ISOLATION, PURIFICATION AND EFFECT

OF LIGANDS

ON THE NICOTINIC CHOLINERGIC RECEPTOR

by

EUGENE ANTHONY KAPP

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ABSTRACT

The nicotinic cholinergic receptor protein of the fish electric organ, *Torpedo fuscomaculata*, has been isolated, purified and shown to represent a true model for the nAChR from other species and higher vertebrates. It is an integral membrane protein composed of four different subunits, tightly associated with other functional, but non-specific proteins.

Purification of the nicotinic cholinergic receptor by chromatofocusing demonstrates an improved method over that of affinity and ion-exchange chromatography. Gel chromatography and SDS-polyacrylamide gel electrophoresis show evidence of four subunits; α(40-44 kDa), β(53 kDa),γ(63 kDa) and δ(66 kDa) despite some degradation of receptor molecules by intracellular proteases.

Spectrophotometric and fluorimetric studies of receptor-ligand interactions, show the functional and chemical integrity of the receptor to remain intact after solubilisation. The effect of cholinergic ligands on purified receptor preparations indicate quenching of the intrinsic fluorescence of the receptor. Agonists, like acetylcholine, bind and cause local conformational transitions, changing the active region from a hydrophobic to a hydrophilic environment. This phenomenon is illustrated by the 10-fold increase in fluorescence when the receptor is in a desensitised state. Antagonists, such as d-Tubocurarine, block this conformational transition.

In vitro rectus abdominis muscle preparations show the nitrosamines, dimethylnitrosamine and diphenylnitrosamine, to be true agonists of the nAChR. However their low affinity and specificity for the receptor precludes them as photoaffinity labelling agents. Photoactivation of dimethylnitrosamine occurs when associated with an acidic hydrogen at the active site of the receptor, suggesting energy-transfer labelling to be more facile than photoaffinity labelling. The membrane-bound receptor, in the presence of these nitrosamines, undergoes conformational transitions regulating the opening and closing of the ion-channel. Desensitisation and receptor activation are shown to involve one and the same molecular transition.

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CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

In 1906, Langley concluded his experiments on the effects of nicotine on muscle contraction in the fowl by postulating that a "receptive substance ... combines with nicotine and curari and is not identical with the substance which contracts". Sixty-five years later, the nicotinic receptor for acetylcholine was isolated as the first in an ever-increasing list of receptors for neurotransmitters (1). The wealth of biochemical information accumulated over the past fifteen years makes the nAChR the best known neurotransmitter receptor and also the most thoroughly investigated membrane protein (2, 3).

Nicotinic cholinergic receptors (nAChR) mediate chemical communication at synapses in many parts of the nervous system of vertebrates, including neuromuscular junctions; autonomic ganglia and certain sites of the brain (2). The receptor recognises and binds specifically the neurotransmitter acetylcholine, released from nerve endings in the presynaptic membrane and converts the chemical signal, acetylcholine, into an electrical effect by allowing an influx of cations through the ion-channel. This is also known as depolarisation of the post-synaptic membrane (3).

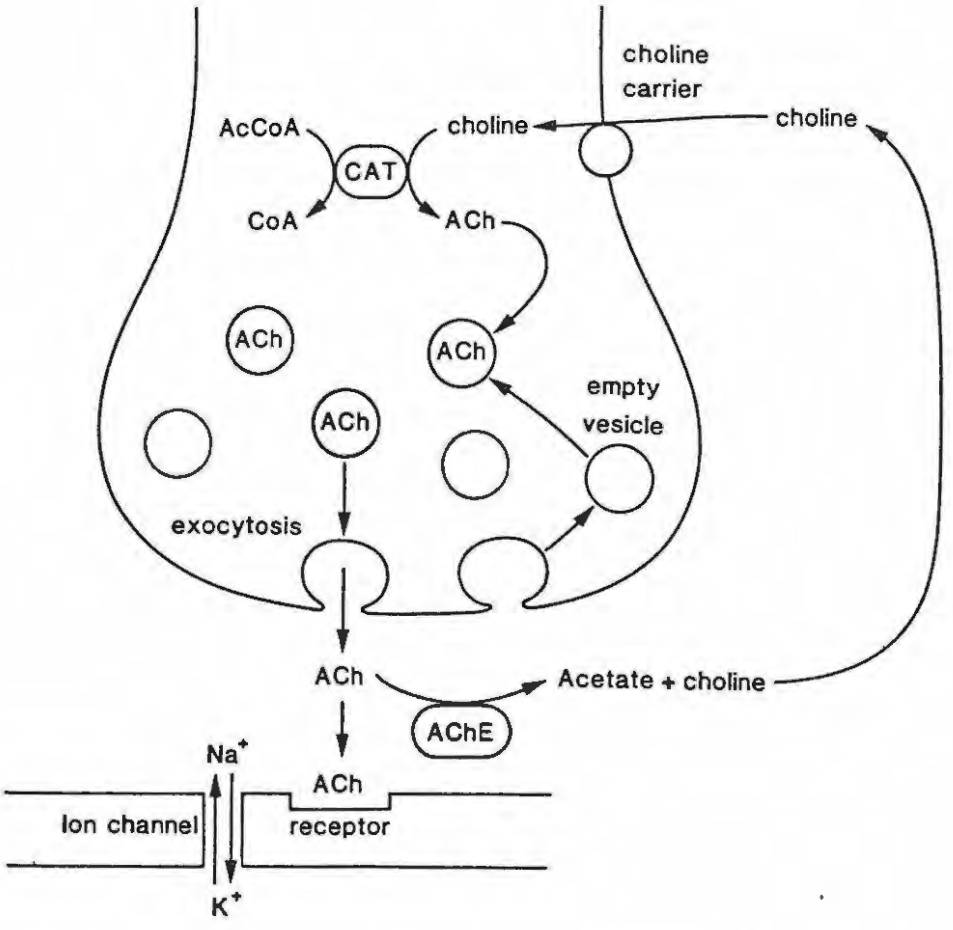


Figure 1.1 Events at a cholinergic synapse: Acetylcholine shown acting post-synaptically on a nicotinic receptor controlling a cation channel (4).

CAT	-	Choline acetyltransferase
AcCoA	-	Acetyl CoA
ACh	-	Acetylcholine
AChE	-	Acetylcholinesterase

1.2 ISOLATION, PURIFICATION AND ASSAY OF THE NICOTINIC CHOLINERGIC RECEPTOR PROTEIN

1.2.1 Isolation

Marine elasmobranchs such as the *Torpedo* ray and the electric eel, *Electrophorus electricus*, provide rich sources of the nicotinic cholinergic receptor protein for studying structural and functional aspects of receptor proteins (2).

The electric tissue of the ray is derived from skeletal muscle in which the original contractile properties have been lost and the capacity to generate electric potentials has been greatly developed (5, 6).

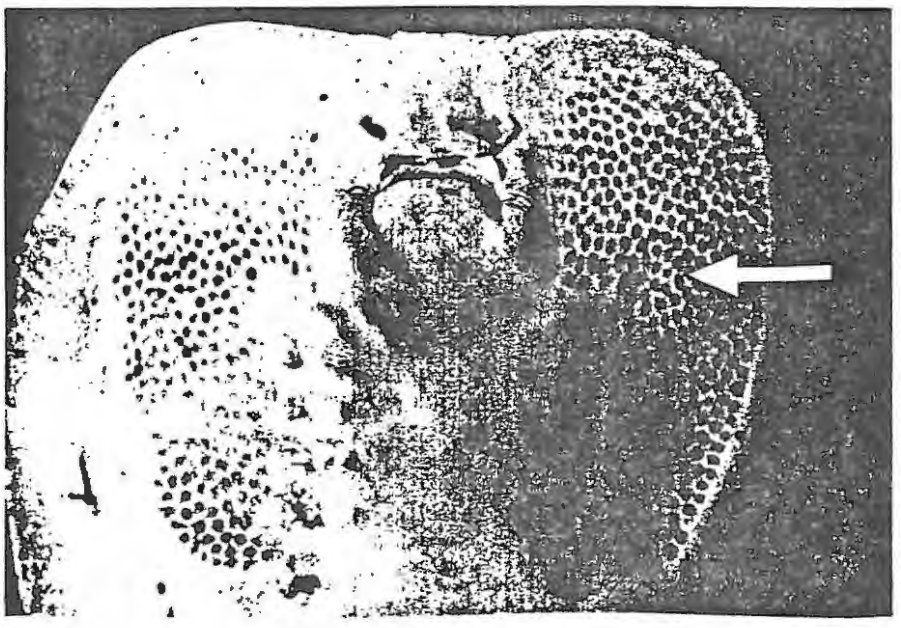


Figure 1.2 Ventral view of a *Torpedo* ray.

The bilateral kidney-shaped honeycomb-like structure is the electric organ (6).

The electric organ is advantageous for the isolation and purification of the nAChR because it represents a homogeneous high density population of synapses, all of which are identical and exclusively of the nicotinic cholinergic type (1, 5).

Electric rays can be stored frozen or alternatively, excised electric organs can be stored under liquid nitrogen at -70^{o}C. Continual freezing and thawing of the tissue releases intracellular proteases which are able to attack the receptor protein and render it structurally altered (7).

Electric organ is trimmed free of adherent muscle, connective and adipose tissue and homogenised in a blender. The sensitivity of nAChR to proteolytic attack is well documented (8-11), therefore homogenisation of the receptor requires the presence of a "cocktail" of protease inhibitors in a buffered system.

Crude homogenate is centrifuged at low speed, removing a pellet of membranous material comprising receptor protein. Solubilisation of this membrane protein requires the presence of non-denaturing detergents such as Triton X-100, Tween-20 or 80, deoxycholate or cholate (12). The concentration and type of detergent, has however, allowed for disparity as regards the functional integrity of the receptor protein. It is suggested that regulatory properties of the receptor are lost upon solubilisation with Triton X-100 and that the affinity for many agonists becomes altered (13).

Recently it has been shown that the release of free fatty acids from the membrane destroys the functional integrity of the receptor (14). It has been suggested (15), that this form represents a desensitised receptor with a higher affinity for many ligands of the cholinergic system.

It is firstly apparent that a critical ratio of detergent to lipid be maintained if active protein is to be released from the membranes (16) and secondly, milder detergents be used, aimed at minimising alteration of structural features (17).

1.2.2 Purification

Attempts at isolating and purifying the receptor protein by conventional methods such as gel filtration, electrophoresis, electrofocusing or density gradient centrifugation met with little success due to either low yields and/or denaturation of receptor protein (6, 18). Several years later however, conventional methods regained popularity since nAChR-rich membranes became available in significant quantities from *Torpedo* electric organ. Centrifugation at low and high speed is sufficient to give the nAChR in a relatively pure form and in milligram quantities from a crude-detergent extract (13).

1.2.2.1 Gel filtration

Purification of biological macromolecules on the basis of size is reliable and simple in comparison to most other techniques, however, the resolving power is limited by chromatographic factors and cannot really yield fractions of discrete molecular size.

Acetylcholinesterase (AChE), the enzyme responsible for the hydrolysis of acetylcholine at the post-synaptic membrane, has a similar molecular weight (250 kDa) to its receptor protein (19), and hence the use of Sephadex G-200 or Sepharose 6B gels have not proved entirely successful (12, 19, 20). A 3.2-fold purification, using Sepharose 6B, for nAChR has been reported, but contamination of this purified material with non-specific proteins is suspected (20).

1.2.2.2 Ion-Exchange Chromatography

The resolving power of this technique is superior to gel filtration in that separation is based on differences in charge rather than on the size of macromolecules. It is however not sufficiently characteristic, allowing certain proteins to elute under similar conditions (21).

DEAE-Sephadex A-25, an anion-exchange gel, has frequently been employed in conjunction with affinity chromatography for purifying the nAChR. Desorption of receptor occurs in the presence of 0.5 M salt (5, 22).

1.2.2.3 Density Gradient Centrifugation

Post-synaptic membranes highly enriched in nicotinic receptors show an unusually high protein to lipid ratio (2:1) (the electric tissue originally been derived from skeletal muscle). These membranes can therefore be isolated from homogenates of electric tissue by equilibrium ultracentrifugation on sucrose density gradients (23). Membranes are separated into two fractions; those sedimenting through the gradient and those retained by the gradient. The membranes retained, have been shown to consist of highly purified nicotinic post-synaptic membranes (23). These membranes do, however, consist of non-receptor proteins as evidenced upon subsequent characterisation. Further purification by alkaline treatment and discontinuous or continuous sucrose gradient centrifugation appeared necessary to obtain a more homogeneous population of receptor molecules (23-26).

1.2.2.4 Isoelectric Focusing and Chromatofocusing

Purification and fractionation of proteins from biological fluids is facilitated by their different isoelectric points. The possibility of producing a pH gradient using an ion-exchange column has provided techniques for separation with resolution and recovery comparable to those of electrophoretic systems. With ampholyte displacement chromatography, a pH gradient is produced by mixing buffers of different pH or by employing the buffering action of an ion-exchanger and a running buffer initially adjusted to one pH through a column, adjusted to another pH (27). This column chromatographic method is also known as chromatofocusing.

Chromatofocusing was first mentioned in 1978, when the popularity of affinity chromatography precluded its use for purifying the receptor. A 6.3-fold purification of the receptor has been achieved using isoelectric focusing (28). This is the highest fold purification reported for the classical biochemical techniques discussed so far.

1.2.2.5 Affinity Chromatography

Since 1972, affinity chromatography has been the method of choice for purifying the receptor. This technique enables the purification of biomacromolecules on the basis of their biological function or individual chemical structure (29). The method is based on the ability of proteins to reversibly bind ligands with high affinity that are covalently attached to a gel matrix (18). The higher the affinity of the binding the more specific, and hence the easier the removal of unwanted material on elution.

1.2.2.5.1 Neurotoxins as Affinity Ligands

The snake α-neurotoxins possess the extraordinary properties of binding with very high affinity and specificity to the nicotinic cholinergic receptor. These neurotoxins are therefore ideal ligands for purifying the receptor in an affinity chromatographic system (5). Two neurotoxins of importance are α-bungarotoxin from *Bungarus multicinctus* and α-cobratoxin from *Naja Naja siamensis*. The former normally being used to detect the receptor because of its irreversible nature of binding (2), while the latter displays a lower affinity, but similar specificity for the receptor and is therefore used in purifying the protein according to its reversible binding. It has been shown firstly, that the toxins have a multipoint attachment to the receptor involving several locally separate structural elements of the toxin and secondly, that they interact only with the receptor and not with the enzyme, acetylcholinesterase as do other cholinergic ligands (30).

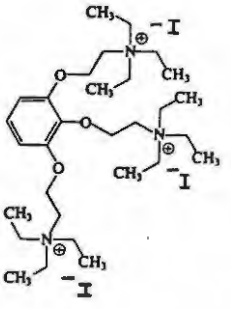
Neurotoxins, covalently linked to a solid support such as Sepharose 4B gel, following activation with cyanogen bromide, allows only 5-10% of the immobilised toxin the ability to bind nAChR (5, 9, 12, 22, 28). Approximately 1 mg/cm^3 of toxin is incubated with the affinity resin prior to adding solubilised receptor material (8). Typical recoveries are : 4-6 mg from an initial 200 g of electric organ (8) and 10 mg from 100 g (31). Disparity amongst the literature makes strict comparisons extremely difficult, however the many excellent reviews that have appeared, show without a doubt, the popularity of affinity chromatography (28, 32-35).

Fold purifications vary from 13.9-fold (20) to 60-fold (22). Obvious disadvantages of this technique are the time taken to perform affinity chromatography (22, 36); the difficulty in reversing or dissociating receptor from bound toxin (22) and obtaining material with modified binding properties once removed from the bound toxin (25). It is evident from electrophoretic criteria that the material ordinarily eluted from the column is not pure (12) and is of low yield (22). Apart from Sepharose-linked neurotoxins many quaternary ammonium compounds have been investigated as possible affinity ligands.

1.2.2.5.2 **Other Affinity ligands**

Many ligands of the cholinergic system show a characteristic quaternary nitrogen atom responsible for the high affinity and specificity for the receptor (37). Among these compounds, quaternary ammonium ligands have been studied and found to interact specifically with the receptor. Eldefrawi and Eldefrawi (18) have used phenyltrimethyl ammonium iodide as an affinity ligand but only achieved a 10-fold purification with very low recovery. Crude receptor protein has been adsorbed onto an affinity column containing an analogue of flaxedil, linked to Sepharose 2B, then eluted with a solution of flaxedil (gallamine trimethiodide) (see below),This method gave a 210-fold purification with a 30% yield representing 6-8 mg of purified material (24). Trimethyl (p-aminophenyl) - ammonium chloride has been covalently attached to Sepharose 4B through two lengths of the arm -$NH(CH_2)_3NH$-$(CH_2)_3$-$NHCO(CH_2)CO$ -; this gave a 3 800-fold purification from mammalian muscle, with biospecific elution using flaxedil (36). These same authors performed a similar purification on mammalian musele (38) but reported low yields despite a 2 400-fold purification.

Gallamine



Electroplax from Narcine ray has been purified 13.9-fold with a 35% recovery, by covalently linking N-(ε-aminohexanoyl) 3- aminophenyl trimethyl ammonium bromide hydrobromide to agarose gel (20).

It is evident that the low yields obtained using these ligands, have favoured the more popular use of α-neurotoxins as affinity ligands.

1.2.3 Assay

Several satisfactory methods have been developed for the detection of receptor sites. Most of these assays are based upon the formation of a radiolabelled toxin-receptor complex (39). The most versatile method, involves the separation of free toxin and toxin-receptor complexes by gel filtration on small columns of Sephadex G-100 or G-50 (5, 6). Another method involves the precipitation of toxin-receptor complexes in 30% ammonium sulphate and collection of precipitate on Millipore filters (5). The former method suffers a disadvantage when extremely small amounts of nAChR are assayed while the latter has the problem of coprecipitation of the free toxin along with the complexes. Assaying in the absence of detergents is necessary since receptors tend to aggregate in the absence of detergents (2, 5, 18, 24), allowing the complexes to be retained on the filters.

A method making use of the receptors intrinsic fluorescence has been used where toxins, binding to the receptor, cause quenching of the proteins fluorescence (40). The fluorescence intensity reaches a plateau, indicating saturation of the binding sites and hence direct determination of the concentration of active receptor sites (40). This method proves reliable and reproducible without many of the difficulties mentioned above.

1.3 PROPERTIES OF THE NICOTINIC CHOLINERGIC RECEPTOR

1.3.1 Molecular Structure

The purified receptor is a pentameric glycoprotein composed of five polypeptide chains of four different but homologous subunits. The resulting quaternary structure $\alpha_2\beta,\gamma,\delta$, represents a functional monomer unit (3, 14).

The molecular weight of the monomer receptor is between 250 kDa and 270 kDa (2, 3, 11, 41). Values vary considerably, from as low as 230 kDa to as high as 430 kDa (1, 6, 13, 36, 42), depending on the amount of detergent and glycosylation present. The receptor can exist as monomers and/or dimers; dimers consisting of two identical monomers, covalently crosslinked by an intermolecular disulphide bridge (1, 3, 13, 15)(Table 1.1).

1.3.2 Criteria of Purity

Several criteria are employed to determine the homogeneity of a purified preparation. These include specific activity, molecular weight, subunit weight, and the isoelectric point.

1.3.2.1 Specific Activity

The α-neurotoxins are used for assaying receptor sites as mentioned in 1.2.3. Values vary, depending on the source of material and/or method employed. Specific Activities are: 3.5μ Moles/g of protein (36), 2-6μ Moles/g (38) and 7.5 μMoles/g (which is a theoretical figure based on a molecular weight of 267 kDa)(2) and 12μ Moles/g of protein have been reported (6).

TABLE 1.1 MOLECULAR PROPERTIES OF nAChR FROM ELECTRIC TISSUE (5)

SPECIES	MOLECULAR WEIGHT BY GEL FILTRATION
Torpedo nobiliana	250 000 (Triton)
Torpedo marmorata	88 000 (Triton)
	180 000
	150 000 (Triton)
	300 000 (Triton)
	450 000 (Triton)
	300 000 (Lubrol)
Torpedo californica	500 000 (Triton)
	330 000 (Triton)
Electrophorus	550 000 (deoxycholate)
electricus	500 000 - 600 000 (Triton)
	360 000 (Triton)

From table 1.1 it can be seen that an accurate assessment of the molecular weight of the receptor is complicated by the presence of detergent in the purified preparations, accounting for the large variations in molecular weight reported (6, 9, 43).

1.3.2.2 **Molecular weight of subunits**

The α_2,β, γ,δ, pentameric structure can only be dissociated under denaturing conditions in the presence of sodium dodecyl sulphate (1, 3).

For many years there has been controversy in the literature as to the subunit composition of the receptor. Some groups have reported four kinds of subunits, whereas others claimed that the nAChR only consists of α -subunits (11, 12). The reason for the disparities is largely due to the different sources of material and because of the variety of electrophoretic conditions chosen (2). Preparations, where it has been feasible to minimise proteolysis, show four major polypeptides with the following molecular sizes: α(40-45 kDa); β(49-53 kDa); γ (53-56 kDa) and δ(57-68 kDa) (2). Others have reported specific values of 40 kDa, 50 kDa, 60 kDa and 65 kDa (42, 44) or 39 kDa, 48 kDa, 58 kDa and 64 kDa (1) for the α, β, $\gamma$and δ subunits respectively. The Laemmli method (45) or the Weber and Osborn (46) method have been used in determining the above values.

Incomplete inhibition of proteolysis during the purification can cause proteolytic nicking of the β, γ and δ-subunits so that only the α-subunit, which appears to be more resistant, is apparent on gel electrophoresis (11).

A polypeptide of approximately 130 kDa has also been identified in several studies (11, 15, 43, 47). This has been shown to be the result of two subunits linked through a disulphide bond, hence *Torpedo* nAChR exists as a dimer in its native membrane state (11).

Table 1.2 gives the subunit molecular weight and specific activities of various species of purified nAChR (13).

TABLE 1.2 SPECIFIC ACTIVITY AND CHAIN COMPOSITION OF nAChR-RICH MEMBRANES AND DETERGENT PURIFIED nAChR (13)

ORGANISM		POLYPEPTIDE CHAIN (MOL.wt.X 10^3)					SPECIFIC ACTIVITY
		α	β	γ	δ		μ M/g)
AChR-rich membranes							
T. californica		41	51	60	64	(105)	4
		40	49	60	69		2.9-4.6
T. marmorata		40 43	(50)		(66)	(100)	4
Purified nAChR							
T. californica	26; 35	42					7
		40	49	60	67		-
		39	48	58	64		4
		40	48	62	66		-
		41	51	60	64		8.8
		40	48	59	67	140	10-12
T. nobiliana	33.5; 35.5;38.5	43.5					12.2
T. ocellata		40	50	61	81		10
T. marmorata		42					9.5
		41.5					6
		46					-
		45	50				2.3
		42	50		70		-
		37	49		74	93; 148	(7)
		40	(50)		(66)		9
Electrophorus electricus							
						160	11
		44	50				4.5
		43					6
		42					7.5
		41					2.4
		42	49				6
			48	54	60	110	3.6
		43	48	54			7.5

From the table it is evident that similarity occurs amongst different species but not between the various subunit molecular weights reported by the different laboratories.

