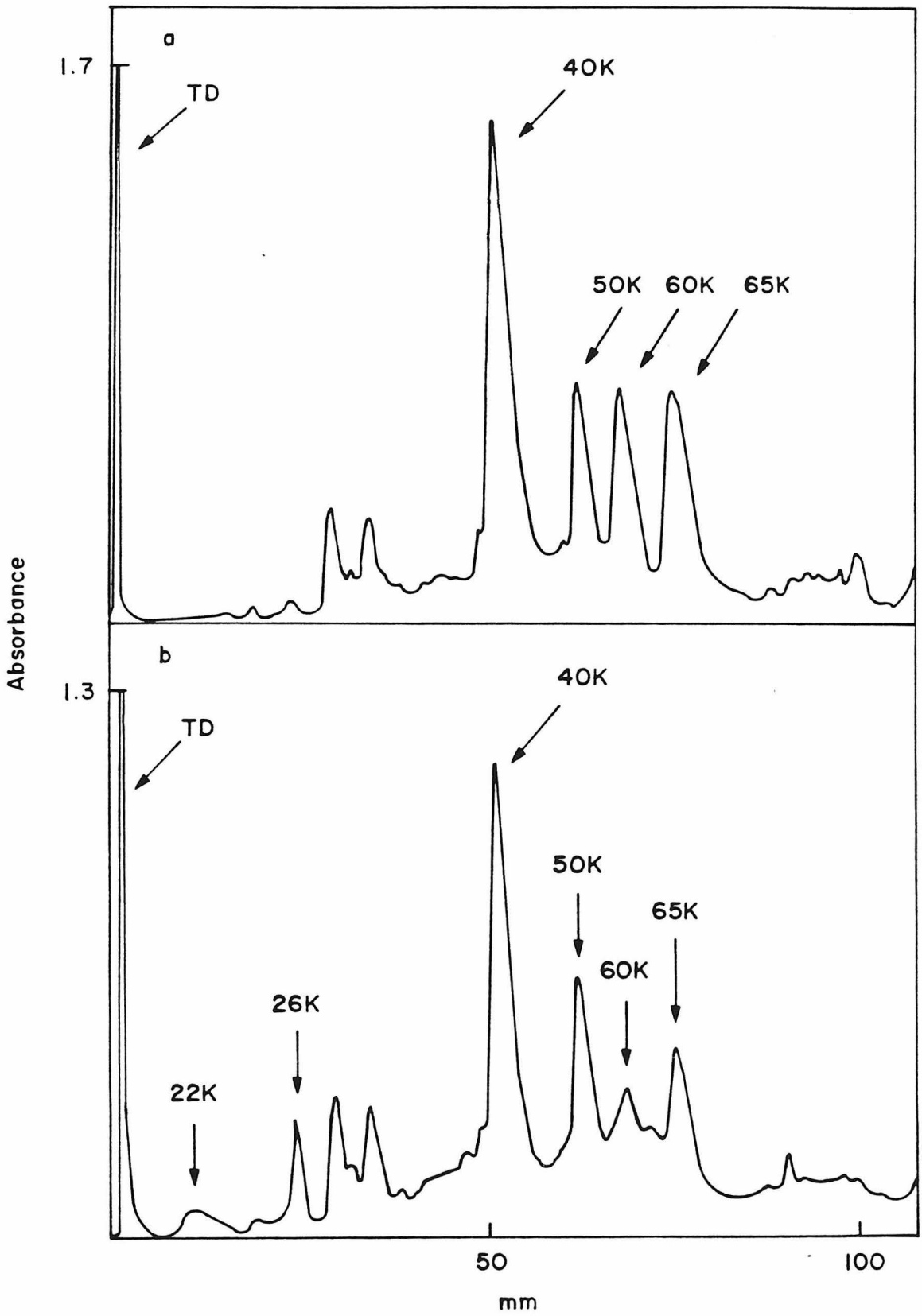
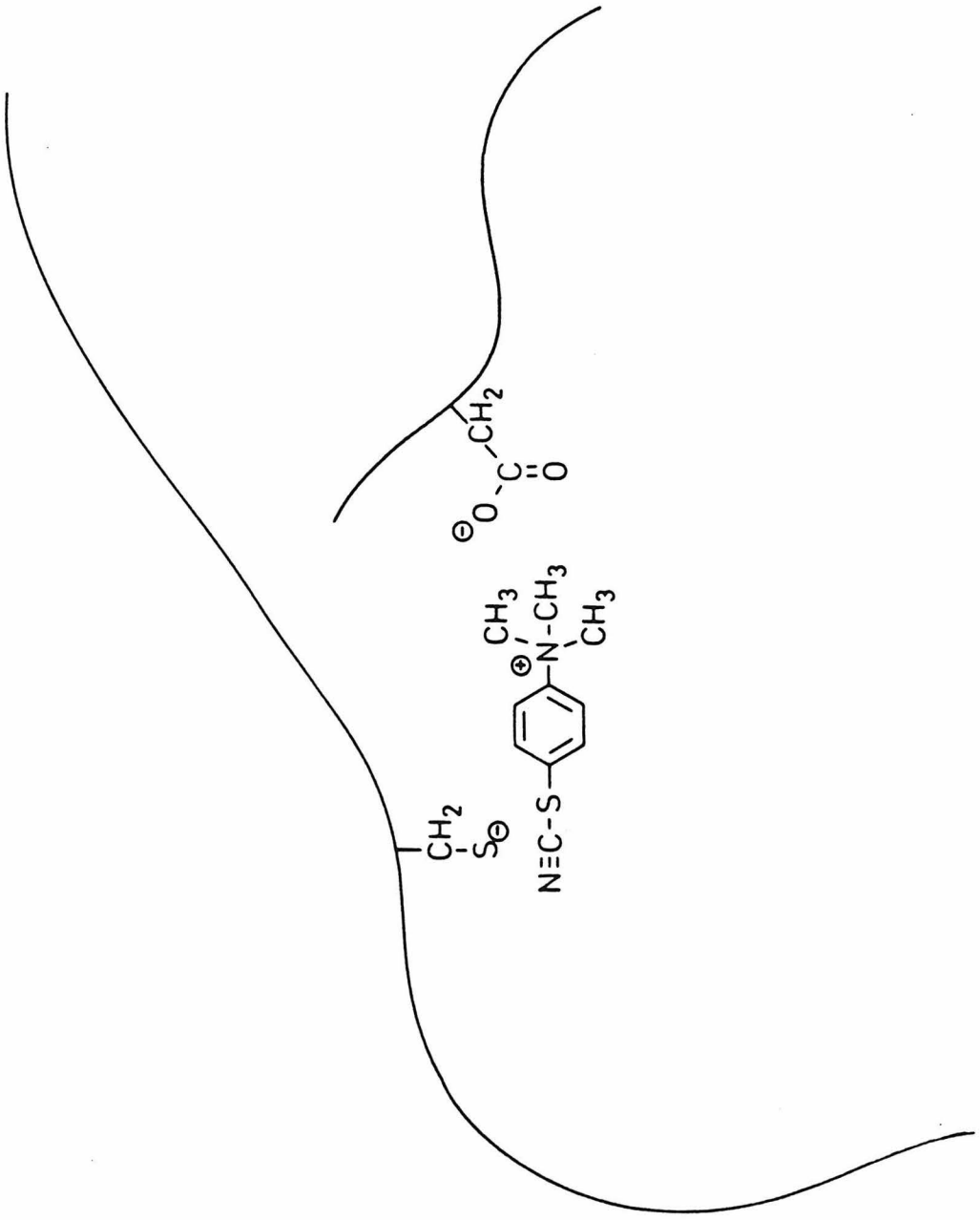