Treatment of purified nAChR with trypsin or chymotrypsin illustrates the effect of proteolytic nicking. All subunits are smaller than 40 kDa after treatment and in cases where severe treatment is performed, the largest components are in the order of 8 kDa (6).

It has been suggested that the 50 kDa chain is derived from the 66 kDa chain by proteolysis (7). Work by Ong and Brady (22) suggested four subunits of molecular weights 43.5 kDa, 38.5 kDa, 35.5 kDa and 33.5 kDa. Subunits of 28 kDa, 38 kDa and 45 kDa have also been reported (20). Failure to inhibit protease attack can therefore result in erroneous estimations of subunit molecular weight.

More recently (44, 48) it has been shown that a suspected cytoskeletal element of molecular weight 43 kDa is simultaneously purified with the receptor. This 43 K protein is thought to participate in anchoring the receptor at the post-synaptic site by linking the "receptor" to the cytoskeleton (48). It is therefore not a general membrane-associated element, but functions through its occurrence, with the acetylcholine receptor.

1.3.2.3 Chemical Properties

The nAChR displays an overall high acidic nature which is reflected in its low isoelectric point. Isoelectric focusing with broad pH gradients, indicate in most cases, homogeneous purified preparations, whereas narrow pH gradients reflect micro-heterogeneity (2, 13). Reported pI values are 5.0 (2); 4.8 (18); 5.3 (25); 4.9 (28), and 5.3 (38).

Narrow pH gradients give values of 4.5-4.8 (6); 4.8-5.0 (9) and 4.8 - 5.2 (13), indicating broad peaks and hence micro-heterogeneous preparations.

Accurate pI values are therefore clearly an indication of purity which, together with molecular weight determinations on PAGE/SDS-PAGE and specific activity assays, give an indication of the homogeneity of a purified receptor preparation.

1.4 PHOTOAFFINITY LABELLING AND PHOTOCHEMISTRY OF POTENTIAL NICOTINIC CHOLINERGIC LIGANDS

1.4.1 Principles and Criteria

A profitable approach to the analysis of receptor structure involves the use of specific, irreversibly acting molecules which will covalently label the receptor site and which, with appropriate labelling techniques, permit the isolation of receptor material (49).

Current techniques include energy-transfer induced labelling and photoaffinity labelling. The former method takes advantage of the fact that most proteins emit light when excited at the upper region of their absorption spectrum. A photosensitive ligand can therefore be decomposed to a reactive species when the ligand is in close proximity to the active site of a receptor. Certain prerequisites have to be met however: the photolabile ligand should be stable at the excitation wavelength (about 290 nm); it should be an efficient fluorescence quencher, and finally, should be photosensitive at the emission wavelength (about 340 nm) (50). There are few ligands however that meet all of the above criteria, and hence behave appropriately in energy-transfer induced reactions leading to covalently modified receptor active centres.

Photoaffinity labelling requires that the ligands have an inherent affinity for a binding site and possess biological activity. The ligands must also contain a photosensitive functional group which, when photoactivated, is capable of forming a covalent bond at or near the binding site of a receptor (51). For high affinity ligand-receptor interactions to take place precise knowledge of the geometry of the binding site must be known (49).

The photoaffinity label should therefore have an affinity for the active site, bind reversibly and only become covalently attached once photoactivated by light. The light source used for photolysis must also be suited to the absorption spectrum of the photoaffinity label (51).

The criteria for active site labelling are based on the relationship between a physiological response and the

specificity of the interaction. The photodecomposed ligand tends to react immediately with its surroundings, implying non-specificity due to high reactivity (52).

Nearly every known neurotransmitter receptor has been photolabelled to date, this facilitating the biochemical and biophysical characterisation and isolation of many cellular molecules (51). The fact that photoaffinity labelling is directly applicable to intact cells or isolated organ preparations (51) lends credence to the pharmacological understanding of ligand-receptor interactions. A major drawback of photoaffinity labelling, which does not apply to energy-transfer labelling is the damaging effects of U.V. light on the preparation. It is imperative then, that a desired level of covalent insertion be achieved with, simultaneously a reduced level of toxicity (51).

A simple scheme for photoaffinity labelling appears as follows (52).

$$L + R \underset{1}{\overset{\text{slow}}{\rightleftharpoons}} L\,R \xrightarrow[2]{h\nu} L^{*}\,R \xrightarrow[3]{\text{fast}} L\text{–}R$$

where (1) represents a slow rate to equilibrium of ligand and receptor (2) photodecomposition by light and (3) instantaneous covalent linkage between reactive species and receptor.

1.4.2 Photoaffinity Labelling of nAChR

The nicotinic cholinergic system had been well characterised prior to the application of photoaffinity or energy-transfer labelling techniques, due to the availability of specific and reversibly-binding ligands with biological activity and high affinity. Since reversibly-bound ligands dissociate from the receptor during chromatographic or electrophoretic procedures, it became imperative to be able to covalently label the active site of the receptor

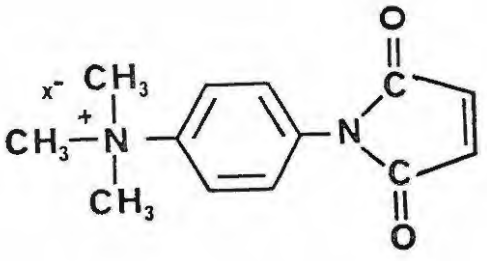
and through this, allow further progress to be made towards the ultimate characterisation of the nAChR.

Early attempts at labelling the receptor resulted in inactivation of the receptor protein and non-specific interactions due to saturation of binding sites (53). These same authors did however show that ammonium aryl azides are potential photoaffinity ligands, decomposing to reactive-nitrenes upon photolysis. The reactive nitrenes irreversibly inactivated the enzyme acetylcholinesterase and the receptor molecule of the intact frog sartorius muscle (53).

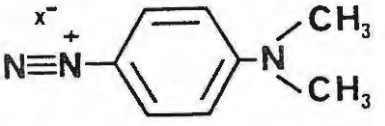
The goal of receptor isolation, an ambitious one in biochemical pharmacology, has made good use of photoaffinity labelling. A list of potential photoaffinity labels and their applications are reviewed (51). More recently, attempts have been made to localise ligand binding sites and hence characterise the structural features of the various polypeptide subunits in the functional monomer (54). The requirement and usage of ligands of high affinity and specificity have resulted in specific covalent interactions, and thus incorporation in particularly functional active sites. It is for this reason that photoaffinity ligands have not replaced the more conventional use of affinity chromatography and related techniques for purifying the receptor. Photoaffinity labelling has been, and still is, utilised as a structural probe of the receptor complex, rather than a form of purification of the receptor molecule.

The use of naturally photolabile underivatised ligands such as α-bungarotoxin, have proved to be extremely useful in labelling the receptor. The main advantage to be gained using natural photolabile ligands over photoaffinity analogues of ligands, is that the bulky photosensitive moeity is not present. Secondly, the potency and efficacy of the compound has not changed as can occur when a ligand is derivatised (51). Raftery *et. al.* (55) derivatised α-bungarotoxin by adding aryl azide side chains to the toxin. This allowed firstly that the polypeptide to which the toxin derivative binds and an adjacent polypeptide be derivatised upon photolysis or secondly, only the adjacent polypeptide be labelled.

Ligands, amongst others, that have proved useful in identifying the acetylcholine binding site are 4-(N-maleimido)-[^{3}H]-benzyltrimethylammonium iodide ([^{3}H]MPTA); p-(N,N-dimethyl amino)-benzene diazonium fluoroborate ([^{3}H]DDF) (see below); [^{3}H] bromoacetylcholine and more recently [^{14}C]chloroacetylcholine perchlorate. The first ligand is a sulphydryl-directed affinity reagent labelling a single α-subunit cyanogen bromide fragment. The second photoaffinity ligand, DDF, offers significant advantages over MPTA in that it labels native nAChR without prior reduction, a modification known to produce marked changes in the conformation of this site. Also the photolabile diazonium moeity may interact directly with the tetraalkylammonium binding site providing a more detailed view of the structure of the acetylcholine binding site, in its native conformation (56).



MPTA



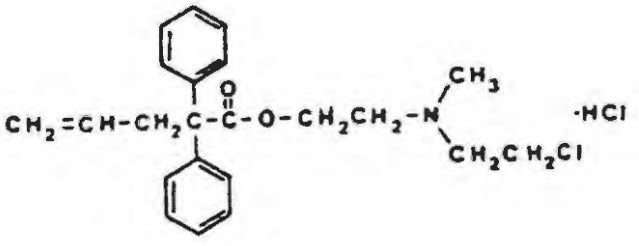
DDF

The alkylating agent, chloroacetylcholine was found to be more applicable in labelling the receptor than bromoacetylcholine affording specific alkylation at the α-subunit. Detection in SDS-PAGE was found to be considerably easier due to the use of [^{14}C] instead of [^{3}H](57). In photoaffinity labelling, not enough protein is radiolabelled for easy detection on a polyacrylamide gel because of the low efficiency of a photolabelling process (52).

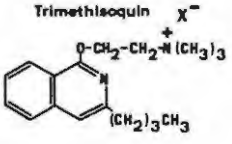
A series of halodibenzo-p-dioxins with the photolabile aryl azide functional group have also been synthesised and screened as potential photoaffinity labels for the nAChR. The labels were directed against rodent liver preparations and led to the characterisation of the receptor in mouse liver cytosol (58).

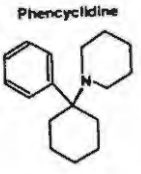
A photosensitive O-nitrobenzyl moeity has been attached directly to the carbamate nitrogen of the well characterised acetylcholine analogue, carbamylcholine. This derivatised compound showed major advantages over other photoactivatable acetylcholine analogues, in that the linkage of the O-nitrobenzyl group to a nitrogen, suggested preparation of photoactivatable derivatives of other neurotransmitters that contain an amino group (59) [^{3}H]-Tubocurarine has also been employed as an affinity label for the agonist-binding site (60), but was found to be incorporated into all the nAChR subunits.

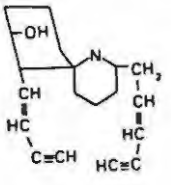
Affinity labels have also been used in attempts to identify the location of the allosteric antagonist binding site, one such compound is meproadifen mustard (see below). This ligand alkylates several polypeptides in addition to nAChR subunits with specific alkylation of the α and β subunits (60, 61).



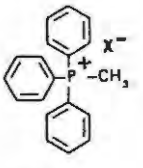
Non-competitive antagonists (see below) also serve as useful tools for the identification of functionally important domains of the receptor (61). For the case of nAChR they include trimethisoquin, phencyclidine, perhydrohistrionicotoxin and chloropromazine. Chloropromazine photolabels specifically the β and δ chains of the receptor in the presence of the agonist carbamylcholine, whilst reduced labelling occurs in the presence of phencyclidine (54). Earlier studies by this group showed that chloropromazine heavily labelled all four polypeptides (α, β, γ, δ). From these studies and others (62-64) the idea that the δ chain is involved in the binding of all of these non-competitive blockers is therefore conclusive. Of the ligands that gave successful labelling, all had a carbon ring with important substituents (62). Triphenylmethyl phosphonium, frequently used as a voltage sensor in membrane systems, binds reversibly to the α-subunit of the receptor but not at the acetylcholine binding site. This same ligand, once U.V. irradiated in the presence of the receptor, alkylates the α-subunit, but, in the presence of carbamylcholine, labels preferentially the α and β -subunits. This can be interpreted as an indication of a rearrangement of the receptor's quaternary structure on binding agonists (65).





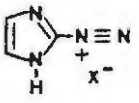


Histrionicotoxin



TPMP⁺

Aryl diazonium salts have been described as irreversible blockers of the phencyclidine binding site. In the presence of carbamylcholine, interaction with all four subunits of the receptor occurs. Among these diazonium salts, an imidazole derivative is unique in that the photoinduced irreversible blocking is only effective when the receptor is in a desensitised state (66).



Recently, Middlemas and Raftery (67) included cholesterol as an affinity probe. Synthesis of [^{3}H] cholesteryl diazoacetate, capable of incorporating itself into lipid bilayers, demonstrated that cholesterol interacts with all four subunits of the nAChR.

The pattern of labelling is dependent on the structure of the photosensitive ligand as well as the state (open, closed etc.) and source of receptor (66). The popularity of an affinity ligand will be governed essentially by its structure and hence reactive products upon photolysis. Of all the ligands, the aryl azides have the most predictable photodecomposition under mild U.V. irradiation (52). Problems of efficiency, stability and specificity in labelling are never completely overcome but the aryl azides on decomposing to aryl nitrenes have proved to be the best (52).

Energy-transfer and photoaffinity labelling of acetylcholinesterase using p(N,N-dimethylamino)- benzene diazonium fluoroborate (DDF) has allowed a direct comparison of these two techniques. Photolysis of the enzyme-DDF complex at 410 nm resulted in less inactivation of the enzyme than when energy-transfer between the enzyme and DDF occurred at 295 nm. The latter method showed an increase in the rate of enzyme inactivation and a better stoichiometry of covalent labelling (68). At high concentrations of inhibitor however, non-specific labelling is increased due to the increase of the amount of uncomplexed ligand (68).

1.4.3 Nitrosamine Photochemistry

Nitrosamines are amongst the most powerful carcinogenic chemicals known and are easily converted, after *in vivo* α-hydroxylation, to alkylating species which may be the diazoalkane (R-CH=$\overset{+}{N}$=N), the diazonium salt (R-CH_2-$\overset{+}{N}$≡N) or the carbonium ion (R-$\overset{+}{C}H_2$) (69, 70).

The nitrosamines are pharmacologically interesting agents capable of highly selective enzyme inactivation (69) and can prove to be selectively toxic to organisms or tissues possessing the requisite activating enzyme.

Extensive interest in the photochemical reactions of nitroso-compounds began in 1964. It was discovered that the N-Nitrosamines remain stable towards U.V. irradiation in neutral solution but undergo various photoreactions in the presence of a dilute acid (pH1-3) (50, 71). It is reasonable to suppose that the dilute acid promoted photolability of the nitrosamines through association with their dipolar character. Structure II (figure 1.3) possesses a 48% contribution of polar resonance form, suggesting a slight favour towards the non-charged resonance structure I (figure 1.3) (71).

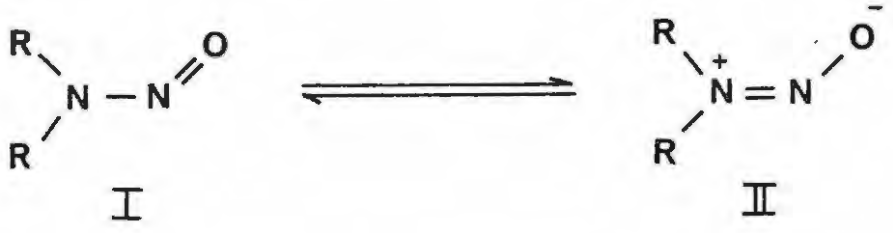


Figure 1.3 **Resonance forms of Nitrosamine compounds.**

Photolysis of III (figure 1.4) results in the formation of nitrosyl and aminium radicals by dissociation of the N-N bond.

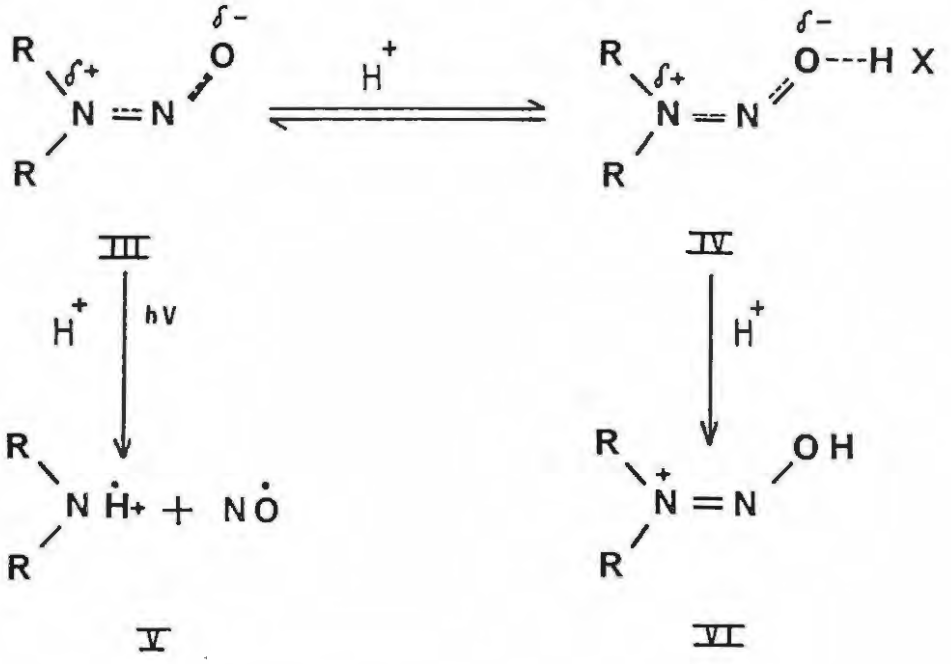


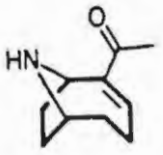
Figure 1.4 **Photochemistry of N-Nitrosamines.**

Ultraviolet spectroscopy studies of nitrosamines in aqueous sulphuric acid have demonstrated that at low concentrations of acid (pH 1-3) association through hydrogen-bonding is the primary mode of interaction (III-IV) (figure 1.4) (71, 72). At higher concentrations of acid, nitrosamines are protonated on the nitroso oxygen (VI). It is the former (IV) but not the latter (VI) that is photolabile (71).

Dimethyl-N-Nitrosamine (DMN) shows absorption maxima at 228 nm and 332 nm in aqueous solution in the absence of acid. An increase in the acidity results in a decrease of the intensity of these absorption maxima, with complete disappearance in concentrated acid (72). It is therefore favourable to mimic a medium in which the nitrosamines are most photosensitive. Mediums of low dielectric constant; non-polar solvents such as heptane or chloroform in the presence of trifluoroacetic acid tend to enhance the photoreactivity of the nitrosamines (50, 73). DMN in non-polar solvents exhibits absorption maxima at 220 nm and 360 nm. Shifts in the absorption maxima are best explained by hydrogen-bonding in a solute-solvent interaction. The amount of

shift appears to be of the order of the hydrogen-bonding ability (not acidity) of the solvent (73).

Anatoxin-a is a potent nicotinic acetylcholine receptor agonist (74).



The geometry of anatoxin-a has been determined by x-ray crystallographic spectroscopy and force field calculations. Comparisons of this conformation with models proposed for acetylcholine receptor activation (75) shows good agreement and allows for further inferences concerning the stereodiscrimination by the receptor. Since a planar configuration is shared by both anatoxin - a and DMN (76), the nitrosamines are considered as potential ligands for the proteins involved in the cholinergic system and as a preamble they are also the simplest in structure.

The stereodiscrimination by the nAChR suggests that in nicotine the role of the carbon skeleton is simply to provide proper stereochemical localisation of the functional group. Good correlation between lone pair directionality and ligand protein binding is essential. The nAChR donates a hydrogen bond to the agonist and recognises the plane defined by the hydrogen-bonding system. The distance between the electrostatic interaction (i.e. quaterinised N) and a hydrogen-bonding acceptor is of paramount importance (50). An additional interesting feature of these molecules is that their $-N_2$ moeity which is responsible for their affinity is also the photolabile function. It may therefore be possible to covalently label the residues belonging to the quaternary ammonium binding site.

It has been suggested (50) that the nitrosamines were specifically photoactivated after association with an acidic hydrogen at the active site (69, 71).

Nitrosamine derivatives have been shown to irreversibly inhibit acetylcholinesterase in the presence of light (69); the inactivation rate being maximal at a photolysis wavelength of about 335 nm. Methyl (acetoxymethyl) nitrosamine (MAN) is a poor competitive inhibitor of this enzyme (Km = 10^{-2}M). AchE inactivation suggests that there is at least one protonated residue in the enzyme active site and that the active site must be hydrophobic in nature. Thirdly there are certain chemical requirements which induce photoactivation when complexed at the active site, based on the fact that only 24% of MAN is degraded after 20 min in an aqueous acidic medium (69).

1.5 MOLECULAR INTERACTIONS BETWEEN LIGANDS AND THE NICOTINIC CHOLINERGIC RECEPTOR

1.5.1 Regulation of ionic permeability

Receptors are coupled to many different types of cellular responses, some of which may be very rapid, i.e. millisecond time scale, or very slow with a time scale of hours. In the case of neurotransmitters, these receptors are located in the plasma membrane and convey the signal through second messenger systems from outside the cell to the inside. Depending on whether the second messenger is an ion or a low-molecular weight compound like cAMP, will decide whether the receptor is coupled to an ion-channel (Type I) or an enzyme catalysing the synthesis of the second messenger (Type II). A prototype of the channel-coupled (Type I) receptor is the nicotinic cholinergic receptor found at the neuromuscular junction (3). Type I exhibits the fastest type of receptor-mediated response, when a neurotransmitter acts on the post-synaptic membrane of a muscle cell, transiently increasing its permeability to particular ions (4). The speed of this response makes it likely that the coupling between receptor and the ionic channel is a direct one (4).

The effect of acetylcholine binding to the receptor on the post-synaptic membrane is to cause a large increase in its permeability to small cations, particularly sodium and potassium ions and, to a lesser extent, calcium ions. Because of the large inwardly-directed electrochemical gradient for sodium ions across the cell membrane, an influx of Na^+ ions occurs, causing depolarisation of the post-synaptic membrane. This transmitter mediated depolarisation is called an endplate potential (epp). In the muscle fibre the localised epp spreads to adjacent electrically excitable parts of the muscle fibre. If its amplitude is sufficient to reach the threshold for excitation an action potential is initiated, which propagates to the rest of the fibre and evokes a contraction (4).

1.5.2 Molecular mechanism of nAChR action

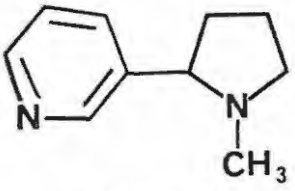
There are three main classes of cholinergic effector molecules: agonists, competitive antagonists and non-competitive antagonists. The term ligand, synonomous with effector molecule is commonly used to describe any substance that binds a particular type of receptor.

1.5.2.1 **Agonists**

Agonists-like the natural neurotransmitter acetylcholine-bind to nAChR and activate it (3).

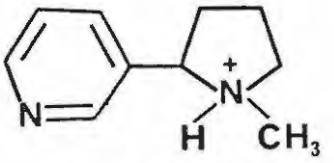
1.5.2.1.1 **Structural - functional aspects**

Nicotine, a tertiary amine, exists mostly as the univalent nicotinium ion at physiological pH (figure 1.5 (i) + (ii)). It is this nicotinium ion that specifically activates the nicotinic receptor (77).



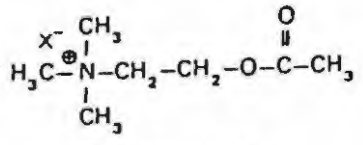
Nicotine

i



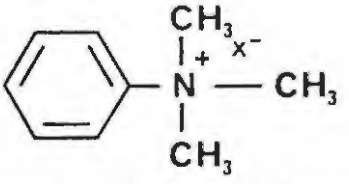
Nicotinium ion

ii



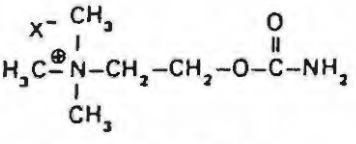
Acetylcholine

iii



Phenyltrimethyl ammonium

iv



Carbamylcholine

v

Figure 1.5 **Structures of nAChR agonists**

Several structural features of agonists are important for recognition of the cholinergic receptor.

(i) **Cationic head:** The quaterinised nitrogen atom is mainly responsible for the affinity for the receptor and agonist activity.

Simple tetramethylammonium ions $(CH_3)_4\overset{+}{N}$ stimulate both nicotinic and muscarinic receptors, although are considerably less potent than acetylcholine. The importance of the positive charge suggests that the initial attraction between acetylcholine and the receptor is an electrostatic one. An increase in the size of the quaterinising groups, i.e. (replacement of methyl groups with longer alkyl chains) results in reduction of activity, but simultaneously an increased affinity for the receptors (77).

(ii) **The ester link:** The ester link is important in that both the carbonyl and ether oxygens of acetylcholine are involved in the combination with the receptor, the carbonyl oxygen being relatively more important for nicotinic activity. This suggests that additional active sites on the receptors (positively charged areas) are present, interacting with the partial negative charge at the "appropriate position" in the active compound (77). Aromatic compounds such as phenyltrimethylammonium (figure 1.5 (iv)) are potent agonists of nicotinic receptors but poor muscarinic agonists. Their increased potency over acetylcholine is due to the partial negative charge on the electron-attracting benzene ring in place of the carbonyl oxygen of acetylcholine (77).

(iii) **The acetyl group:** Choline possesses all of the pharmacological properties of acetylcholine but is 100-100 000 times less potent. The reason being, that the hydroxyl group of choline impairs its reaction with the receptor. Possibly, hydrogen-bonding between the hydroxyl group and water molecules prevents its close approach to the receptor (77). The absence of the acetyl group in carbamylcholine (figure 1.5 (v)), which stimulates both nicotinic and muscarinic receptors, emphasises the importance of this group for specific nicotinic recognition.

(iv) **The alkylamine chain:** Branching of the chain alters the activity of acetylcholine-like molecules. The addition of a methyl group to the β-carbon atom for example, results in almost complete loss of activity. Optimum length of the chain is five atoms (cf acetylcholine), but is not a strict rule for nicotinic agonists.

From all of the above structural features, it would appear that the cationic head and the carbonyl oxygen atom are the most important features necessary for nicotinic recognition.

1.5.2.1.2 **Cooperative interactions and conformational changes**

In 1937, Clarke pointed out that; "not only must a ligand bind on a receptor in order that a response be observed," but "must also produce an effect" (78), i.e., a specific structural change must be induced in the receptor.

Methods of studying cellular responses have been by several means: mechanical, which is the simplest method involving muscle contractions; electrophysiological methods eg. patch-clamp techniques, and many biochemical binding assays. One drawback of the first two methods is that association and dissociation constants for agonists cannot be accurately determined because the concentration of ligand present at the receptor is unknown. Dose-response curves in the presence of agonists give unreliable estimates of Ka and Kd for receptors because the relationship between receptor occupancy and response is usually non-linear (4, 79).

The study of response as a function of agonist concentration is a direct and very widely employed strategy for the study of receptor activation. These kind of studies have provided evidence that activation of the acetylcholine receptors and probability that the channel is open, is greatest when two ligand binding sites are occupied (1, 6, 80-82). It has been suggested that singly liganded receptors which exist at low agonist concentration make a small contribution (82, 83).

Positive cooperativity has been detected, i.e. binding of one molecule to the receptor enhances the binding of the second molecule to the receptor (6). Evidence for this, comes from calculation of the Hill coefficient which was found to be between 1.6 and 2.0 indicating two binding sites interacting allosterically. Two early models attempting to explain similar observations are those of Monod, where the protein oscillates spontaneously between two conformations and those of Koshland's induced fit model where binding of a ligand to one subunit of the protein induces changes in the conformation of the protein which make it easier (or more difficult!) for a second molecule to bind to the other subunit (6).

The present assumption is that binding of Ach to it's receptor induces a "change in the conformation" of the

receptor, which in turn allows a second molecule of Ach to bind causing the channel to open and hence ions to flow through it. Not only does the receptor change its conformation upon binding of an agonist, but it appears also to change into a third conformation when in a desensitised state (6).

Jackson (82) gained significant insight into the mechanism of receptor activation using the agonist, carbamylcholine. "The first ligand binds tightly, but accelerates the rate of channel opening by only a factor of 40, and increases the channel mean open time by a factor of 5 . The second ligand binds less tightly than the first, but accelerates the rate of channel opening by an additional factor of 2 500 . Binding of the second ligand increases the mean open time by another factor of 5." The major effect of ligand binding is destabilisation of the closed state as opposed to stabilisation of the open state. Jackson deduced that the ligand-receptor interactions at the two binding sites are similar in strength when the channel is open, but substantially different when the channel is closed (3, 80-83). The difference in binding affinities for an agonist is a new observation that was not evident in previously reported macroscopic dose-response curves (82).

One can picture the second ligand binding site as a poor complement to the ligand when the channel is closed, forming incomplete contacts with the ligand and therefore becoming more easily dislodged. If the channel opens, the binding site changes its configuration to complement better the structure of the ligand, and thus strengthen the ligand-receptor contacts (82).

In summary, three fundamental steps appear to be involved in the transduction process: (a) ordered binding of two Ach molecules to the α-subunits at the receptor site that controls the formation of transmembrane channels. (b) The conformation of the receptor changes from a closed-channel state to an open-channel state that allows ions to flow through the receptor channel, and (c) a conformational change of the receptor, from an active to an inactive (desensitised) form occurs, due to the continued presence of an agonist (3, 84).

The channel is said to be either open or closed i.e. "an all or nothing event" (3, 4). The mean open time of the channel depends on temperature, membrane potential, location in cell membrane and on the agonist. Values of 0.9 ms for carbamylcholine and 2.4 ms for acetylcholine have been cited (3). The fraction of the time for which the channel is open (γ) increases with increasing concentration of agonist indicating that channel opening is closely linked to agonist binding (1, 85).

1.5.2.1.3 Role of Ca^{2+} and the ion-channel

There is no doubt that the ion-channel is an integral part of the $\alpha_2\beta\gamma\delta$ pentameric structure of the receptor (3). The channel is selective to cations and is a water-filled pore; permeating ions probably interact almost exclusively with water ions rather than with protein side chains (3).

Triggle's work, cited in (6), on the guinea-pig ileum led them to suggest that the intrinsic activity of an agonist was a direct reflection of its ability in the agonist-receptor complex to activate the membrane-bound Ca^{2+} translocation system. The source of mobilised Ca^{2+} was uncertain at this stage but later developments ascertained that the receptor has a very high affinity for Ca^{2+} ions and therefore, these ions played a role in receptor-ligand interactions. Prinz and Maelicke (86) established that binding of Ca^{2+} inhibited receptor binding of a fluorescent ligand with an inhibition constant (Ki) in the mM range. It is now known that for every acetylcholine molecule bound, 2 - 3 Ca^{2+} ions are displaced, suggesting a direct responsible role in receptor-mediated permeability changes (6). One view is that Ca^{2+} release results in a change in the dipole moment of the ion-channel leading to opening (6).

1.5.2.1.4 Desensitisation phenomenon

The term desensitisation, first used by Monod in 1963 for describing allosteric interactions of regulatory enzymes, is a totally different phenomenon from what neuropharmacologists describe today (87).

Desensitisation is the loss of response to a previously effective dose of agonist. It results from exposure to a high dosage of agonist or prolonged exposure to low depolarising dosage. It occurs in response to externally applied acetylcholine or other ligands or by excessive nerve stimulation (6). Whereas for receptor activation, where two agonist molecules are necessary, binding of only one molecule is sufficient for desensitisation.

The following scheme outlines this :-

$$\begin{array}{ccccccc} R + L \rightleftharpoons & RL & + L \rightleftharpoons & RL_2 & \rightarrow & AL_2 \\ & \Downarrow\Uparrow & & \Downarrow\Uparrow & & \\ & DL & & DL_2 & & \end{array}$$

where R - receptor resting state

L - ligand (agonist)

A - active receptor, channel open

D - desensitised receptor, channel closed (3).

The rate and extent of desensitisation varies with the structure and concentration of agonist used and is enhanced by Ca^{2+} and other cations as well as by high temperature (6). Desensitisation is known to occur within tens of milliseconds, allosterically shifting the receptor into a stable state that does not conduct ions, but binds agonists with increased affinity (15, 88). At least two such "desensitised" states have been described, with affinities for acetylcholine in the range of 1 μM to 10 nM respectively, as opposed to 100 μM for the non-desensitised state (15). Solubilisation of membrane-bound nAChR also converts them into a desensitised form (15), suggesting that most binding studies involve desensitised nAChR's, rather than resting nAChR's.

Four general functional states of the receptor can therefore be distinguished: resting, open, fast-onset desensitised, and slow-onset desensitised. The resting state is characterised by relatively low affinity and a

closed channel. The open state is an active state with channel open, affinity unknown or intermediate. The receptor desensitises in two steps, fast and slow; in each step the affinity for agonist increases by two orders of magnitude (89). This scheme is further complicated by the fact that the ion-channel may fluctuate between two distinct open states and even between resting and desensitised states which both represent closed-channel states. Katz and Thesleff, cited in (1), proposed the following model :

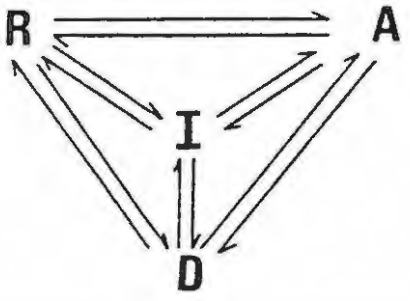


Figure 1.6 **Allosteric transitions proposed by Katz and Thesleff**

A - active state, channel open.

I and D - rapidly and slowly desensitising states.

The same two Ach sites are involved in each state, but their affinity varies from R to D via A and I. All these states are interconvertible, and, for some of them, present before ligand binding.

The high concentration of agonist required to produce desensitisation would suggest that it occurs through binding to a low affinity site. It was therefore proposed that, in the membrane at rest, the receptor is present in a state with low affinity for agonists, and that agonists stabilised the receptor in a conformation having a high affinity for agonists, a conformation that corresponded to desensitisation of the system (6).

Whether stabilisation (6) or destabilisation (90) of the state occurs has yet to be clarified. The notion that, "ligand binding displaces bound water" and therefore, "it is the replacement of water by ligand" that destabilises the closed state, appears to be acceptable (78, 82).

Prinz and Maelicke (80) (1983) indicated from electrophysiological studies that two separate reaction sequences for receptor reactivation and desensitisation occur: both processes are reversible but, compared with activation, desensitisation requires longer periods of incubation of agonist and receptor. Neither formation nor breakdown of the desensitised state necessarily proceeds via the active form of receptor which supports the model proposed by Katz and Thesleff .

Studies by Udgaonkar and Hess, in attempting to obtain information about the dissociation constants of the receptor-ligand complexes before and after receptor desensitisation, and rate coefficients for desensitisation and recovery from it, showed, using a quenched flow technique that desensitisation actually occurred at 100 times the rate estimated from electrophysiological techniques (83). Frog (*Xenopus*) muscle and *Torpedo* receptors exhibit an additional desensitisation process that occurs in the second-time region (83).

Based on structural specificities of agonists, it has been suggested that the conversion from the low to high affinity state *in vitro* and receptor desensitisation involve the same molecular transition (91). Since the transition in receptor state can occur with ligands having widely different capabilities to activate the receptor, the relationship between receptor desensitisation and activation might be considered in terms of the respective rates of conversion in state for the various ligands (91).

A disulphide-interchange model (92) proposed by Moore and Raftery is depicted as follows:

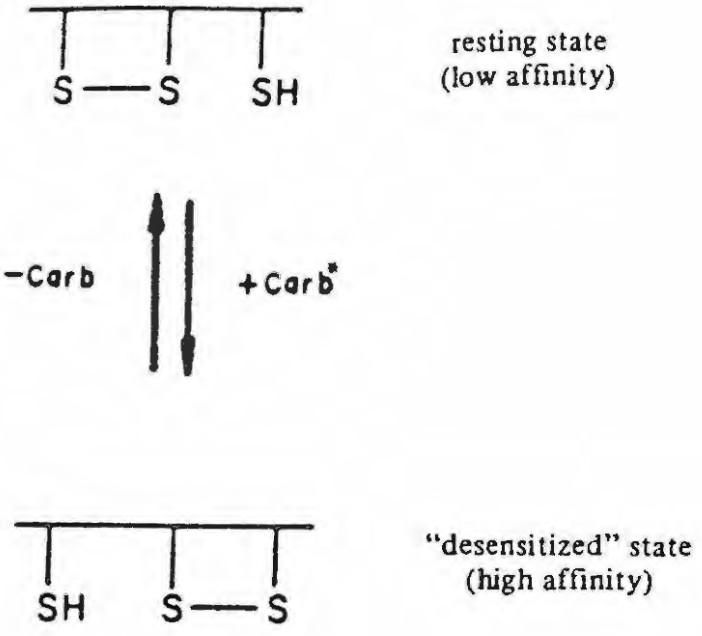


Figure 1.7 **Disulphide - interchange model**

*Carb - carbamylcholine

The agonist binding site is now thought to be in the region of Cys 192 - Cys 193 (93, 94). Others have reported a readily reducible disulphide bond, 1 nm from the negative subsite of the Ach binding site (89). Thus, this disulphide appears to be intimately involved in the local conformational changes at the acetylcholine binding site leading to activation (94). The application of sulphydryl alkylating or acylating agents after disulphide reduction leads to irreversible modification of the receptor response (95) Recently, it was proposed (96) that the free cysteine is associated with a hydrophobic pocket in the receptor, which, when alkylated, blocks channel activity.

The interactions of forskolin, with the nAChR - ion channel complex has been studied (97). It was found that low concentrations (nM) induced receptor desensitisation but had no effect on the properties of Ach - activated single channel currents, suggesting a mechanism of phosphorylation of the nAChR. Indeed phosphorylation of the nAChR could occur either in the resting state or in states with bound Ach. Such phosphorylation would enhance pathways leading to possible desensitised, non-conducting states (97).

Desensitisation may not be evident under physiological conditions since the quantity of acetylcholine released during repetitive nerve firing does not appear likely to be sufficient to induce desensitisation. However, desensitisation of the receptor may serve as an autoregulatory function, protecting the region against excessive phosphorylation (97). It is known that protein phosphorylation by specific protein kinases often have an autoregulatory role.

More recently, it has been shown that acetylcholine also binds to a regulatory site, known as the isosteric site, inhibiting the formation of transmembrane channels. Isosteric inhibition (also known as voltage-dependent regulation), in combination with desensitisation appear important in the regulation of receptor function (84, 98).

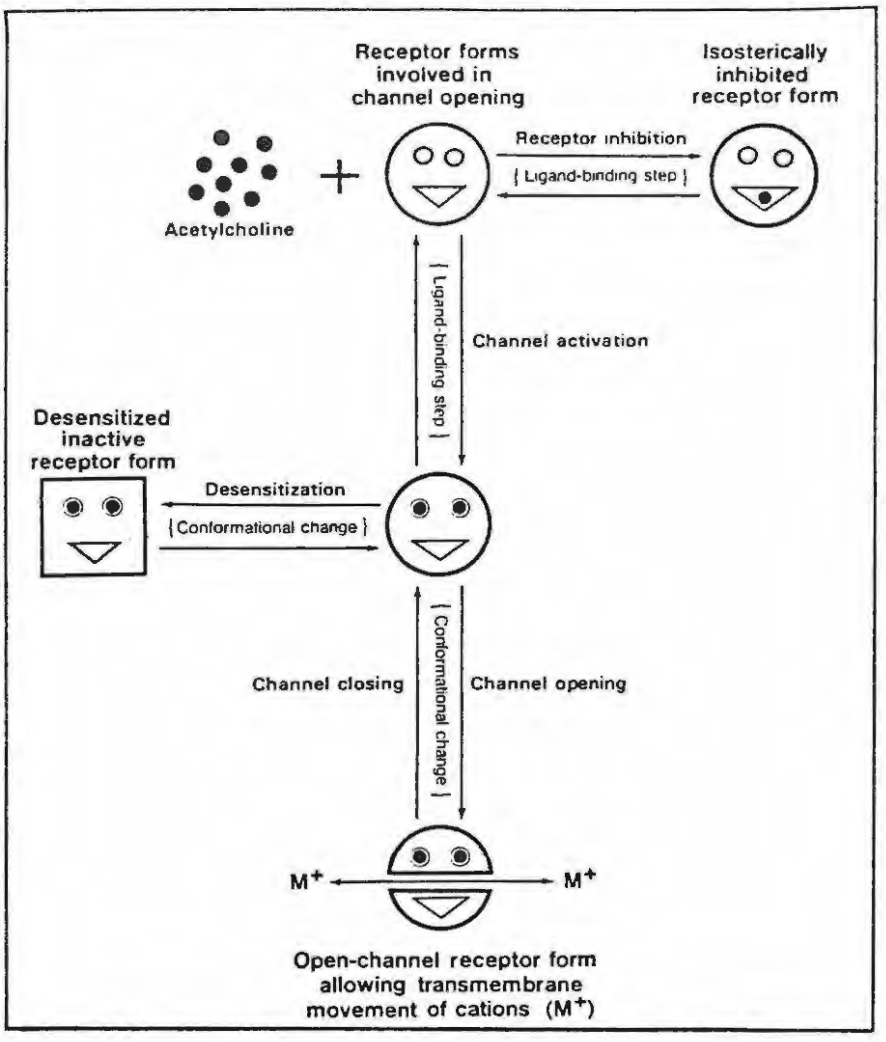


Figure 1.8 **Schematic representation of regulatory mechanisms at synaptic acetylcholine receptor**

Isosteric regulation appears to occur on the same time scale as signal transmission, regulating the fraction of receptors in the membrane that can form transmembrane channels and thereby make the signal transmission process sensitive to small fluctuations in both transmembrane voltage and the concentration of acetylcholine (98). Similarly, fast-onset desensitisation regulates by blocking only a few percent of the receptors, but more importantly, because the rate of recovery is twenty-times slower than desensitisation itself, subsequent signal-transmission is blocked to the extent that a critical concentration of receptors are only effective, determining whether a signal is transmitted or not.

Finally, the effects of membrane perturbants on the receptor should be mentioned. Thermal perturbation studies using differential scanning calorimetry have shown that structural alterations of the receptor do occur in the presence of cholinergic agonists and that the release of free fatty acids by phospholipase A_2 treatment from the membrane, result in destabilisation of the receptor and loss of conductivity upon exposure to agonists. Structural probes at the lipid/proteins interface also cause perturbations, in analogy with the release of "fluid" free fatty acids (14). Solubilising concentrations of detergents also produce a destabilised receptor, which in some cases can be reversed upon dialysis (17).

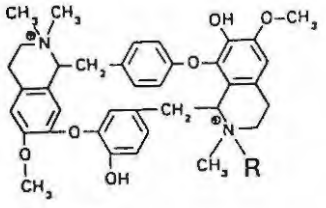
Hence it can be seen that conformational changes of the receptor, which are vital to the functioning of the receptor can also become irreversibly altered states with an ultimate loss of activity devoid of any voltage dependency.

1.5.2.2 Antagonists

1.5.2.2.1 Competitive

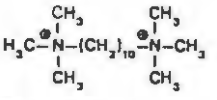
Claud Bernard showed in 1856 that "curare", a South American arrow poison, caused paralysis by blocking

neuromuscular transmission (4). A specific curare agent, d-tubocurarine (d-TbC) (figure 1.9 (i) R = H), the essential structure of which was established by King in 1935, has helped tremendously in elucidating many of the functional aspects of the nAChR. Until recently it was assumed to be a bis-quaternary ammonium compound, however it is now known that one of the nitrogen atoms constitute a tertiary amine (37). A semi-synthetic derivative, dimethyl tubocurarine (figure 1.9 (i) R = CH_3) is almost three times as potent as d-TbC. Because of the large number of "curare"-like compounds and synthetic derivatives known, it will suffice to deal with only a few.



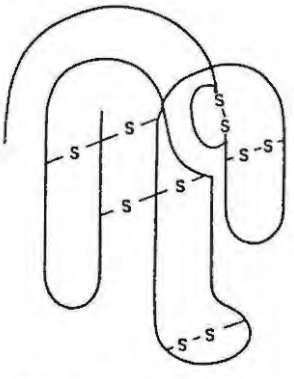
d-Tubocurarine

i



Decamethonium

ii



α-Bungarotoxin

iii

Figure 1.9 **Antagonists of nAChR**

d-Tbc is a specific non-depolarising competitive neuromuscular blocking agent with little or no other actions,

other than interacting with the enzyme, acetylcholinesterase (37, 78). Many well-known alkaloids such as atropine, strychnine, quinine etc, show a marked increase in curaremimetic potency when their nitrogen atoms are quaterinised. Essential factors such as electrostatic attraction, steric influences, and the lipophilic-hydrophilic balance of the molecule determines its activity (37). The concentration of charge on the nitrogen atoms also undoubtedly constitutes to potency. Optical isomerism is an additional factor; the d-form of tubocurarine being 20-60 times more potent than the l-isomer. Bulkiness and rigidity of the molecules are characteristic features, and the distance between the quaternary nitrogens (12.5 Å for d-TbC) indicates a critical feature for curariform action.