a
1.7
TD
40K
50K
60K
65K
b
1.3
TD
40K
50K
65K
60K
26K
22K
Absorbance
50
100
mm

FIGURE 17: A model depicting the interaction of the cationic TPMA with an anionic binding site.



the reaction with free cysteine. This reaction is not blocked by the alkylation of the AChR receptor with iodoacetamide during the preparation. Therefore, the cysteine residue that reacts with TPMA is not exposed to the aqueous phase. It is either buried in a hydrophobic pocket of the protein or the lipid bilayer, either of which could be near or contribute to an agonist binding site.

Labeling of the *Torpedo californica* AChR with N-phenylmaleimide inhibits the ion-channel activity of the AChR (Yee et al. 1986). As treatment with iodoacetamide was also included in the preparation of the AChR, this site is not exposed to the aqueous phase. The 60K (γ) subunit incorporated the most label during the reaction with [^{3}H]N-phenylmaleimide. This preferential labeling of the 60K subunit with a hydrophobic sulfhydryl labeling reagent may be the same site at which TPMA is reacting.

Although the affinity labeling studies with BrACh and MBTA have conclusively demonstrated the existence of agonist binding sites on the 40K subunit, the dependence of labeling on the presence of an easily reducible disulfide bond may not allow labeling of all agonist binding sites. Kao & Karlin (1986) have demonstrated cysteine residues 192 and 193 in the 40K subunit form a disulfide bond which, after reduction, are the residues specifically labeled by MBTA. Witzemann and Raftery (1977), using the more general photoaffinity label, DAPA, found both the 40K and the 60K subunits were labeled. This labeling was specifically blocked by both α-bungarotoxin and DAP. Our results are consistent with this earlier finding.

Mishina et al. (1986), by expression of AChR in a Xenopus oocyte system, have found that receptors with different conductance and gating properties result when oocytes are injected with subunit-specific mRNA for the α, β, γ, and δ subunits or the α, β, γ, and ϵ subunits of calf. These results indicate the γ subunit (in calf) is involved in determining gating and conductance properties. The possibility arises that a low-affinity agonist binding site on the 60K subunit may also be involved in gating properties of the AChR. The simplest explanation is the occupation of this site may be linked to channel opening.

The finding by Catsimpoolas & Wood (1966) that scission of a disulfide bond by cyanide yields a sulfhydryl and a thiocyano group, which subsequently effects cleavage of the peptide bond, prompted the development of a reagent which could cyanylate cysteine residues in high yield. NTCB cyanylates cysteine residues in high yield, since the resonance stabilized thiolate is a good leaving group (Figure 18). A mechanism for the cleavage of the peptide bond, which is catalyzed by hydroxide ion, has been proposed by Jacobsen et al. (1973) (Figure 19).

The demonstration of sequence homology between all four of the AChR subunits (Raftery et al. 1980; Noda et al., 1982, 1983a,b) supports the notion that multiple agonist binding sites exist. Evidence for sites other than those labeled by the affinity reagents, BrACh and MBTA, has been aquired by monitoring fluorescent changes of a covalently bound probe (Dunn & Raftery, 1982). Specific labeling of both the 40K and 60K subunits by the photoaffinity probe, DAPA, indicates there may be a binding site on the 60K subunit. In this communication, the cleavage of a peptide bond by an affinity cleavage reagent provides evidence for a low-affinity binding site on the 60K subunit.

FIGURE 18: Cyanylation and subsequent cleavage of a protein with NTCB or TPMA.