Synthesis of methonium compounds with varied polymethylene chain lengths (figure 1.9 (ii)) have helped in deducing the arrangement of the cholinergic site, which is now thought to be tetrameric (37). Thus the interaction of two anionic centres with antagonists appears to prevent any structural rearrangements of the receptor, normally associated with depolarising agents (agonists) (37).

The snake venom toxins, α-bungarotoxin (see figure 1.9 (iii)) and cobratoxin have largely been responsible for the elucidation of the α-subunit of the receptor. The toxins are the "exceptions to the rule" in that they do not interact with acetylcholinesterase as do other nAChR ligands (78). Both are relatively small polypeptides (8 kDa) and are classified as competitive antagonists in that they inhibit the regulatory activity of the receptor complex. Pharmacologically however, they are poorly understood due to their near-irreversible action.

Interaction with nAChR monoclonal antibodies show five-toxin binding regions on the α-subunit, one region being that of the acetylcholine binding site (99). However it is not known whether the five toxin-binding regions form distinct binding sites or are faces of one binding site (99). Recently it has been conclusively shown, using monoclonal antibodies, that α-toxin binding overlaps with both d-TbC sites and agonist binding sites, and that the two α-toxin binding sites are non-equivalent (100).

Binding of the classical antagonist, d-TbC, is complicated by the fact that two differing affinities occur for this ligand by the receptor, i.e. (two binding sites as for α-toxins and Ach but two different affinities) (44, 101, 102). It has been proposed that the two widely differing affinities, for eg. 20 nM and 5μM at equilibrium is a result of

the bifunctionality of the ligand showing a biphasic binding pattern, however other ligands have also shown this. No evidence has been obtained for an interaction of d-TbC with a site other than the Ach binding site (44).

Agonist affinity alkylation of nAChR with bromoacetylcholine has provided evidence that the site which is easily labelled is the site with higher affinity for d-TbC, whilst the other low-affinity site for d-TbC shows lower reactivity towards bromoacetylcholine, and is only labelled under more extreme conditions (103).

Direct binding of d-TbC to purified and membrane-bound nAChR has been studied in detail by Neubig and Cohen (102). They clearly showed that pretreatment with excess α-bungarotoxin or 100 μ M cold d-TbC, completely blocked binding of radiolabelled d-TbC to the receptor; also the interaction between Ach and d-TbC is simply competitive, once the two d-TbC binding affinities are taken into account. However the low-affinity binding component of Ach necessitates very high concentrations of d-TbC (100 μM) to produce total inhibition.

The biphasic nature of binding of d-TbC is insensitive to conditions that alter equilibria between monomeric and dimeric forms (102).

This same study identified a further binding component for d-TbC, that of a non-specific interaction with the membranes characterised as non-saturable (102). Interaction with the ion-channel in the presence of agonist has not been ruled out (4). The issue of whether d-TbC inhibits the receptor by competitive or non-competitive mechanisms has long been controversial and poorly understood (104). Measuring the rate of ion-flux prior to inactivations of the receptor over a wide range of ligand concentrations has shown that d-TbC inhibits both competitively and non-competitively (104). The two widely separate affinities involve the high affinity site, competitively inhibiting Ach binding and the other low affinity site, non-competitively. It seems likely that d-TbC competes for both competitive binding sites and in the absence of agonist does not result in any detectable activation or inactivation of the receptor (104). Evidence has been presented suggesting that d-TbC binds to the same non-competitive inhibitory site on active receptors as cocaine, and not exclusively with the open-channel form as previously postulated (104).

The multitude of effects of acetylcholine and antagonists can be viewed as follows:-

The inhibitory site is different from the regulatory site for activating ligands. Inhibitors binding to the inhibitory site may affect the rate of receptor inactivation (desensitisation) or have no effect on the rate of inactivation (d-TbC). In addition, an inhibitor like d-TbC can bind both to the inhibitory site and the channel-activating ligand binding site, i.e. acetylcholine binding site (104).

Finally mention of a group of antagonists, called the metaphilic antagonists eg. diphenyldecamethonium, convert the receptor to a higher affinity state (desensitised). The rate of conversion appears to be independent of the ligands capacity to activate the receptor; enhanced blockage of receptors occuring in the presence of agonist (79, 89, 91).

1.5.2.2.2 **Non-Competitive**

The third class of cholinergic effectors, the non-competitive antagonists/inhibitors (NCI) are an extremely heterogeneous group of compounds (3, 15). They are defined as ligands that block the nicotinic permeability response without preventing the binding of agonists.

They comprise, aminated local anaesthetics such as procaine, lidocaine, the alkaloid histrionicotoxin (HTX); the hallucinogen phencyclidine (PCP); the neuroleptic chloropromazine, the lipophilic cation triphenylmethyl phosphonium salt ($TPMP^+$); detergents below their critical micelle concentration and fatty acids; finally alcohols and general anaesthetics (the latter are NCI because of their desensitising action, acting probably by affecting the lipid phase surrounding the nAChR) (3, 62).

The following properties are exhibited by many of these compounds: (a) permeation through the open channel, (b) increased antagonistic effect in the presence of increasing amounts of agonists; voltage-sensitivity of antagonism, thought to reflect the effect of transmembrane voltage on these charged compounds as they penetrate the channel, (c) decrease of the mean channel open time; (d) occupation of a single site per

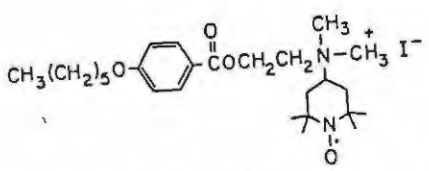
nAChR monomer in contact with all constituent subunits and trapping of the ligand inside the channel on closing before dissociating (1, 15,89, 105).

Two properties that appear to prevail amongst these compounds are the positive charge and hydrophobicity, suggesting certain structural requirements for binding to the NCI binding site (66). Tertiary amine local anaesthetics have pKa's in the region of 8-9 and therefore bear positive charges at physiological pH (105). Benzocaine, an uncharged local anaesthetic only slightly decreases agonist-induced currents (105). It appears therefore that voltage affects the relative potencies of local anaesthetics in blocking agonist-induced currents. At low negative membrane potentials the potency of the anaesthetics parallels their lipid solubility, but at high negative membrane potentials this correlation disappears. The results suggest a dual mode of action: an indirect interaction with the lipid surrounding the receptor, and a direct, voltage-dependent interaction with the receptor-channel complex (105, 106). The functional effects of the more lipophilic agents are poorly understood in comparison to the hydrophilic agents (93).

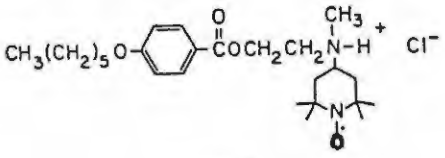
Electron spin resonance has been used to contrast the accessibility of tertiary and quaternary local anaesthetics to their high affinity binding site in the desensitised nAChR (106). Two ligands, C6SLMeI (permanently charged) and C6SL (pH-dependent charge of high hydrophobicity) (see below) have shown the following:

(a) A charged gate in the channel mouth, presumably restricting entry of permanently charged quaternary amine local anaesthetics to the NCI site in the desensitised receptor. Conversely, the uncharged form of the tertiary amine enters with no barrier, and after entering can re-establish its charged state, depending on local solvent polarity and pH, allowing it to bind.

(b) The uncharged form, C6SL, is able to penetrate the lipid bilayer and gain access to the ion-channel. The existence of this hydrophobic path of entry would suggest a delayed onset effect (106).



C6SLMeI



C6SL

In conclusion, NCI bind to sites regulating the ion-channel sterically or allosterically (3).

1.5.3 Ligand accessibility and receptor dynamics

Studies of the effect of a specific ligand on the conformation and dynamics of a protein molecule are of considerable interest. Most non-polar groups in proteins are buried in the interior of the molecule. However some are found on the surface or in pockets in proteins. These may form hydrophobic sites of interaction with small effector molecules, becoming exposed as a result of biologically significant conformational changes in the proteins or during the process of denaturation (107).

Spectroscopic methods have been widely employed in studying these changes; intrinsic fluorescence, as well as fluorescence quenchers have proved potentially useful (107, 108). The advantages of using intrinsic protein fluorescence in studies are twofold: Firstly there is minimal perturbation of the system; and secondly, natural ligands can be used at high concentration without loss of specificity or sensitivity (109).

The nAChR of electric organs has strong intrinsic fluorescence due to its content of 1.4-2.4 mol% L-tryptophan. When excited at 290 nm, the receptor emits fluorescence with a maximum at 336 nm (6). The binding of neurotoxins to nAChR induces a fluorescence change of the receptor because the decrease in fluorescence intensity is much larger than the intrinsic fluorescence of neurotoxin itself. This change is thought to involve a conformational change, enhancing the affinity of the neurotoxin and the receptor by an optimum rearrangement of their local structures and/or allowing them to produce a mutually locked complex (40).

It is well accepted that agonists cause a conformational change of the receptor, and therefore, would alter the intrinsic fluorescence of the receptor (40). However, there are many contrasting findings as to whether interacting cholinergic ligands induce fluorescence changes or not. No changes in intrinsic fluorescence have been reported (90); small changes (4-10%) with agonists (110) and others suggest more marked changes with agonists and antagonists (40). It is important to bear in mind that regulatory functions of the receptor observed *in vivo* might be lost upon solubilising (110), and as previously stated, the formation of an inactive solubilised receptor complex.

Kaneda, N. *et. al.* (110) suggest a 7-8% decrease in fluorescence on binding acetylcholine; 4-5% with carbamylcholine and no change with d-TbC or decamethonium. They observed maximum emission at 345 nm, in contrast to 336 nm previously reported (6, 90), suggesting a hydrophilic rather than hydrophobic environment.

The dynamics of proteins (i.e. fluctuations that facilitate the penetration of a ligand or quencher) will always be coupled to and damped by the surrounding solvent (108). Solvent viscosity is thought to influence the rate of formation of protein complexes (111).

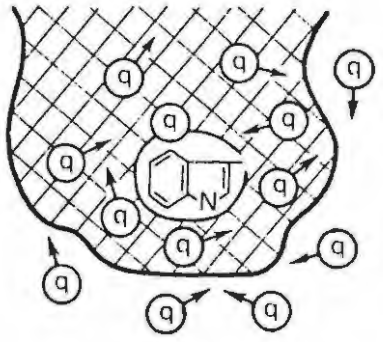


Figure 1.10 **Fluorescence quenching: Dynamic quenching of a fluorophore buried inside the protein globule requires diffusion inside the globule through densely packed groups of atoms (112)**

In addition to sensing the degree of exposure of tryptophanyl residues, quenching studies can be used to reveal certain details concerning the microenvironment of the residue; for example, altering the pH can result in modifications of amino acid side chains and hence altered interactions. It appears then that the accessibility of buried chromophores of proteins to quenchers is based on the dynamic perturbation in protein structure (the dynamic accessibility). These structural perturbations are assumed to be governed by some diffusion - limited processes in the solvent surrounding the protein molecule (112). The fact that at least one tryptophanyl residue exists at the region neighbouring the binding site for cholinergic ligands allows one to further investigate the nAChR.

CHAPTER 2

ISOLATION AND PURIFICATION OF THE NICOTINIC CHOLINERGIC RECEPTOR

2.1 INTRODUCTION

The nicotinic cholinergic receptor of the ray, *Torpedo fuscomaculata*, has,as yet not been isolated and purified. Although few species differences have been noted by our overseas counterparts (5), it nevertheless provided an opportunity to study the structural and functional aspects of this receptor complex.

A pre-requisite for obtaining receptor preparations functioning both in ligand recognition and ion-channel gating is a purification under conditions which protect the receptor from proteolytic attack (11, 25). Homogenisation of the electroplax tissue in the presence of ethylene diamine tetraacetic acid (EDTA) and sulphydryl blocking agents such as N. ethyl maleimide or iodoacetamide has been recommended (11). While this procedure apparently preserves the chemical integrity of the receptor protein, this may not necessarily be the case for its functional integrity (13).

Current techniques for the preparative isolation of proteins use the chromatographic recognition of a variety of physical parameters. The ideal separation method employs an easily identifiable physical parameter unique to the protein of interest (113). Gel filtration, affinity chromatography with biospecific or group specific adsorbents, ion-exchange chromatography and preparative electrophoresis have proved to be versatile separation techniques. Seldom does any one of these procedures provide sufficient resolution or characterisation to obtain a homogeneous protein from a complex biological material. For this reason purification procedures normally combine some or all of these techniques to increase purity in a series of chromatographic/ electrophoretic steps.

It should be noted, that unlike the isolation of proteins with enzymatic function where the activity of the enzyme can be monitored throughout a purification, receptor isolation is accompanied by a concomitant loss of physiological activity, a process dependent upon the integrity of its membrane components (5).

As part of a study in our laboratories on the nicotinic cholinergic receptor it has been our intention firstly, to improve upon the purification of the receptor and secondly, with the advent of new and improved products for protein purification, to re-focus our attention on the classical biochemical techniques such as ion-exchange chromatography and chromatofocusing. Finally, purification by these methods and those of affinity chromatography allow for a comparison of the techniques.

2.2 MATERIALS AND METHODS

2.2.1 Materials

All reagent solutions were freshly prepared in doubly distilled/deionised water or appropriate buffer.

Buffer-solutions (ll4):

Tris-HCl (0.1 M/l at pH 6.6; 7.6 and 8.8). Sodium phosphate (0.1 M/l, pH 8.0). Sodium acetate (0.1 M/l, pH 4.0 containing 0.5 M/l NaCl) and Imidazole-HCl (0.025 M/l, pH 7.4) were prepared.

Reagents

Pure *Naja Naja* venom (α-cobratoxin) was purchased from the Council for Scientific and Industrial Research, Pretoria, South Africa.

Sodium azide and ethylene diamine tetraacetic acid (EDTA) were obtained from Aldrich Chemical Company, Ltd. Gillingham, England.

Tween-20 and Coomassie brilliant blue were obtained from Merck, Darmstadt, West Germany.

Polybuffer exchanger (PBE 94); Polybuffer 74; Sephacryl S-400, S-200 and cyanogen bromide Sepharose 4B

were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

Phenylmethylsulphonyl fluoride; carbamylcholine; DEAE-Sephadex A-50; DEAE-Sepharose 6B; sodium dodecyl sulphate; acetylthiocholine chloride; molecular weight standards and d-tubocurarine were obtained from Sigma Chemical Company, St. Louis, U.S.A.

Triton X-100; bromophenol blue and β-mercaptoethanol were obtained from BDH Chemicals, Ltd. Poole, England.

Albumin fraction V (Bovine serum); 5,5-dithiobis-(2-nitrobenzoic acid (DTNB) and acetylcholinesterase were obtained from Boehringer Mannheim, West Germany.

N, N, N′, N′-tetramethylenediamine (TEMED); acrylamide and N,N- methylene-bis-acrylamide were obtained from Fluka AG, Chemical Fabriek, Switzerland.

Dialysis membrane tubing with molecular weight cut-off 6000-8000 Da was obtained from Spectropor, Los Angeles, U.S.A.

Ammonium sulphate and ammonium persulphate were obtained from Koch-Light laboratories Ltd. South Africa.

Glycine was obtained from Saarchem (Pty) Ltd. South Africa.

2.2.2 Methods

Live electric rays (*Torpedo fuscomaculata* (115)) (see figure 2.1) were caught in the Bushman's estuary off the South East coast of South Africa by the author. Figure 2.2 shows the necessary equipment and figure 2.3 a *Torpedo* ray demonstrating its defence mechanisms.

Figure 2.1

Figure 2.2

Figure 2.3

2.2.2.1 Extraction of Receptor

Electric organs were excised from a freshly killed *Torpedo* ray and stored at -80°C until required. Electric tissue (120 g) was homogenised (2 minutes) in either imidazole buffer (0.025 M/l, pH 7.4, 150 cm^3) or Tris-HCl buffer (0.1M/l, pH 7.6, 150 cm^3) containing phenylmethylsulphonyl fluoride (0.1 mM), EDTA (10 mM) and sodium azide (0.01%) (8). The homogenate was then centrifuged (20 000 x g, 60 min, 4°C) and the supernatant discarded. The pellet was resuspended in the same buffer solution and Triton X-100 added to a final concentration of 1%. The mixture was stirred (18 h, 4°C) and then centrifuged (100 000 x g, 60 min, 4°C), the pellet resuspended in the above buffer, centrifuged again and the supernatants pooled. Receptor activity and protein content were determined.

2.2.2.2 Affinity Chromatography

The affinity resin CNBr-activated Sepharose 4B (40cm^3) was incubated with α-cobratoxin (15 mg) for 18 h at 4°C (29). This was treated with 0.2 M/l glycine pH 8.0 and then washed alternatively with coupling buffer ($NaHCO_3$ 0.1M, pH 8.3 containing 0.5 M NaCl) and acetate buffer (0.1 M, pH 4.O containing 0.5 M NaCl). This was poured into a column (7.5 x 2.6 cms) and washed with Tris-HCl (0.1 M/l, pH 7.6). 10 cm^3 of partially purified receptor material was allowed to filter through the gel matrix. After all the receptor material had been applied, an incubation period of 2 hours at 20°C was allowed. Three washing cycles were then used to remove unwanted material: extraction buffer containing 0.1% Triton X-100 alternating with 1M NaCl. Fractions (3.0 cm^3) were collected and monitored for absorbance at 280 nm. Carbamylcholine (1M, 20 cm^3) in Tris-HCl buffer was then passed into the column, incubated (5 hours) and fractions (3.0 cm^3) collected and monitored for absorbance at 280 nm. Receptor activity, enzyme activity and protein content were determined (6, 8).

2.2.2.3 Ion-Exchange Chromatography

The resins (DEAE - Sephadex A-50 and DEAE - Sepharose 6B) were equilibrated in Tris-HCl buffer (0.1M/l, pH 7.6) and poured into separate columns (10 x 2.6 cms). The partially purified receptor (10 cm^3) in Tris-HCl buffer (0.1 M/l) was filtered through the gel matrix at a rate of 80 cm^3/h, fractions (3.0 cm^3) collected and monitored for absorbance at 280 nm. After all the receptor solution had been applied the column was washed with Tris-HCl buffer (0.1 M, pH 7.6) until the absorbance of the effluent had dropped to base-line levels. A linear gradient of sodium chloride was then started with a flow rate of 80 cm^3/h, fractions (3.0 cm^3) collected and monitored for absorbance at 280 nm. Receptor activity, enzyme activity and protein content were determined.

2.2.2.4 Chromatofocusing

The receptor extract (10 cm^3) in imidazole buffer (0,025 M/l, pH 7.4) was added to a chromatofocusing column (11 x 1.6 cms) previously equilibrated and washed successively with imidazole buffer (0.025 M/l, pH 7.4) and Polybuffer 74 (5 cm^3, pH 4.0) (27). The column was eluted with Polybuffer 74 (200 cm^3, pH 4.0) at 54 cm^3/h, fractions (3.0 cm^3) collected and monitored for absorbance at 280 nm. Finally the column was washed with sodium chloride (1M, 50 cm^3) to elute proteins not displaced by low ionic strength. Receptor activity, enzyme activity and protein content were determined.

A pH calibration curve was prepared using cytochrome C (pI = 10.1) (8 mg) and Bovine carbonic anhydrase (pI = 6.57) (8 mg).

2.2.2.5 Ammonium Sulphate Fractionation

Ammonium sulphate was added to the receptor active fractions from each of the affinity chromatographic, ion-exchange and chromatofocusing stages. 100% saturation was achieved, stirring on ice for 3 hours. The precipitate was collected by centrifugation (10 000 g/ 10 mins) redissolved in Tris-HCl buffer (0.1 M/l, pH 7.6, 1.0 cm^3), dialysed against the same buffer (2 l, 4°C, 4 h) and finally lyophilised.

2.2.2.6 Molecular weight determinations

(a) Gel chromatography

The molecular weight of the material obtained from the different methods was determined by chromatography on Sephacryl S-200 and S-400 in Tris-HCl (0.1M/l, pH 7.6). The S-400 column (40 x 1.6 cms) was standardised with Blue dextran (2 x 10^6 Da), lactate dehydrogenase (140 000 Da), DNase (63 000 Da) and carboxypeptidase A (34 300 Da) (Appendices 1 and 2). Purified material (15 mg) in 1.0 cm^3 was applied to the column and eluted with the same buffer, at a rate of 33 cm^3/h, fractions (3.0 cm^3) collected and monitored for absorbance at 280 nm. The S-200 column (30 x 2.6 cms) was standardised with the above standards excluding DNase at a flow rate of 75 cm^3/h.

(b) Disc-Polyacrylamide gel electrophoresis

Electrophoresis of the purified material was carried out using the Laemmli method (45) on SDS slab gels. Resolving gel (10%) and stacking gel (4%) were prepared from the acrylamide-bis acrylamide mixture in 0.1 M/l Tris-HCl, pH 8.8 and pH 6.8 respectively, polymerised using 0.025 % TEMED and ammonium persulphate (1.5% W/V). The upper and lower buffer compartments were filled with 0.1% SDS - TRIS-HCl buffer. Standards ovalbumen(45 000 Da), BSA (68 000 Da), catalase (60 000 Da), LDH (36 000 Da) and carboxypeptidase A(34 300 Da) were used at 1 mg/ cm^3 to prepare a standard curve (appendix 3).

The sample (50 µl) was treated with dissociation buffer (50 µl) and applied to the gel. A potential of 100 mV was applied for 30 min and then 150 mV for 3 h until the tracking dye was within 5 mm of the bottom of the gel. Protein was stained with a 0.2 % solution of Coomassie Blue in a mixture of methanol-water-glacial acetic acid. Destaining was achieved by extensive washing in methanol-water-glacial acetic acid. The gels were vacuum dried (4h) and stored.

2.2.2.7 **Protein Determination**

Protein content was determined by the modification of the Lowry method (116) using bovine serum albumen as the standard (appendix 4).

2.2.2.8 **Assay for acetylcholinesterase**

The method of Ellman (117) was used. Acetylthiocholine is hydrolysed by AChE to acetic acid and thiocholine, which then reacts with DTNB (see materials) to form a yellow anion. The increase in absorbance is monitored spectrophotometrically at 410 nm.