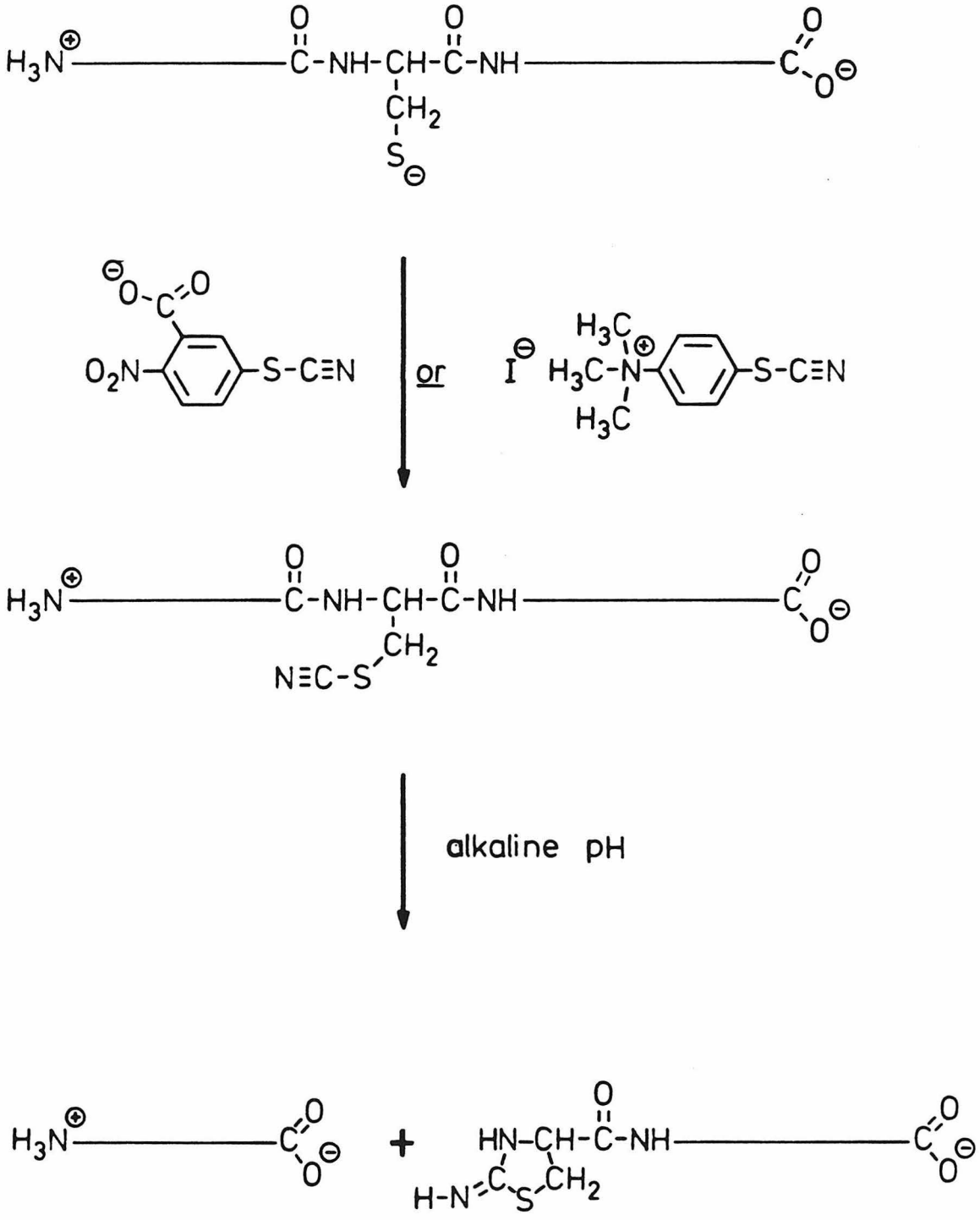
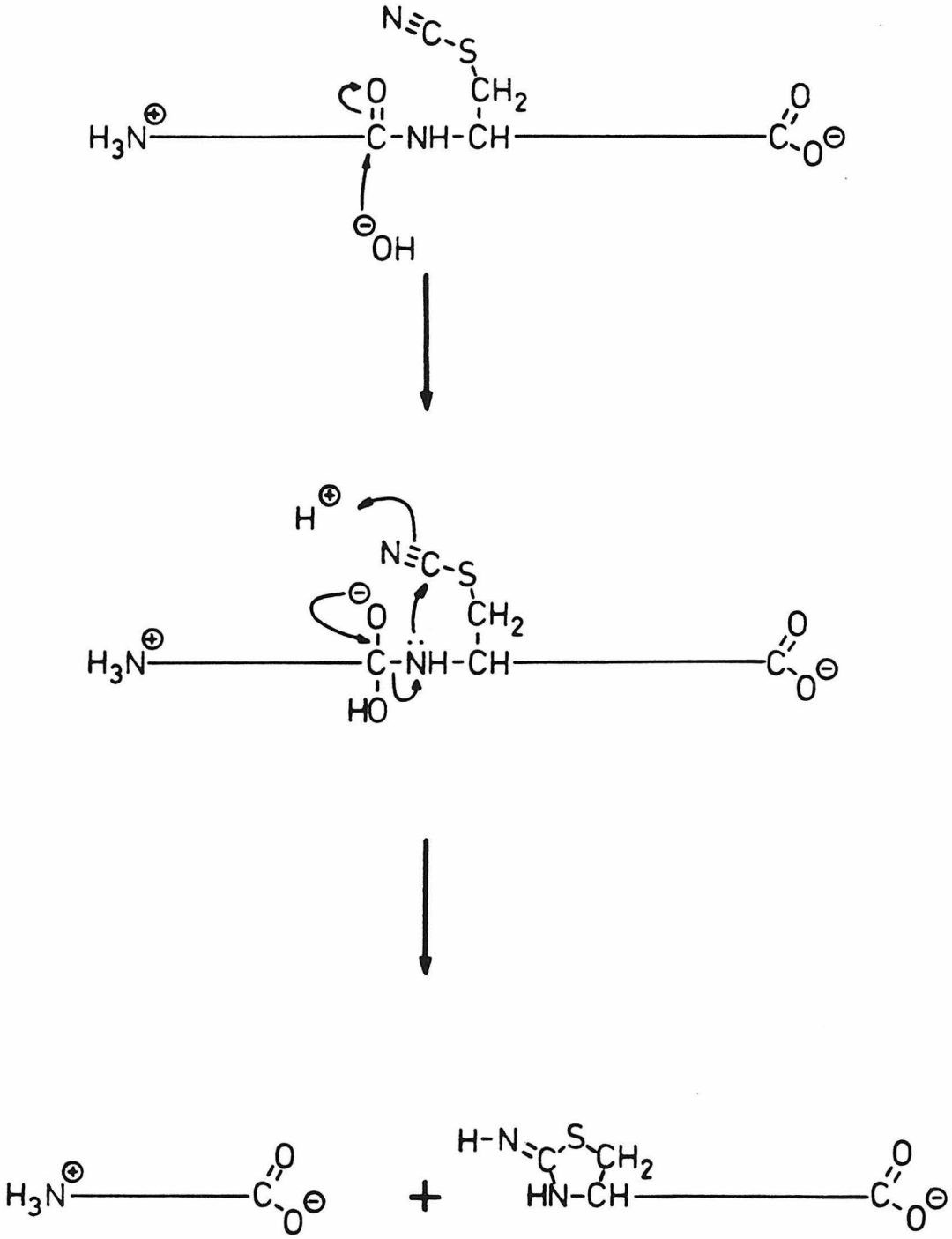

or
alkaline pH

FIGURE 19: A mechanism for the cleavage reaction proposed by Vanaman & Stark (1970).



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