2.2.2.9 **Assay for Receptor**

A fluorescence titration assay is described here which allows a rapid and reproducible means for determining receptor concentrations without many of the difficulties associated with the use of radiochemicals (102). d-Tubocurarine, a specific competitive antagonist of the nAChR, shows similar affinities for both membrane-

bound and solubilised receptor and hence includes its use as a fluorescence quenching ligand (24). Since the excitation light decreases along the path due to the absorption by d-TbC, the decrease in the tryptophanyl fluorescence is corrected by dividing the apparent intensity (Fa) by the light intensity in the sample cell, as

$$Fc = Fa/10^{Eca}$$

where Fc is the corrected fluorescence intensity, E and c are molar extinction and molar concentration of d-TbC, and "a" is an instrumental constant which was found to be 0.588 (110).

Fluorescence measurements were made using a Hitachi fluorescence spectrophotometer with excitation light source from a Xenon 150 lamp. The spectra were taken at an angle of 90^{o} to the exciting light. A quartz cuvette consisting of sample (3.0 cm^3) in appropriate buffer was titrated against increasing d-TbC concentrations until Fc reached a maximum indicating saturation of binding sites and hence concentration of receptor. An excitation wavelength of 290 nm and emission wavelength of 340 nm were chosen.

2.3 RESULTS

2.3.1 Extraction of Receptor

The non-denaturing detergent Triton X-100 was effective in solubilising the receptor after an incubation period of 18 h. From an initial 120 g of electric organ, 2.52 g of material was removed by low speed centrifugation with a specific activity of 0.53 μM/g. High speed centrifugation, in the presence of Triton X-100, resulted in 1.41 g of protein with a specific activity of 0.84 μM/g. This gave a 30% yield of semi-purified receptor material.

2.3.2 Affinity Chromatography

Extraneous protein material was removed by adding alternatively Tris-HCl buffer/0.1% Triton X-100 and 1 M NaCl (elution profile not shown).

Carbamylcholine proved successful in recovering free nAChR from the Sepharose column in 20 cm^3. 76 mg of protein with a specific activity of 3.03 μM/g was recovered. The receptor active fractions were found to be devoid of any enzyme activity.

Affinity chromatography results in a 7.1 fold purification and a 5.84 % yield. Saturation with ammonium sulphate and lyophilisation improved this fold purification to 12 with a 1.5% overall yield and 5 μM/g specific activity.

Figure 2.4 represents a gel chromatogram for the determination of the molecular weight of the purified receptor on a Sephacryl S-400 column. A major broad band with a peak maximum at 57 000 ± 1000 and Ve of 81 cm^3 is present. A small amount of material (400 000) appears in the void volume (V_o = 31 cm^3) (Appendix 1)

Figure 2.5 is an electrophoretogram of SDS-PAGE analysis of the purified receptor.Three major bands are prominent (i) 66 000 ± 1 000; (ii) 63 000 ± 1 000 and (iii) 40 000 ± 1 000. A minor band appears at 52 000 ± 2 000.

2.3.3 Ion-Exchange Chromatography

It can be seen that the receptor adsorbs tightly to the anion-exchange gel (figure 2.6). Removal of extraneous protein and Triton X-100 is achieved at low ionic strength. Increasing the NaCl concentration produced two peaks, the first of low receptor activity corresponding to a low molecular weight species of about 36 000 while the second (0.6 M NaCl) had higher activity. Fractions of the second peak (24 cm^3) consisted of 60 mg of protein with a specific activity of 2.0 μM/g. This fraction was found to have negligible enzyme activity.

Other ion-exchange resins such as DEAE-Sephadex A-50 were found to be totally inadequate in purifying the receptor protein. They gave broad elution peaks over a wide range of ionic strength with poor resolution.

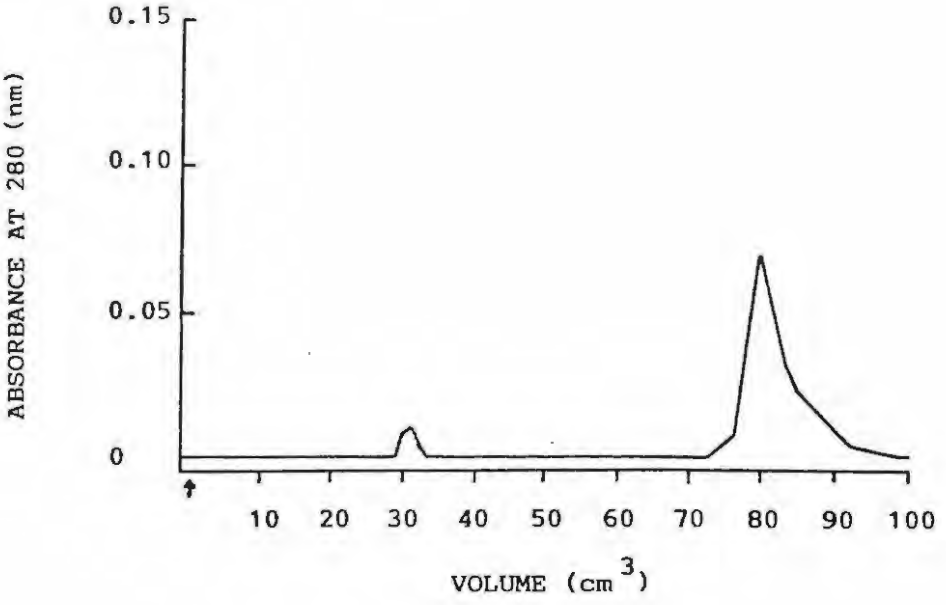


Figure 2.4 **Gel chromatogram of lyophilised sample from Affinity chromatography on Sephacryl S-400 gel.**

↑ - application of sample (1.0 cm^3)

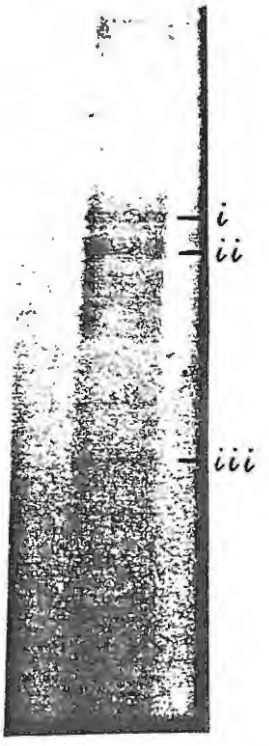


Figure 2.5 **SDS-PAGE electrophoretogram.**

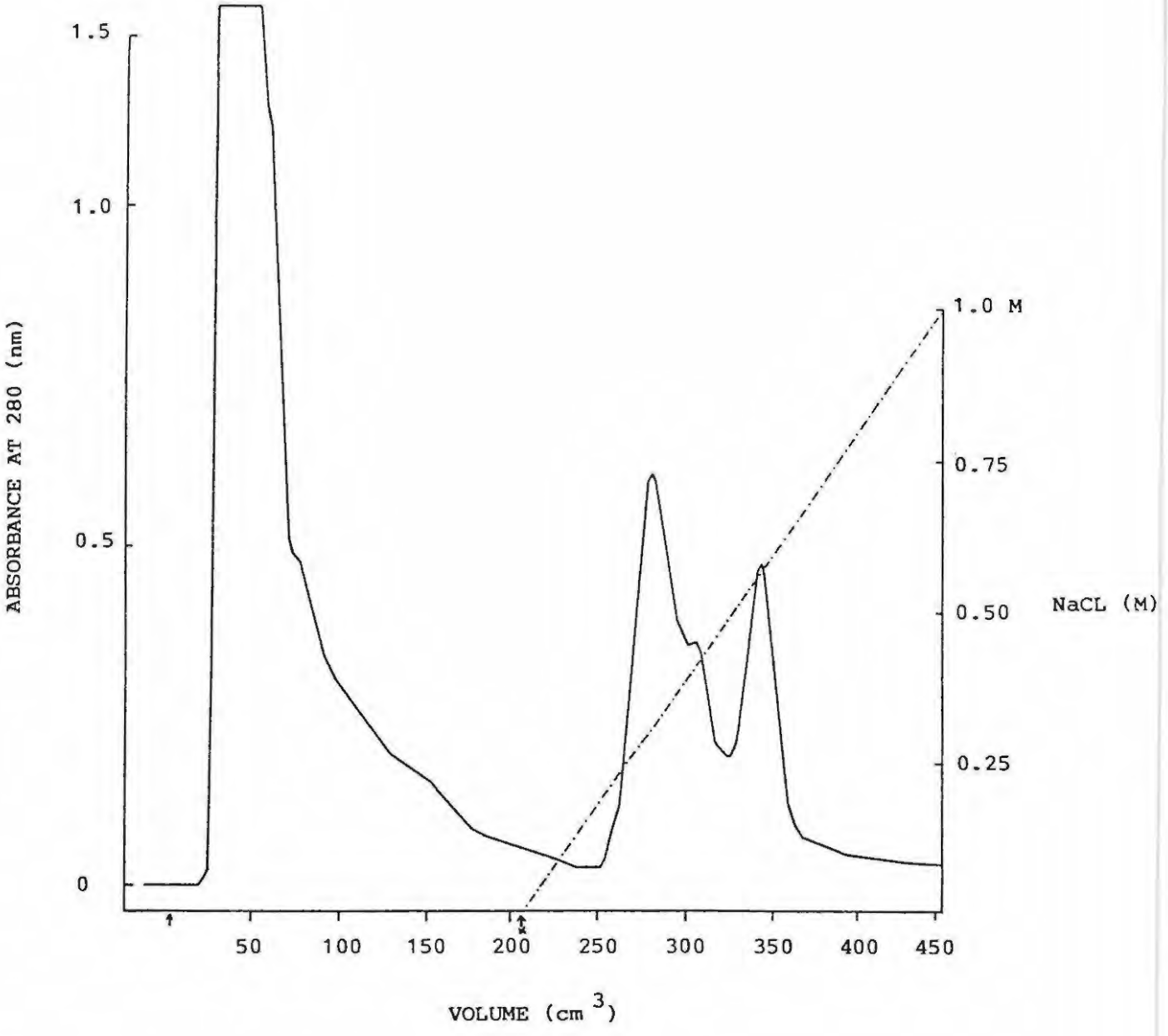


Figure 2.6 **Ion-Exchange chromatography of solubilised nicotinic cholinergic receptor.**

Column dimensions (10 x 2.6 cms) application of sample (10 cm^3)

↑- application of sample (10 cm^3)

----↑ Gradient (0.1M - 1.0M)
x

Ion-exchange chromatography gave a fold purification of 5.0 and a 3% yield. Saturation with ammonium sulphate and lyophilisation increased the fold purification to 7 with a 1% yield. The specific activity was 3μM/g.

Figure 2.7 shows the gel chromatogram on Sephacryl S-400 and figure 2.8 the SDS-PAGE electrophoretogram.

In figure 2.7 two major molecular weight species are observed - 125 000 ± 1000 at 72 cm^3 Ve and 44 000 ± 1000 at 88 cm^3 Ve. A minor peak at 81.5 cm^3 Ve represents a 66 000 component and a small peak in the void volume accounts for receptor dimer. The electrophoretogram in figure 2.8 shows several bands - (i) 66 000 ± 1000; (ii) 63 000 ± 1000; (iii) 53 000 ± 1000 and (iv) 40 000 ± 1000. Bands below 40 000 and between 80 000 and 100 000 are also present.

2.3.4 **Chromatofocusing**

The results of the chromatographic separations are shown in figure 2.9. It can be seen that the removal of Triton X-100 and extraneous protein occurs rapidly. Elution of the active receptor in a sharp peak is readily accomplished by a step increment in ionic strength to 1M NaCl. The chromatofocusing step (16 cm^3) consisted of 132 mg of protein with a specific activity of 3.2 μM/g.

The pH of the receptor active fractions was found to be 5.16 (not shown in figure 2.9) indicating the need for an increase in ionic strength for elution of receptor protein. A 7.5 fold purification and a 10% yield is improved to a 12.5 fold purification and a 2.3% yield after ammonium sulphate treatment and lyophilisation. Negligible enzyme activity was indicated.

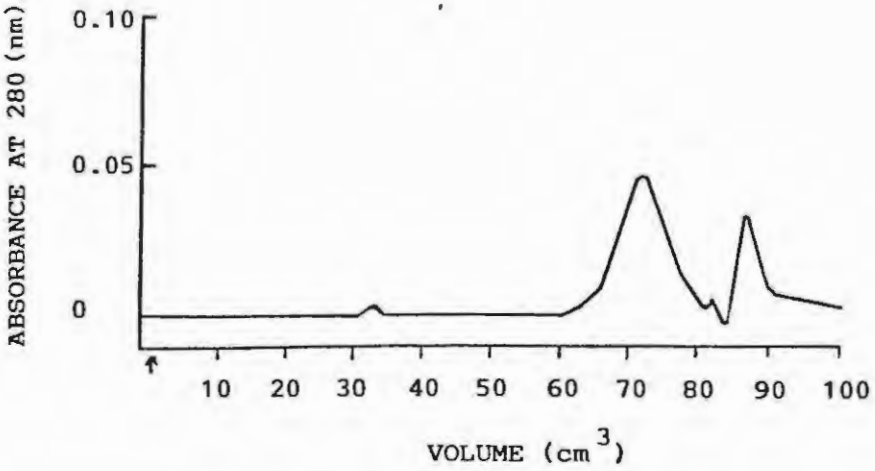


Figure 2.7 **Gel chromatogram of lyophilised sample from Ion-exchange chromatography on Sephacryl S-400 gel.**

↑ - application of sample (1.0 cm^3)

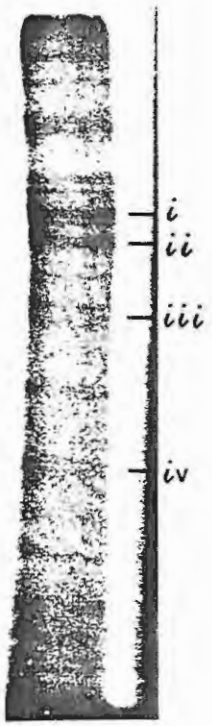


Figure 2.8 **SDS-PAGE electrophoretogram**

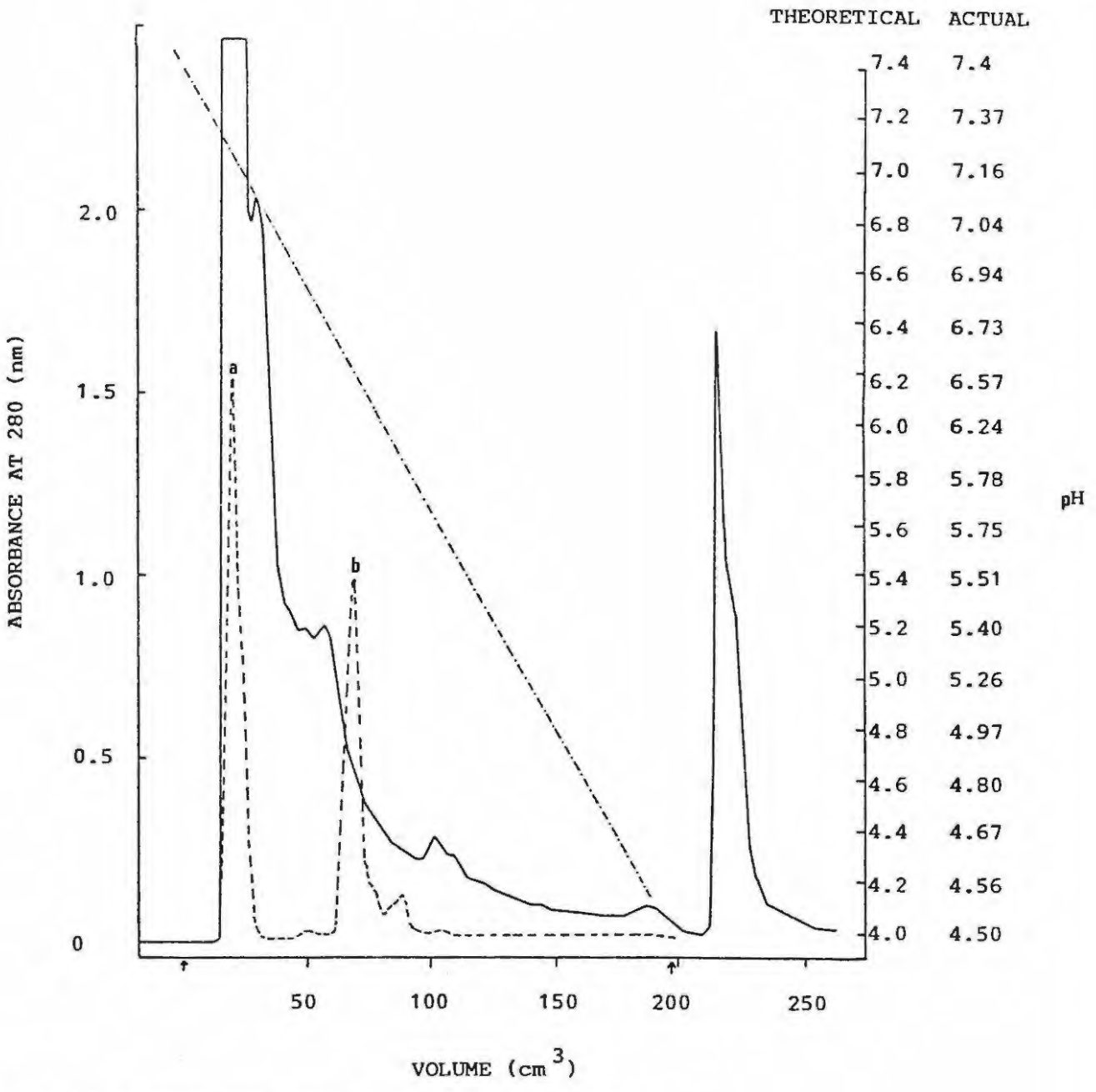


Figure 2.9 **Chromatofocusing on PBE 94 gel of solubilised nicotinic cholinergic receptor.**

Column Dimensions (11 x 1.6 cms)

↑ - application of sample (10 cm^3)

↑ - 1M NaCl (50 cm^3)

-·-·- pH gradient

---- pH Calibration curve

[a] cytochrome C

[b] Bovine carbonic anhydrase

The gel chromatogram (figure 2.10) demonstrates 3 major peaks - 123 000 ± 1000 at 73 cm^3 Ve; 63 000 ± 1000 at 81 cm^3 Ve and 56 000 ± 1000 at 82.5 cm^3 Ve. A minor peak at 89 cm^3 Ve represents a 40 000 molecular weight unit. Two smaller peaks, one in the void volume represents receptor dimer and another at 65 cm^3 Ve a molecular weight of 230 000.

SDS-PAGE analysis (figure 2.11) shows several bands (i) 95 000 - 100 000; (ii) 66 000 ± 1000; (iii) 63 000 ± 1000; (iv) 53 000 ± 1000 and (v) 44 000 ± 1000. Other bands present are those of (vi) which represents material of 8000 - 10 000 molecular weight and 2 - 3 bands between 75 000 and 95 000.

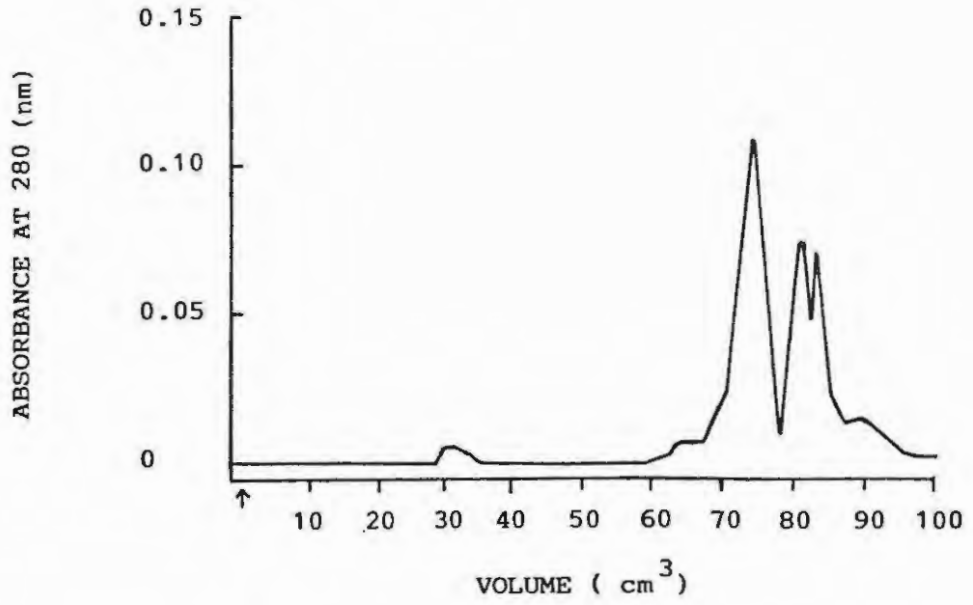


Figure 2.10 **Gel chromatogram of lyophilised sample from Chromatofocusing on Sephacryl S-400 gel.**

↑- application of sample (1.0 cm^3)

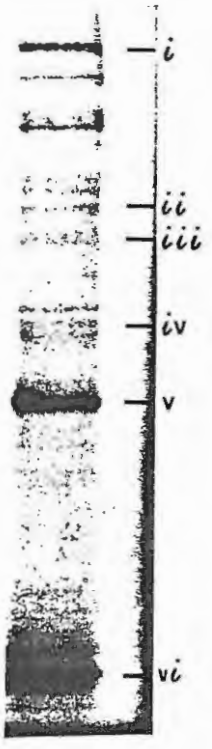


Figure 2.11 **SDS-PAGE electrophoretogram**

2.4 DISCUSSION

The electric organ of the *Torpedo* ray is advantageous for the isolation of the nicotinic cholinergic receptor because it represents a homogeneous high density population of synapses all of which use the cholinergic mechanism. The electric organ is also the richest source of acetylcholine receptor known (1, 5).

Freezing and thawing of tissue was kept to a minimum during the extraction procedures since the probability of proteolytic attack by intracellular proteases during these cycles is great (7). No noticeable differences were observed however when organs which had been stored at -20^{o}C for several months, were used instead of fresh ones.

The high affinity and specificity of the α-neurotoxins for the receptor (18) facilitate the removal of unwanted material on an affinity chromatographic system. Only 5-10% of the toxin retains its ability to bind receptor protein (5, 9, 12, 22, 28), resulting in a low recovery.

The amount of receptor protein recovered compares well with 4-6 mg from 200 g (8). A 5.0 μM/g specific activity is also in agreement with the literature (2.0-12.0 μM/g) (2, 6, 36, 38). It should be noted that the activity of the receptor preparations have been monitored by the receptors ability to quench the fluorescence of d-TbC and not that of the α-neurotoxins. The slightly lower specific activity is possibly due to some non-specific interactions between d-TbC and the crude-receptor extract.

A fold purification of 7.1 is low in comparison to other reported findings. After ammonium sulphate treatment and lyophilisation there was an increase in purification to 12-fold which is in agreement with the literature (5). It is reasonable to suppose that the popularity of affinity chromatography stems from its ability to achieve high fold purifications of proteins. Disadvantages are clearly the low recovery of receptor material (5.84% yield), the time taken to perform affinity chromatography (2 days) and possible modified binding properties of the protein once recovered (25).

Estimation of the molecular weight of the receptor protein under non-denaturing conditions revealed a small amount of receptor dimer linked through a disulphide bond between δ-δ subunits. This is to be expected since the receptor exists in a dimeric state in the membrane (13). The absence of monomer units (250-270 kDa) (2, 3, 11) appears to indicate proteolysis of receptor material into individual subunits. This is surprising since maximum precautions were taken. All solutions contained Triton X-100, for preventing aggregation of receptor protein, and certain protease inhibitors such as EDTA and phenylmethylsulphonyl fluoride. Sodium azide was also included to inhibit bacterial growth.

The broad band (figure 2.4) at 81 cm^3 Ve indicates poor resolving power of the system. Investigation of the gel chromatograms of both ion-exchange and chromatofocusing however suggest this not to be the case. It appears to indicate a number of subunits that are intimately but non-covalently associated.

Under denaturing conditions, i.e. in the presence of SDS, the δ-subunit (66 kDa), γ-subunit (63 kDa) and α-subunit (40 kDa) are represented. The sensitivity of the β-subunit (53 kDa) to degradation is evident.

Purification of the receptor by ion-exchange chromatography has in the past proved poor in comparison to techniques such as affinity chromatography. Elution of receptor protein in the presence of 0.5 M NaCl, using DEAE-Sephadex A-25 in conjunction with affinity chromatography, had proved however more advantageous (5, 22). DEAE-Sepharose 6B (an anion-exchange gel) has shown that recovery and purification of receptor is nearly as efficient as affinity chromatography. This, and the fact that poor resolution was obtained using DEAE-Sephadex A-50, clearly demonstrates an improved product for protein purification allowing for increased resolution.

Recovery of 2.5 mg of protein at 0.6 M NaCl is only slightly lower than that of the affinity chromatographic step, however a 3 μM/g specific activity reflects a lower fold purification.

The removal of receptor protein material within the first peak (figure 2.6), indicates extensive proteolysis of the receptor in the absence of protease inhibitors and Triton X-100. It was envisaged that the increased ionic strength would minimise proteolysis and simultaneously prevent any clustering of receptor protein. The lower

recovery (1%) after ammonium sulphate and lyophilisation is therefore a result of proteolytic nicking, while the lower fold purification is due to the recovery of non-specific proteins with the receptor.

Ion-exchange chromatography is inefficient in purification of proteins since it allows many proteins to elute under similar conditions and large volumes of eluting buffer are required to cause elution of receptor.

Molecular weight determinations under non-denaturing conditions reveal receptor-dimer and a component of approximately 130 kDa (δ-δsubunits). This is in agreement with many other findings (11, 15, 43, 47). Similarly, as was found with affinity chromatography, receptor-monomer units are absent from the chromatogram. Increased presence of individual unit (figure 2.7) over those of figure 2.4 show that the extent of proteolysis was greater using ion-exchange chromatography than for affinity chromatography. The δ-subunit (66 kDa) and the α- subunit (44 kDa) are represented in the chromatograms.

Under denaturing conditions, all four bands of the receptor are observed: α (40 kDa), β (53 kDa), γ (63 kDa) and δ (66 kDa). The results, however, show considerable impurities whether by contamination of the gels (118) or by proteolytic action from within the receptor samples themselves. Even though the latter appears to be true the former possibility cannot be ignored. Samples could easily have been subjected to contamination during experiments and lyophilisation. Ochs (118) has reported many artifacts and confusing results observed on SDS-PAGE due to contaminant proteins such as skin keratins.

Another explanation of the heterogeneity of the gels is the concept that the nAChR protein is highly associated in the membrane with other specific binding proteins (22). Treatment with Triton X-100 might lead to co-solubilisation such that the integrity of the protein-protein interactions are undisturbed. The nAChR can be eluted from the ion-exchange chromatography with the associated proteins still attached.

The presence of a cytoskeletal element (43 K protein), which is functionally associated with the receptor (48), is also suspected.

Of the classical biochemical techniques, isoelectric focusing had proved to be the best for purifying the

receptor protein achieving a 6.3-fold purification (28). Chromatcfocusing provides an alternative and offers advantages in that: (a) no special equipment is needed, (b) the method saves time, (c) no problems exist with cooling, (d) special care is taken to attain low optical density in the buffers used and so rapid removal of Triton X-100 and extraneous protein, and (e) different proteins are separated according to isoelectric points without cross contamination.

The use of 1 M NaCl proves useful in that clustering of receptor monomers is prevented in the absence of Triton X-100, furthermore it allows rapid recovery with small volumes of the protein. The fact that the receptor protein does not elute during the salt gradient is because of its low pI value and also its tendency to adsorb to the gel. One would require an extremely low pH to elute this protein at low ionic strength buffer.

Recovery of 6 mg of receptor protein of 5.5 μM/g specific activity is better than that achieved by both affinity and ion-exchange chromatography and in good agreement with the literature (8).

Molecular weight analysis under non-denaturing conditions reveals similar species to those from ion-exchange chromatography. This suggests that the purifications exhibit nearly identical patterns on Sephacryl S-400 chromatography strongly arguing that a unique population of protein molecules is being selectively isolated. The presence of receptor monomer, even after extensive proteolysis, indicates an increased amount of purified receptor material. This is not surprising since a step-increment in ionic-strength facilitates removal of all adsorbed receptor material in a small volume. All the subunits (α, β, γ and δ) are represented with molecular weights in excellent agreement with other reported findings (2, 42, 44). That extensive degradation of the receptor has occurred is clearly evident in figure 2.10. The low fold purification and recovery is evidently related to the extent of proteolysis, since the activity of proteolytically degraded receptor monomer/dimer units would be very low. Competitive antagonists, such as d-TbC, preferentially interact with receptors in a resting rather than desensitised (inactive) receptors.

Receptor material in the presence of SDS reveals the α (44 kDa), β (53 kDa), γ (63 kDa) and δ (66 kDa) subunits. Similarly, as for ion-exchange chromatography, the presence of several bands indicates impurities whether by contamination or by proteolytic attack. The latter may possibly arise from incomplete inhibition of

the proteases during purification so that on SDS-PAGE electrophoresis only the α-subunits (40-45 kDa) are apparent (11). Nicking of the β, γ and δ-subunits might also account for the considerable number of minor bands in molecular weight range of 54-57 kDa and 65-68 kDa observed in the gel electrophoretogram.

The presence of molecular weight units greater than 68 kDa indicates insufficient purity of the receptor preparation (3). Integral enzymes such as protein kinases and/or proteases are suspected. Units below 40 kDa i.e. (band (vi) figure 2.11) indicate severe proteolysis. This can be compared to the treatment of receptor with trypsin or chymotrypsin rendering components in the order of 8 kDa and less (6).

A comparison of all three chromatographic procedures (i.e. ion-exchange chromatography, chromatofocusing and affinity chromatography) shows chromatofocusing to be more advantageous, firstly because of the time and cost that is saved and secondly, since all three methods use commercial Sepharose as the gel-matrix; nAChR adsorbing to the gel would be removed by 1M NaCl. Only tightly bound receptor-neurotoxin complexes would be retained in affinity chromatography resulting in extremely low recoveries.

The disadvantages of both ion-exchange chromatography and chromatofocusing is that non-specific proteins are removed simultaneously with the receptor. Purification of proteins on the basis of their isoelectric points is superior to variations in salt concentrations. The difference in fold purification and recoveries of the two methods show this to be the case.

The estimated degree of purity, based upon electrophoretic analysis, before and after chromatofocusing was judged to be greater than 90%. The extent of proteolysis however suggests the requirement of a "cocktail" of protease inhibitors present throughout the purification. For chromatofocusing and ion-exchange chromatography dilution of these inhibitors has occurred resulting in the observed electrophoretograms.

The fact that cloning of the receptor has recently been made possible (15), has allowed an accurate assessment of the subunit molecular weights of the receptor; α-subunit (50116 Da), β (53681 Da), γ (56279 Da) and δ-subunits (57565 Da). The differences between these values and those of SDS-PAGE are best explained by the amount of glycosylation (10-11% on subunits) and detergent present (Triton X-100)-(11%). Since both add

weight to the subunits, the differing molecular weights reported by the three methods can be attributed, firstly to these factors and also to the characteristics of the individual techniques for purifying the receptor.

From chromatofocusing a pI value of 4.5-5.0 can be assumed for the receptor. Based on the strict criteria for assessing purity however, our findings reveal a heterogeneous receptor preparation.

Whether a true receptor, (i.e. a protein with its essential functional associating elements) can be isolated, is not completely satisfied. Chromatofocusing in the presence of substantial protease inhibitors, proves to be the method of choice for purifying the receptor.

CHAPTER 3

PHOTOCHEMISTRY OF NITROSAMINES AND RECEPTOR - LIGAND INTERACTIONS

3.1 INTRODUCTION

Research in our laboratories and others (69) has shown the nitrosamines to be weak competitive inhibitors of acetylcholinesterase. Like most acetylcholinesterase ligands the nitrosamines should therefore interact with the receptor (85).

Their potential in photoaffinity or energy-transfer labelling has been discussed in chapter 1 . The criteria, necessary for active site labelling by photoaffinity or energy-transfer mechanisms appear to be met by these compounds.

It was envisaged that these compounds and/or derivatives might improve detection of the solubilised receptor through covalent linkage of residues at the acetylcholine binding site. It was hoped, depending on the specificity and reactivity of these ligands, that an improved purification of the receptor could be achieved.

In this chapter, the chemistry of the nitrosamines is dealt with firstly. Secondly, the interaction with semi-purified and purified receptor material and finally the proposition of synthesising and preparing derivatives of these compounds. The aim: that of a highly specific and photosensitive ligand of low Kd and with considerable structural rigidity such that it possesses the geometrical requirements for receptor activation.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Buffer solutions (114):

Tris-HCl (0.1 M/l, pH 7.6) and Imidazole-HCl (0.025 M/l, pH 7.4)

Reagents

Dimethylnitrosamine (DMN) was obtained from Eastman Kodak Company, Rochester, New York, U.S.A. Purity checked by infra-red spectroscopy.

Acetylcholine chloride and diphenylnitrosamine (DPN) (mpt. 66-67^{o}C) were obtained from Sigma Chemical Company, St. Louis, U.S.A.

Trifluoroacetic acid was obtained from Holpro Analytics (Pty) Ltd. South Africa.

3.5 dinitrobenzoylchloride was obtained from Merck, Schuchardt, West Germany.

Electric organ acetone powder (*Electrophorus electricus*) was purchased from Sigma Chemical Company, St. Louis, U.S.A. A 1% Triton X-100 extract was prepared by centrifuging at 10 000 x g/10 mins at 4^{o}C. The pellet was discarded and supernatant kept for further studies.

All other reagent solutions were prepared as in chapter 2.

3.2.2 Methods

3.2.2.1 Spectroscopy and Photolysis

A multiwavelength Bausch and Lomb 1001 Spectrophotometer measured the changes in absorbance of dimethylnitrosamine and diphenylnitrosamine at 12 programmed wavelengths from 230 nm to 380 nm. 3.0 cm^3 samples in a quartz cuvette, consisted of DMN (454μM/l) or DPN (100μM/l), heptane and CF_3COOH (0.43 M/l). A reference cuvette contained heptane and/or CF_3COOH for determining the absorbance of DMN and DPN.

A similar scan was performed using a Beckman U.V. 5240 with dual reference and sample holders allowing an accurate determination of maximum and minimum absorbances with graphical display.

The U.V. spectra of DMN (136 mM/l) in imidazole buffer (pH 7.4) and in dilute acid (pH 1-3) were examined using the 1001 Spectrophotometer. Irradiation of these 3.0 cm^3 samples for 5 mins and 30 mins was carried out with a 125 W Hanovia (Hg) lamp (Slough, England), placed at a distance of 5 cm from the quartz cuvette.

Fractions consisting of suspected active receptor from the chromatofocusing column (chapter 2) were subjected to photolysis (5 mins) from the above lamp, in the presence of DMN (2.83 mM/l). Control and experimental samples (3.0 cm^3) were observed spectroscopically as above. These same samples were then dialysed for 5 h against 0.025 M/l imidazole buffer pH 7.4 and U.V. spectra recorded.

Partially purified electric organ acetone powder (20 mg) in 3.0 cm^3 Tris-HCl buffer, pH 7.6 was suspended with 1% Triton X-100 or 1% Tween-20 (18 h, 4°C). This was centrifuged at 10 000 x g/10 mins and the supernatant retained. 100 µl samples (approximately 100 $\mu g/cm^3$) of receptor material and DMN (10 mM/l) were treated as for the chromatofocusing-DMN samples as above. The irradiated and non-irradiated samples (3.0 cm^3) were then applied to a Sephacryl S-200 column (as in chapter 2), eluent monitored at 280 nm.

3.2.2.2 Fluorimetry

Fluorimetric observations of the receptors intrinsic fluorescence in the presence of DMN and other cholinergic ligands were investigated using the Hitachi fluorescence spectrophotometer (cf. 2.2.2.9). Purified receptor material (100 $\mu g/cm^3$) in 3.0 cm^3 was excited at 290 nm with emission at 340 nm. The absorption of d-TbC at this wavelength was taken into account (cf. 2.2.2.9).

3.2.2.3 Organic synthesis

An attempt was made at derivatising DMN (100 mM/l) with 3,5 dinitrobenzoyl chloride (100 mM/l). The reaction was carried out in dry ethanol or tetrahydrofuran under nitrogen with stirring and heating (50°C). Samples were taken at various time intervals for a period of 72 h. Analysis was carried out using thin layer chromatography (TLC) (0.2 mm $^{60}F_{254}$ aluminum sheets, silica gel; ethylacetate: hexane)(1:1).

3.3 RESULTS

3.3.1 Photochemistry

From table 3.1 it can be seen that dimethylnitrosamine absorbs maximally between 230 and 240 nm, and shows maxima in the region of 360 nm. Scanning spectroscopy indicated 4 peaks: 233 nm (maximum); 351, 362 and 374 nm. In the presence of saturating concentrations of CF_3COOH new maxima at 240 nm and 325 nm were established.

Table 3.2 gives the absorbance maxima for DPN at 230 nm and between 280 and 300 nm. In the presence of CF_3COOH an immediate reaction occurred, visible by the colour change from yellow to blue. A peak in absorbance at 260 nm and a maximum absorbance in the visible region were established. Due to the hydrophobic character of DPN further experiments were carried out using DMN.

In table 3.3 the conditions for DMN decomposition were investigated. At pH 7.6 no decomposition resulted after 30 mins of U.V. exposure, however in an acidic environment (pH 1-3) decomposition occurred after 5 mins. No decomposition of the sample occurs when irradiated in pyrex glassware instead of quartz.

TABLE 3.1 ABSORBANCE OF DIMETHYLNITROSAMINE IN HEPTANE IN THE ABSENCE AND PRESENCE OF TRIFLUOROACETIC ACID

Wavelength (nm)	DMN (454μM)	DMN + CF_3COOH (0.43 M)
230	0.820	0.040
240	**0.928**	**0.600**
250	0.564	0.280
260	0.200	0.080
280	0.004	0.002
300	0.004	0.004
320	0.020	**0.119**
340	0.072	0.072
360	**0.132**	0.020
380	0.082	0.016

TABLE 3.2 ABSORBANCE OF DIPHENYLNITROSAMINE IN HEPTANE IN THE ABSENCE AND PRESENCE OF TRIFLUOROACETIC ACID

Wavelength (nm)	DPN (100μM)	DPN(100μM) + CF_3COOH(0.43 M)
230	**1.239**	**1.354**
250	0.802	0.950
260	0.503	**0.971**
280	0.554	0.803
300	**0.582**	0.786
320	0.377	0.710
340	0.150	0.718
360	0.045	0.869
380	0.022	**1.068**

TABLE 3.3 ABSORBANCE OF DIMETHYLNITROSAMINE[a] IN AQUEOUS BUFFERED pH AND ACIDIC pH UNDER IRRADIATING/NON-IRRADIATING CONDITIONS

	pH 7.6		pH 1-3	
Wavelength (nm)	Control	Experimental[b]	Control	Experimental
220	2.936	2.945	2.966	1.976
230	3.066	3.067	3.080	0.937
240	3.103	3.105	3.123	0.431
250	3.060	3.060	3.083	0.213
260	2.524	2.437	2.504	0.119
280	0.086	0.092	0.138	0.070
300	0.111	0.115	0.165	0.073
330	0.420	0.395	0.449	0.064
365	0.056	0.061	0.063	0.049

[a]DMN at 136 mM/l

[b]Irradiation of sample for 5 mins (Hg Lamp).

3.3.2 Receptor - Ligand interactions

Table 3.4 shows the interaction of DMN and the nicotinic cholinergic receptor. Receptor fractions from the chromatofocusing column (pH = 5.16) in the presence of DMN experienced a decrease in absorbance in the far U.V. region. Dialysis served to remove DMN as seen in "c". In "d", changes in the properties of the receptor were observed upon irradiation. Control experiments where receptor has been irradiated for 5 mins show the effect of the ultra-violet light to be minimal for this time period. Longer periods (10 mins) irreversibly denature the protein. A maximum absorbance between 280 - 300 nm appears to indicate a structural change. Removal of DMN by dialysis ("e") does not alter this structural change significantly, but allows for an increase in absorbance in the far U.V. region, as observed in control "c".

The interactions of DMN with semi-purified receptor from acetone powder were found to be similar to those above. A reversible interaction between DMN and the solubiliser, Triton X-100 became apparent, firstly because quenching of the absorbance at 238 nm was no longer present when using the detergent Tween - 20, and secondly because quenching of the receptors absorbance in the far U.V. region was not concentration dependent.

Using gel chromatography (Sephacryl S-200), receptor protein elutes in the void volume whilst DMN elutes at V_T. Elution of this sample after 5 mins irradiation shows; firstly, a marked increase in absorbance of the void volume fraction at 280 nm and 235 nm and secondly, a reduced absorbance at 235 nm at V_T (results not shown).

TABLE 3.4 ABSORBANCE OF nAChR IN THE ABSENCE AND PRESENCE OF DMN UNDER IRRADIATING/NON-IRRADIATING CONDITIONS BEFORE AND AFTER DIALYSIS

Wavelength (nm)	R[a]	R + DMN[b]	R + DMN[c]	R + DMN[d]	R + DMN[e]
230	**1.521**	0.101	**1.496**	0.404	1.288
250	0.188	0.223	0.194	0.252	0.165
260	0.166	**0.349**	0.052	0.166	0.090
280	0.159	0.183	0.054	3.380	0.519
300	0.095	0.100	0.050	**3.411**	2.782
320	0.041	0.061	0.027	1.981	**3.008**
340	0.020	0.032	0.020	0.078	0.842

[a]R = nAChR.

[b]DMN at 2.83 mM/l.

[c]5 hours dialysis against 0.025 M/l Imidazole buffer, pH 7.4 (2 l).

[d]irradiation of sample for 5 mins.

[e]5 hours dialysis of irradiated sample.

Table 3.5 shows DMN quenching the intrinsic fluorescence of the receptor protein resulting in near non-specific saturation, however after 5 hours of dialysis, this fluorescence is enhanced approximately 10-fold. Similar results have been obtained with the enzyme (AChE) by co-workers in our laboratory (unpublished results). The effects of DMN in the presence of a saturating concentration of d-TbC, show clearly that the enhancement in fluorescence previously found is now no longer present. The above findings were established at both pH 7.4 and pH 4.5, with no significant difference at these pH values.

From table 3.6, it can be seen that d-TbC quenches the fluorescence of the receptor by approximately 30% while acetylcholine quenches 4%. DMN in the presence of d-TbC results in 50% quenching and in the presence of saturating concentrations of acetylcholine 55% quenching.

Attempts to derivatise DMN, under the conditions employed, proved unsuccessful. TLC analysis showed the formation of acid derivatives of 3,5 dinitrobenzoylchloride with no change in the R_F of DMN.

TABLE 3.5 EFFECT OF CHOLINERGIC LIGANDS ON THE INTRINSIC FLUORESCENCE INTENSITY OF nAChR BEFORE AND AFTER DIALYSIS

Ligand	Relative Fluorescence Intensity	Relative Fluorescence Intensity After Dialysis[b]	n
None	100	125.7 ± 7.25	2
DMN (17.5 mM)	28.42 ± 0.707[a]	269.1 ± 11.03	3
DMN (17.5 mM) + d-TbC (50μM)	31.45 ± 0.778	123.3 ± 9.27	3

n = number of experiments

[a]mean ± SD

[b]5 hours dialysis against 0.025 M/l Imidazole buffer, pH 7.4 (2 l).

TABLE 3.6 EFFECT OF VARIOUS CHOLINERGIC LIGANDS ON THE INTRINSIC FLUORESCENCE INTENSITY OF nAChR

Ligand	Relative Fluorescence Intensity	n
None	100	
DMN (8.28 mM)	42 ± 0.515[a]	3
d-TbC (50μM)	79.71 ± 1.690	2
Ach (55μM)	96 ± 0.540	2
[b]DMN + d-TbC	51.75 ± 0.353	3
[c]DMN + Ach	45.04 ± 1.06	2

n represents the number of experiments

[a]mean ± SD

[b]pretreatment of sample with d-Tubocurarine (50μM)

[c]treatment of sample with saturating Ach.

3.4 Discussion

The ultraviolet spectrum of dimethylnitrosamine shows two distinct bands in non-polar solvents. A strong band identified as a π - π^* transition (230-240 nm) and a weaker band due to a n - π^* transition (360 nm) (73). An increase in the polarity of the solvent results in a less pronounced structure as observed by the shift of the wavelength maximum (n - π^* transition) to shorter wavelengths (325 nm). The formation of a new species is indicated by this spectral change and is therefore a function of the acidity of the solvent as shown by the interaction of DMN with CF_3COOH. This interaction allows the formation of a hydrogen-bonded complex containing one equivalent of nitrosamine and one of CF_3COOH (figure 1.4(IV)) (71).

Based on the similarities in structure of DMN and DPN one can assume similar reactions. This is indeed the case when DPN and CF_3COOH react. The observed colour change indicates extensive conjugation of the DPN-CF_3COOH complex, forming structure (VI) in figure 1.4 with a characteristic quaternary nitrogen. The importance of this is that (IV) as for DMN is photolabile whereas (VI) is not (71).

In conclusion both nitrosamines are involved in hydrogen-bonded reactions in a slightly acidic environment. The reactivity of DPN however does not lend itself to photodecomposition due to the presence of the quaternary nitrogen.

The U.V. spectrum of DMN in water (Table 3.3) is almost identical to the spectrum in non-polar solvents. Spectral evidence clearly shows the sensitivity of nitrosamines to different solvents, as demonstrated by the stability of DMN towards ultraviolet irradiation in neutral aqueous solutions (table 3.3) (50,71). The reason for the stability is that DMN is undoubtedly, extensively solvated by hydrogen bonding in the presence of water (72) and secondly, water is a poor hydrogen-atom-donating-solvent (71). An increase in the acidity of the aqueous solvent allows a hydrogen-bonding reaction to take place between the proton of the acid and DMN, since the hydrogen bond is probably stronger than that with any one water molecule. This hydrogen-bonded-DMN complex is however less photosensitive than when in a non-polar solvent (50), hence requiring ultraviolet

irradiation for photodecomposition. The spectrum after irradiation and the fact that decomposition of DMN does not result when irradiated in pyrex glassware allows one to conclude that the N-N bond has been broken with subsequent radical formation (figure 1.4 (IV)).

The interaction of DMN with purified and semi-purified receptor material under non-irradiating conditions clearly shows DMN interacts non-specifically with the receptor and with low affinity. The fact that energy-transfer labelling does not occur under these conditions is not suprising, since decomposition of DMN does not readily occur in an aqueous environment. It was hoped that the active site region of the receptor, which is hydrophobic in nature, would mimic the medium in which nitrosamines are most photosensitive (50), however this does not appear to be the case. Ultra-violet irradiation of the DMN-receptor complex i.e. photoaffinity labelling, induces a structural change of the receptor protein. This conformational change is irreversible suggesting an inactivated complex.

For photoaffinity labelling to occur, DMN must possess the characteristic quaternary nitrogen but simultaneously retain its photolability (figure 1.4 (IV)). It is for this reason that high concentrations of nitrosamine are required.

Several factors may explain the irreversible conformational change, i.e. a change of the active site from a hydrophobic to a hydrophilic environment. Firstly it may be envisaged as a function of the photolabelling process. Secondly receptors, in the presence of high concentrations of agonists, are known to undergo conformational changes (desensitisation) (3). This could therefore be the case for the receptor in the presence of DMN, even though desensitisation is known to be a reversible process (3). Thirdly, phosphorylation of the receptor in the presence of DMN (97). Finally denaturation of the receptor in the presence of U.V. light. Control experiments where receptor protein is irradiated in the absence of DMN exclude this possibility.

The finding that DMN interacts with the solubiliser, Triton X-100, conclusively shows its non-specificity.

The above shows evidence of a protonated residue in the vicinity of the active site of the receptor. This is based on the displaced ultra-violet spectra of the nitrosamines in the presence of CF_3COOH, which is in

agreement with the literature (50, 69). It appears reasonable to suggest the presence of a tryptophan residue, (residing in a hydrophobic pocket of the receptor) which becomes exposed, in the presence of a nitrosamine, to a hydrophilic environment, and in doing so increases the residues accessibility.

Fluorimetric observations of the interactions of DMN and the receptor support all of the spectrophotometric findings. The intrinsic fluorescence of the receptor is non-specifically quenched by DMN, emission observed at 340 nm rather than 336 nm (6) or 345 nm (90). The difference in emission wavelength appears to depend on the solvent system and/or state of the purified receptor (active/inactive).

Results show that energy-transfer labelling occurs in the presence of DMN with excitation wavelength at 290 nm. This is in agreement with previous findings, showing energy-transfer labelling to be more facile than photoaffinity labelling (50). The fact that the hydrogen-bonding ability and not acidity of the solvent is important, is supported by the fluorimetric observations. Labelling occurred at physiological pH and in dilute acid conclusively showing that the receptor has specific physiochemical characteristics, prerequisite for efficient energy-transfer labelling.

d-Tubocurarine, a competitive antagonist of the receptor blocks any specific interaction of DMN with the receptor. This supports the idea that the nitrosamines are potential nicotinic receptor agonists, causing conformational changes of the receptor. This conformational change is the formation of a desensitised (inactive) receptor, with a higher affinity for agonists (1). No mention has as yet been made as to the spectroscopic properties of a desensitised nicotinic receptor. It has been shown that known agonists such as acetylcholine cause slight quenching of the receptors intrinsic fluorescence, in agreement with the literature (110). d-Tubocurarine similarly causes quenching, more marked however than other known agonists, suggesting some non-specific interactions (102). The fact that the α-cobratoxins also cause marked quenching (40) supports the findings with d-TbC.

Competition at the active site of the receptor is clearly evident when both acetylcholine and DMN are present, supporting the postulation that the nitrosamines are potential agonists. The non-specific interactions of the nitrosamines would appear to be due to the oxygen atom of the molecule. Any derivitisation of this atom would

produce structure (VI) in figure 1.4. The presence of the quaternary nitrogen would increase the affinity and specificity of the ligand for the receptor with, however a simultaneous loss of photolability.

In the presence of DMN it can be seen that the reversible (desensitised) and irreversible conformational changes of the receptor improve its detection. The non-specificity of DMN however precludes its use for purifying the receptor.

CHAPTER 4

ISOLATED ORGAN STUDIES OF XENOPUS LAEVIS RECTUS ABDOMINIS MUSCLE

4.1 INTRODUCTION

The simplest method used to study cellular responses triggered by nicotinic cholinergic receptors is the recording of a mechanical response, namely muscle contraction (4). A widely employed preparation is the frog rectus abdominis muscle, consisting of nicotinic receptors, i.e. the receptors recognise the agonist, nicotine and the antagonist, d-Tubocurarine (37). This method allows for the determination of agonistic properties of ligands and simultaneously improves our understanding of the structural - functional aspects of this receptor.

Ligands of the nicotinic system, invariably are characterised by a quaternary nitrogen atom at physiological pH and are usually hydrophilic (37). The investigation of pH-dependent charged ligands such as dimethylnitrosamine (DMN) and diphenylnitrosamine (DPN) – which is lipophilic – allows inferences concerning the structural requirements and accessibility of cholinergic ligands.

Interactions in the absence and presence of light (photoaffinity or energy-transfer labelling) on the nAChR preparation have been undertaken. Competitive displacement studies and dose-response curves are presented.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Ringers solution prepared as follows:

	g/l
NaCl	6.5
glucose	2.0
KCl	0.14
$CaCl_2$	0.12
$NaH_2PO_42H_2O$	0.034
$NaHCO_3$	0.2

Reagents:

Phenyltrimethyl ammonium iodide was obtained from Hopkins and Williams Ltd. England.

Polyethyleneglycol (PEG) 400 obtained from May and Baker Ltd. Dagenham, England.

Diphenylnitrosamine solutions prepared in PEG 400: ethanol (3:7).

2, 3, 5-Triphenyl-tetrazolium chloride was obtained from BDH Ltd. England.

p-Nitroso-N,N-dimethylaniline. Prepared by associates in our laboratories (mpt. 55-60^{o}C).

All other reagent solutions were prepared as in chapter 2 and 3.

4.2.2 Methods

4.2.2.1 Dissection and attachment of muscle

Female *Xenopus laevis* were swiftly stunned and pithed. Dissection and removal of the muscle was carried out immediately and placed in Ringers solution at physiological pH. The muscle was then attached to a transducer and suspended in a 50 cm^3 organ chamber (see figure 4.1), with constant aeration - 95% O_2 and 5% CO_2 at 20^{o}C (119) and allowed to equilibrate (30 mins).

4.2.2.2 Experiments performed under non-irradiating conditions

After equilibration of the muscle, small doses (10-500µl) of the ligand/s of interest were added directly to the chamber and the response monitored graphically. Calculation of the volume of ligand to be added to the chamber, for the cumulative dose-response curves, can be seen in appendix 5.

At the end of an experiment the chamber was drained, washed twice and then allowed to re-equilibrate until base-line levels were obtained.

4.2.2.3 Photolysis experiments

Photolysis of the chamber contents was carried out using the Hanovia (Hg) lamp placed 5 cms from the quartz chamber. A maximum of 5 mins exposure was allowed due to the damaging effects of U.V. light on muscle preparations. pH changes of the Ringers solution were brought about by adding small amounts of HCl (50-200μl).

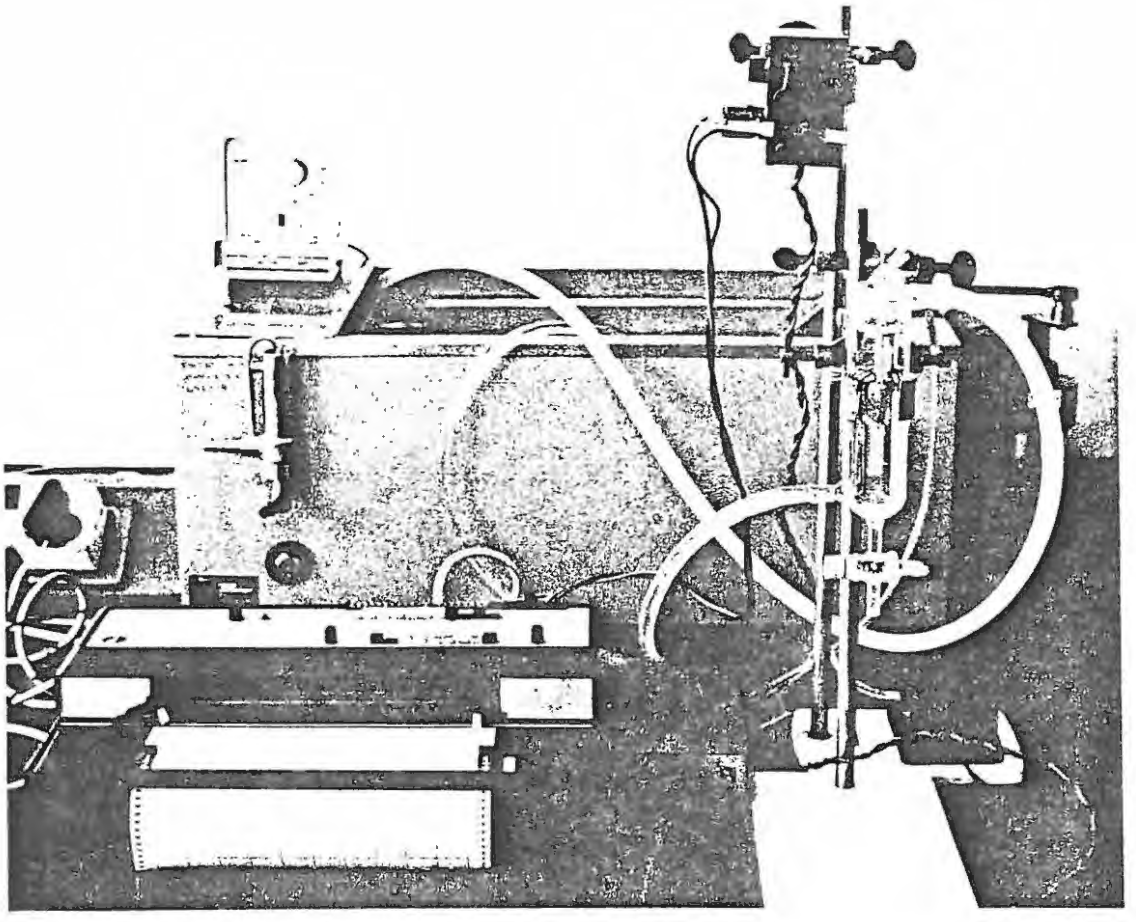


Figure 4.1 **Equipment used for isolated organ chamber studies**

4.3 RESULTS

4.3.1 Experiments performed under non-irradiating conditions

In figure 4.2 the response of equimolar concentrations of the agonists, acetylcholine (a) and phenyltrimethyl ammonium (PTMA) (b) can be seen. PTMA exhibits a longer open channel time and is a more efficient agonist with a 3.3 times greater response.

The cumulative dose-response curve for DMN (figure 4.3) gives an ED_{50} value (i.e. concentration of ligand giving half maximal response) of 56 mM. This is considerably higher than that for acetylcholine (398 μM) (results not shown).

In the presence of the competitive antagonist, d-TbC, the ED_{50} value for DMN increases to 100 mM at 2 μM d-TbC and 177 mM at 6 μM d-TbC. This increase is indicative of competition at the active site of the receptor. Complete blockage and reversal of the DMN response with d-TbC is seen in figures 4.4 and 4.5 respectively. A 6 μM concentration of d-TbC is sufficient to block the DMN response whereas a 200 μM concentration is required to cause complete reversal.

It can also be seen (figure 4.5) that the response elicited by DMN is much slower than that of acetylcholine.

Control experiments consisting of ethanol and PEG 400 were carried out for the cumulative dose response curves for DPN, (figure 4.6) but were found to have a negligible effect on the muscle. From figure 4.6 the ED_{50} value for DPN was found to be 790 μM. In the presence of d-TbC this value increased as follows: at 10μM d-TbC, ED_{50} is 1 mM DPN; at 50 μM d-TbC, ED_{50} is 1.58 mM DPN and at 100 μM d-TbC, ED_{50} is 2.5 mM DPN. Again this increase is indicative of competition at a common interacting site.

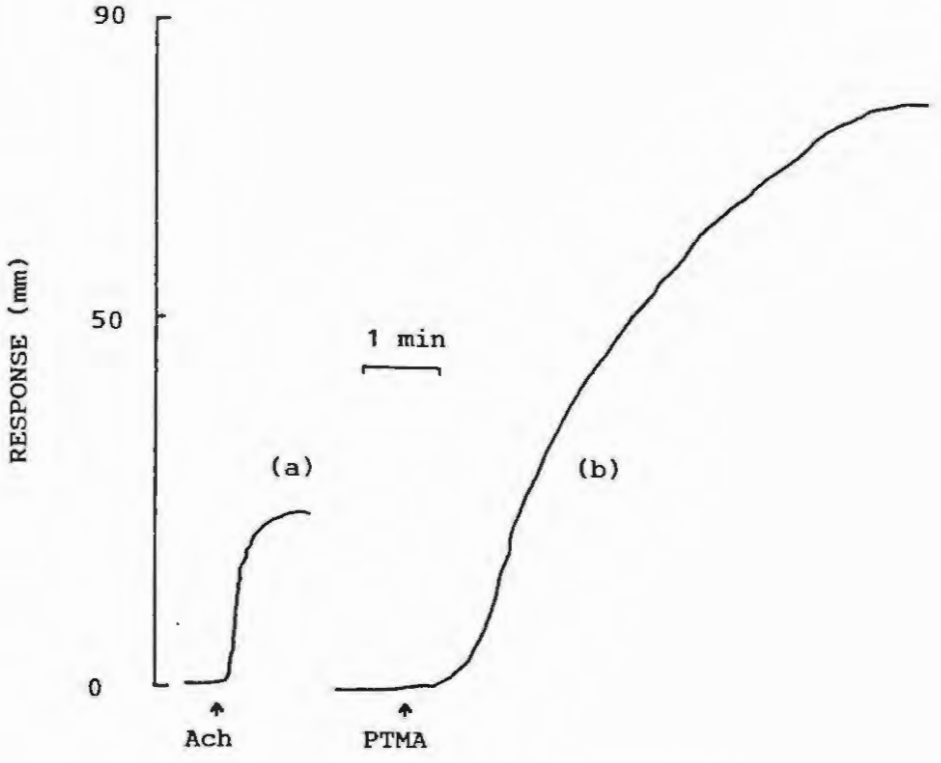


Figure 4.2 **Response of rectus abdominis muscle (a) on addition of acetylcholine (3×10^{-5} M/l) and (b) addition of phenyltrimethyl ammonium iodide (3×10^{-5} M/l)**

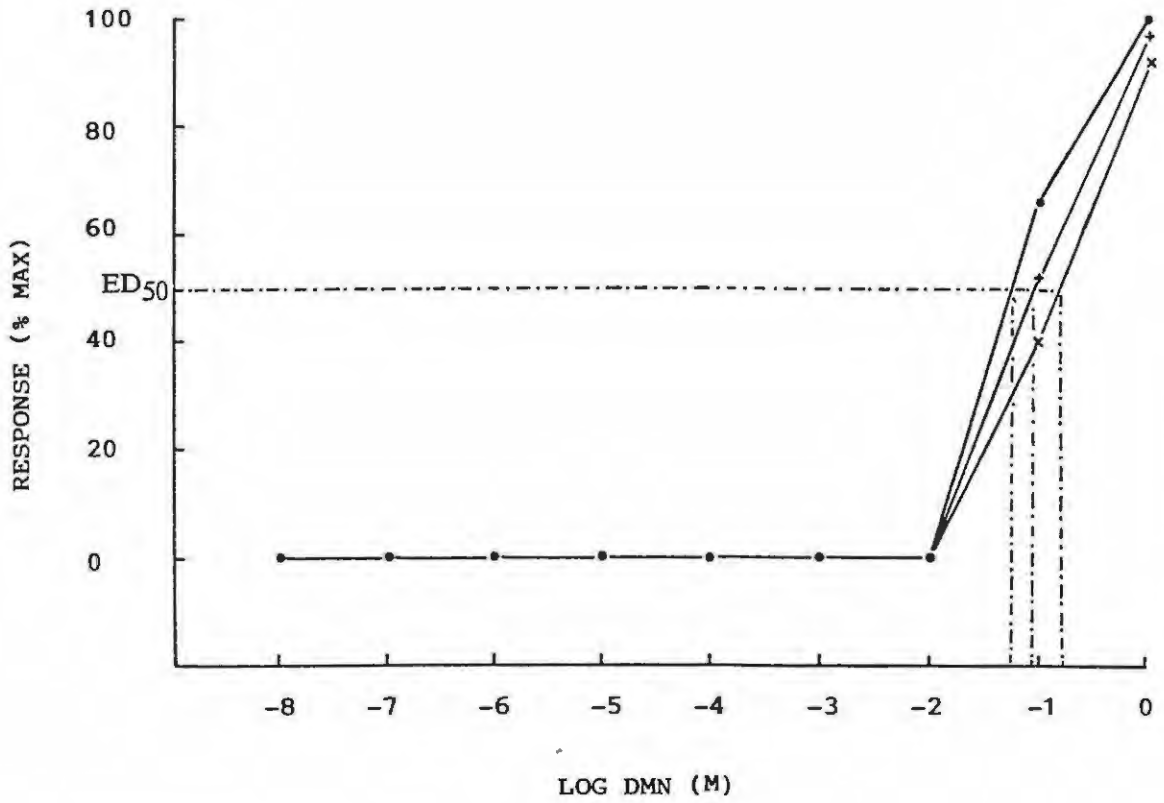


Figure 4.3 **Cumulative dose-response curves showing the effect of DMN (•), DMN plus d-TbC (2×10^{-6} M/l) (+) and DMN plus d-TbC (6×10^{-6} M/l) (×) on the rectus abdominis muscle.**

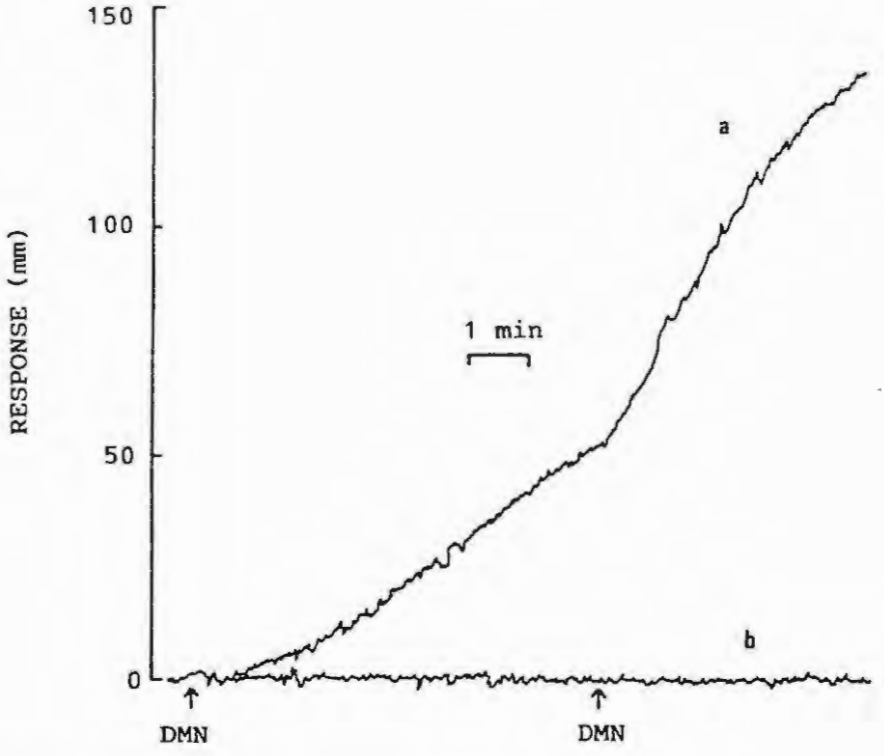


Figure 4.4 Response of rectus abdominis muscle (a) on addition of DMN (1.3×10^{-2} M/l) and (b) pretreated with d-TbC (6×10^{-6} M/l).

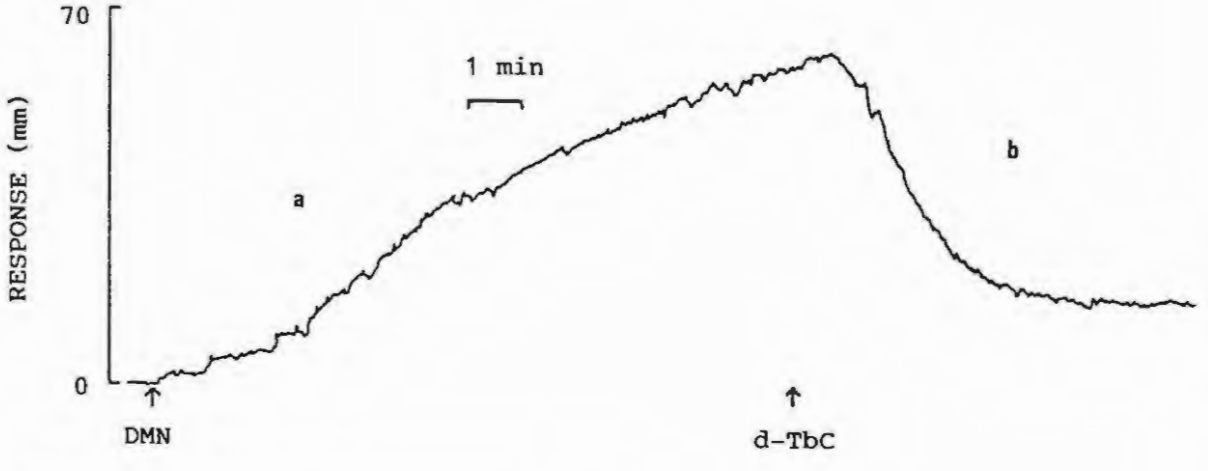


Figure 4.5 Response of rectus abdominis muscle (a) on addition of DMN (1.3×10^{-2} M/l) and (b) treated with d-TbC (2×10^{-4} M/l).

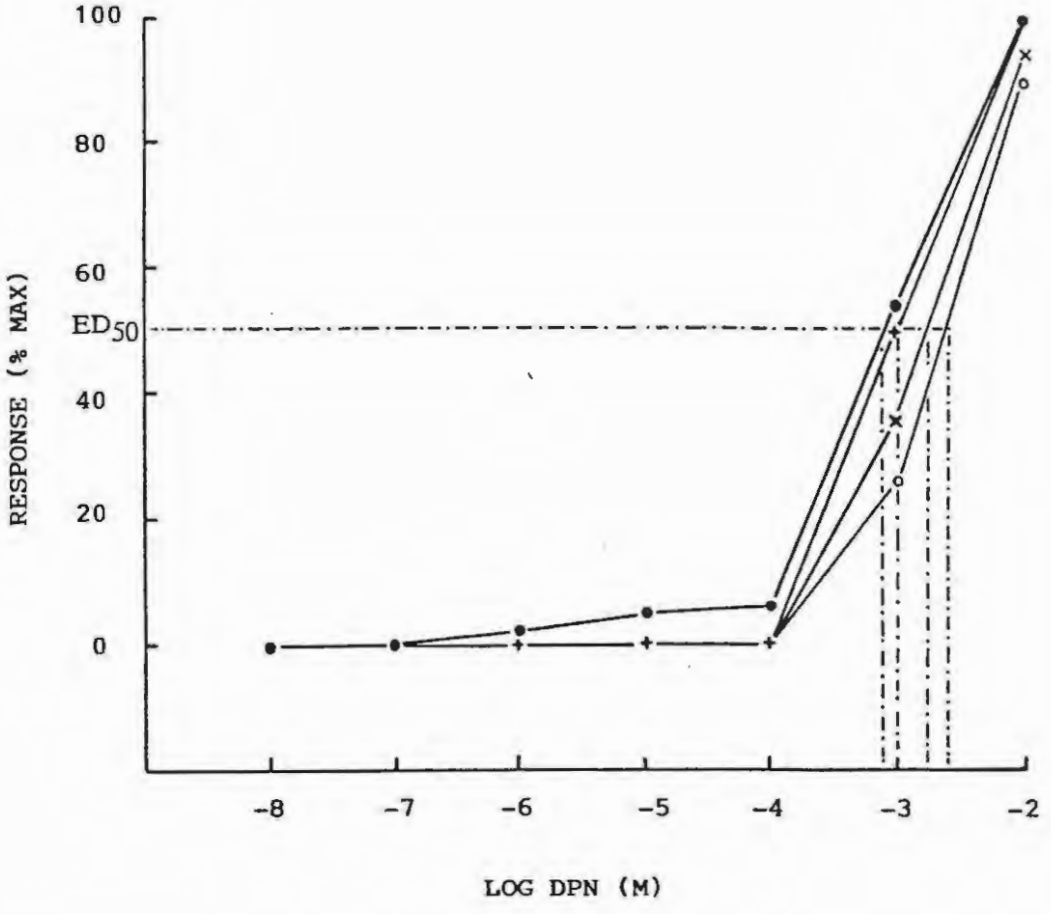


Figure 4.6 **Cumulative dose-response curves showing the effect of DPN (•), DPN plus d-TbC (10^{-5} M/l) (+) DPN plus d-TbC (5×10^{-5} M/l) (×) and DPN plus d-TbC (10^{-4} M/l) (○) on the rectus abdominis muscle.**

Interactions of acetylcholine and DMN on the muscle were investigated. From figure 4.7 it can be seen that a 10 mM DMN dose does not elicit any response (b), however in the presence of acetylcholine (a) a gradual response occurs. In (b) the acetylcholine response is potentiated by a factor of 2.

Figure 4.8 shows the interactions of DPN and acetylcholine with the muscle to be similar to those of DMN and acetylcholine (figure 4.7). However, at 1 mM DPN a response is elicited resulting in only a slight potentiation (b) due to competition at the active site.

Compounds such as 2-3-5 Triphenyl-tetrazolium chloride and p-Nitroso- N,N-dimethylaniline were also tested for nicotinic receptor activity. These however did not cause any muscle contraction.

All of the above interactions were further investigated at a pH of 5.6. It was shown that both the known agonists and the nitrosamines produced an increased response(2-fold) at this pH.

4.3.2 Photolysis experiments

Ultra-violet irradiation of the DMN-receptor complex (5 mins, pH 5.6) (figure 4.9), allows only 66% of the original acetylcholine response. This indicates inactivation of a number of active sites previously available to acetylcholine.

Control experiments in the absence of ligands show firstly, that ultra-violet exposure does not result in any inactivation of the muscle preparation and secondly, only a slight increase in bath temperature ($2^{o}C$) occurs over a period of 5 minutes. Under similar conditions in the presence of DPN no change in the acetylcholine response was observed.

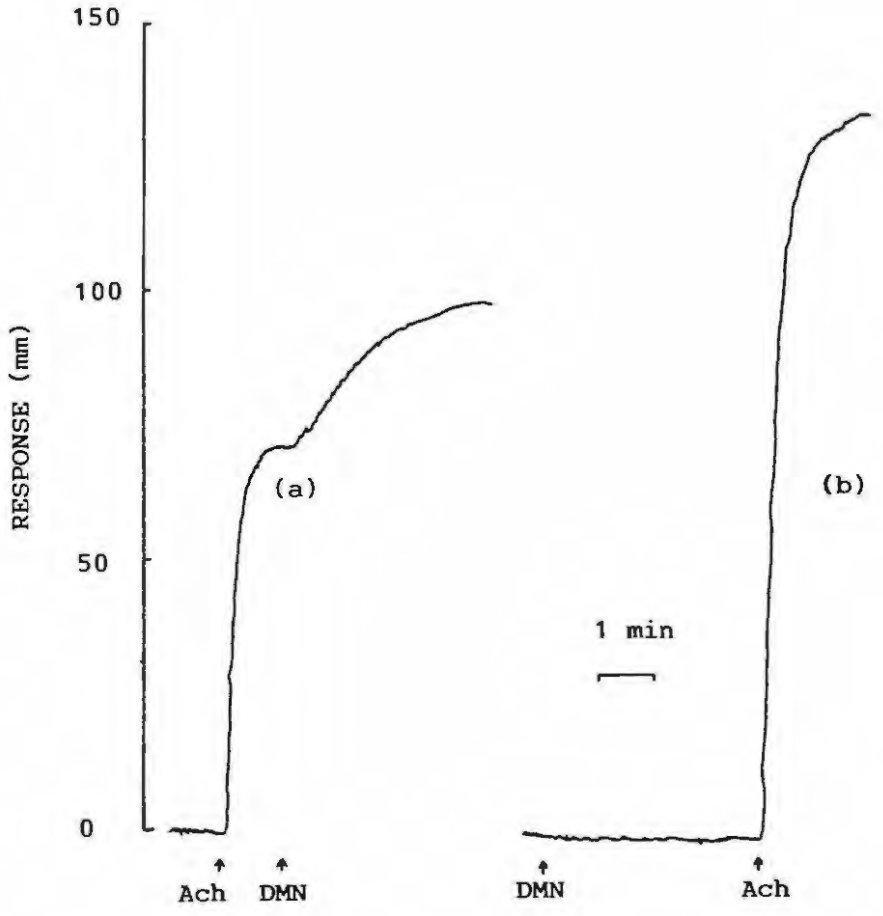


Figure 4.7 **Response of rectus abdominis muscle (a) on addition of acetylcholine (10^{-4} M/l) plus DMN (10^{-2} M/l), and (b) pretreated with DMN (10^{-2} M/l).**

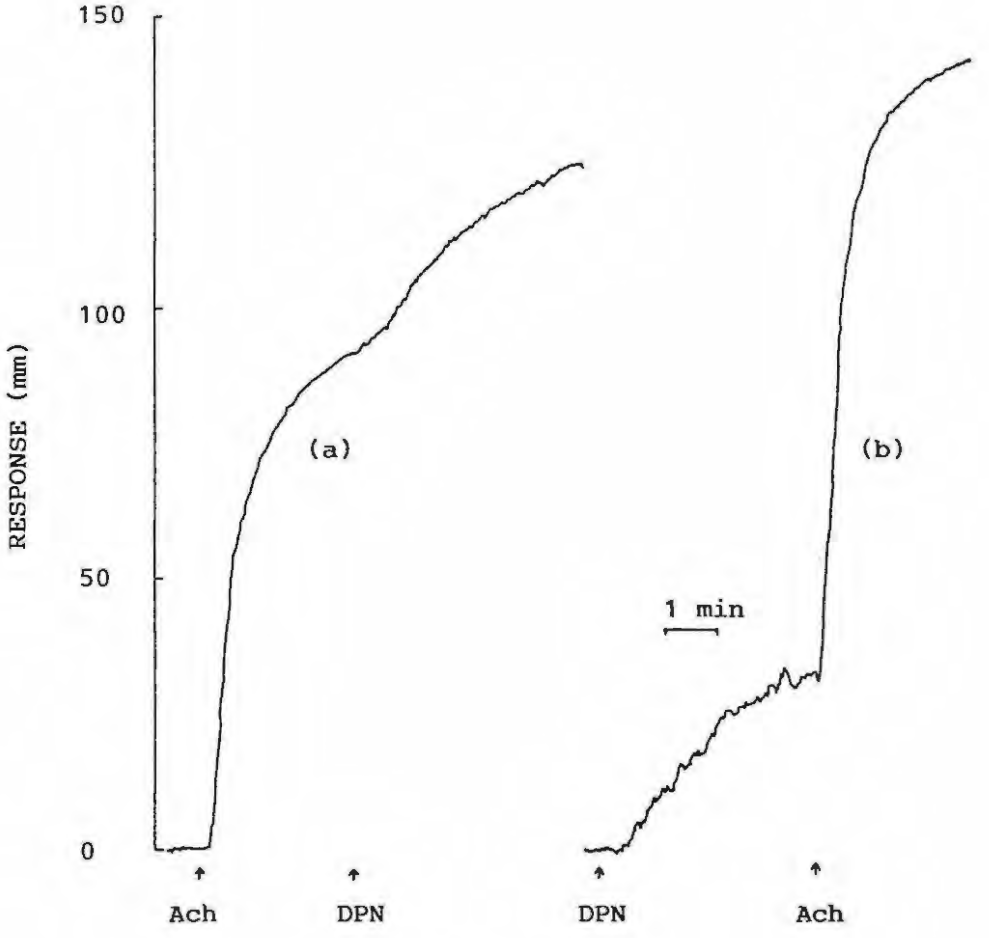


Figure 4.8 **Response of rectus abdominis muscle (a) on addition of acetylcholine (10^{-4} M/l) plus DPN (10^{-3} M/l), and (b) pretreated with DPN (10^{-3} M/l).**

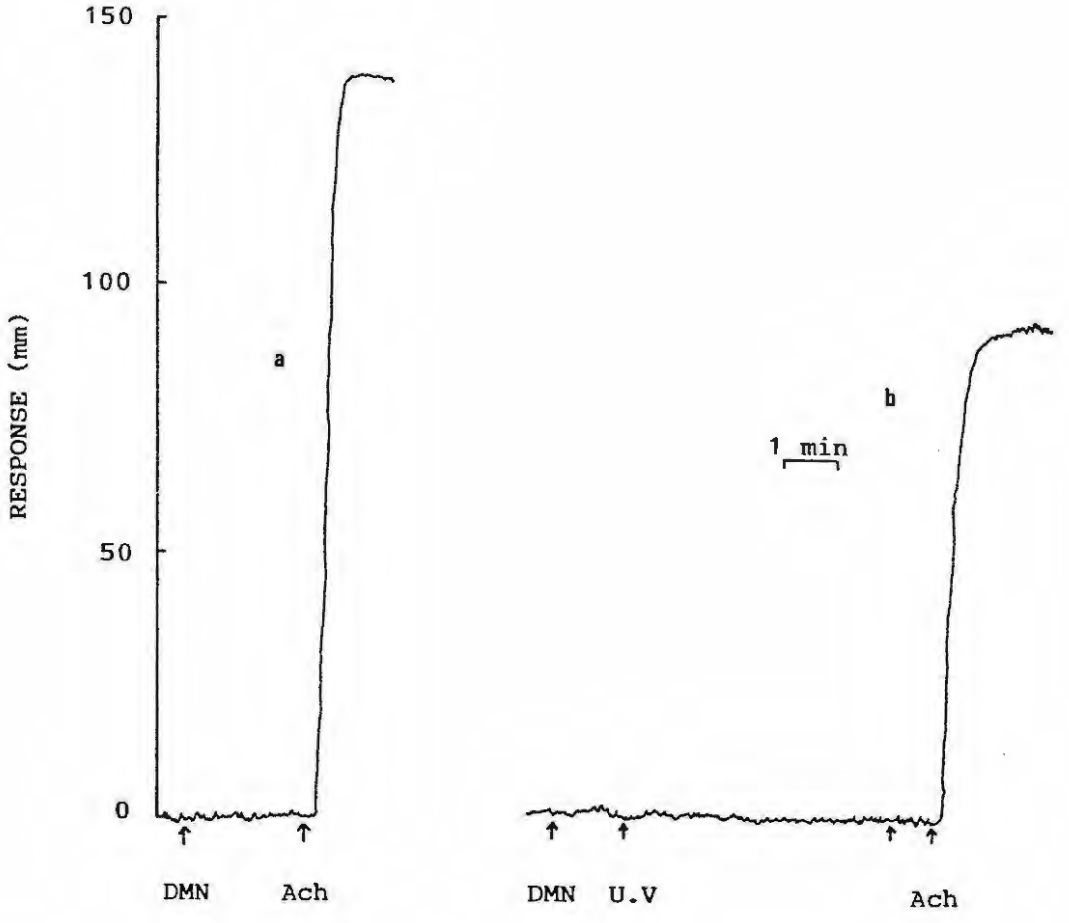


Figure 4.9 **Response of rectus abdominis muscle (a) in the presence of DMN (10^{-2} M/l) and acetylcholine (10^{-4} M/l), and (b) photolysis of chamber (5 mins) in the presence of DMN.**

4.4 DISCUSSION

Known agonists of the nAChR; acetylcholine (figure 1.5 (iii)) and phenyltrimethyl ammonium (figure 1.5 (iv)) activate the receptor-cation channel causing muscle contraction (3). The observed difference in efficiency (i.e. intrinsic activity) of these agonists is due to the partial negative charge on the electron-attracting benzene ring of PTMA substituting for the carbonyl oxygen of acetylcholine (77).

The ability of DMN to act as an agonist of the receptor is confirmed in figure 4.3. This result shows the following : Firstly, DMN is a "full agonist" (i.e. capable of producing a maximal response at a sufficiently high concentration), secondly, it is a very weak agonist and has a low affinity for the receptor reflected in its high ED_{50} value. Thirdly, its interaction with the receptor is reversible and therefore "ionic" in nature. Finally, competition with antagonists such as d-TbC, allow one to conclude a common interacting site.

The fact that it is a weak agonist and of low affinity can be attributed to the lack of a permanent quaternary nitrogen, suggesting non-specificity.

The finding in our laboratory that DMN causes contraction of intestinal smooth muscle (i.e. interacts with muscarinic receptors) confirms the non-specific nature of its interaction. The distance between the quaternary nitrogen and the oxygen atom (figure 1.5 (ii)) suggests the possibility of two DMN molecules interacting simultaneously with one acetylcholine binding site.

Since ionic interactions are the major forces involved in ligand-receptor recognition, it is not surprising that these same forces are involved in DMN recognition.

The competition between DMN and d-TbC for the active site (figures 4.3, 4.4 and 4.5) shows firstly, that DMN is a true agonist; secondly, the presence of DMN results in desensitisation of the receptor and thirdly, d-TbC

binds preferentially to a receptor in a resting state (non-desensitised) and does not result in any detectable activation of the receptor (104).

The accessibility of DMN to the active site of the receptor is controlled by factors such as solvent polarity, pH and the state of the ligand (i.e. charged or uncharged). Since a 1:1 ratio of charged to uncharged species occur (figure 1.4 (I) and (II)), it can be assumed that DMN reaches the active site of the receptor via an aqueous and/or hydrophobic path.

The delayed response of the muscle in the presence of DMN can be observed in figure 4.5. This is due to the fact that DMN is a weak agonist and interacts poorly with the receptor. Also, the interaction requires the displacement of water (82) and/or Ca^{2+} ions (6) to allow for destabilisation of the closed channel state.

From chapter 3, it was assumed that the high reactivity of DPN with CF_3COOH would enhance its affinity for the receptor ($-N^+$) but simultaneously due to steric influences reduce its intrinsic activity (37). The results clearly show DPN to be as efficient an agonist as DMN. DPN however displays a higher affinity for the receptor evident by the lower ED_{50} value. Since the ED_{50} value for DPN is higher than that for acetylcholine, one can assume that the bulky phenyl groups interfere sterically and that the distance between the quaternary nitrogen atom and the oxygen is not optimal (as for DMN).

DPN, is therefore a "full agonist", interacts through ionic forces and competes with d-TbC for the active site. A combination of steric and inductive effects account for its higher reactivity.

The interactions of DPN and d-TbC with the receptor reflect the increased potency of DPN. Whereas low

concentrations of d-TbC are capable of blocking a response by DMN much higher concentrations are required to prevent a response by DPN.

A delayed response by DPN (not shown) appears to indicate that it reaches the active site via a hydrophobic path. Since the rate of muscle contraction by both DMN and DPN are similar, it would appear that DPN also has access through an aqueous path, suggesting the presence of a high percentage of charged DPN molecules (in agreement with results).

Pretreatment of the rectus muscle with acetylcholine (figure 4.7 (a)), and subsequent addition of DMN, (at a concentration not effective in eliciting a contraction) indicates firstly, that a conformational change of the receptor is important for receptor activation and secondly, that DMN at this concentration interacts with only one binding site.

In (b), potentiation of the previous acetylcholine response in (a), reinforces the fact that a conformational change is important for activation.

Positive cooperativity has been suggested i.e., a molecule binding to one site of the receptor induces structural changes enhancing the binding of a second molecule (6). The second ligand binding site can be thought of as a poor complement to the ligand, forming incomplete contacts with the receptor when it is in a resting state (82).

From the results it can be seen that DMN binds with very low affinity to the receptor and because of this is capable of only interacting with the more accessible binding site. This interaction results in a conformational change, which is necessary for receptor activation. Higher concentrations of DMN are however required to interact with the second ligand binding site. This second site controls the receptor-cation channel and thus the response. It is well documented (6, 15, 80-82), that receptor activation is greatest when both agonist binding sites are occupied. The results support this and the notion that the two binding sites are of equal affinity when the channel is open (3, 80, 82, 83).

In conclusion then, it can be seen that the potentiated response in figure 4.7 (b) is firstly due to a

conformational change of the receptor in the presence of DMN, and secondly allows acetylcholine to interact with the second ligand binding site, activating the cation-channel.

The interactions of DPN in the presence of acetylcholine (figure 4.8) (a) and (b), can be explained in much the same way as for those of DMN. The response (i.e. muscle contraction) by DPN (b) however results in competition at the active site evident by the less marked potentiation normally associated with the presence of two agonists.

It is suggested that desensitisation of the receptor can either occur in a fast-step in the millisecond time region or a slow-step in the second time region (89).

The different states of the receptor (i.e. resting, active and desensitised) are proposed to be interconvertible allosteric transitions (1). One can therefore view the transition from low affinity to high affinity state as being the same molecular transition as desensitisation (91).

The fact that two agonist molecules are necessary for receptor activation whereas only one molecule is sufficient for desensitisation (3) is clearly supported by the results.

The non-specificity of DMN and DPN would suggest that these ligands are not only agonists but are also capable of interacting with the membrane and/or ion-channel complex. Since non-competitive inhibitors are a very heterogeneous group (105) interacting with the ion-channel, DMN and DPN could possibly be viewed as N.C.I., however, from the results this does not appear to be the case.

The presence of a quaternary nitrogen does not imply that a ligand must bear agonistic properties. Specific geometrical requirements are necessary for nicotinic recognition and activation.

A drop in the pH of the medium, from a physiological pH to a pH of 5.6 results in marked changes. The effect

of an increase in hydrogen ions on the receptor-channel complex appears controversial. The finding that an increased response occurred at a lower pH is in contradiction with other findings, which suggest that hydrogen ions block the ion-channel (3).

More importantly however, photolysis of the muscle preparation at pH 5.6, in the presence of DMN results in an irreversible modification of receptor response (figure 4.9). Only 66% of an original acetylcholine response is permitted which suggests that an irreversible desensitised conformation is established through covalent insertion (alkylation), or that increased desensitisation of receptors occurs due to the continued presence of DMN (83). Control experiments suggest the former to be correct. Since DMN at this concentration (figure 4.7) does not exhibit any biological activity, photoaffinity labelling of the receptor indicates that similar mechanisms control both activation and desensitisation. This is in agreement with the literature which states that a disulphide bond, which is intimately involved in the local conformational changes at the active site leads to activation (94) and desensitisation (95).

The irreversibly modified response, in our findings, can be compared to that of other findings (69), where only 24% decomposition of a derivatised nitrosamine occurred in the presence of the enzyme, acetylcholinesterase.

The fact that photolabelling of the receptor by DPN does not occur can be extended to its lack of photolability in the presence of the receptor.

CHAPTER 5

DISCUSSION AND CONCLUSIONS

Despite the nicotinic cholinergic receptor being the most well-characteristed neurotransmitter receptor, there are still some basic problems which remain unsolved. Firstly, a crucial one is the difference between agonists (which bind and trigger an effect) and competitive antagonists (which bind with comparable affinity but do not gate the ion-channel). From the mere chemical structure of the effector molecules this difference is not obvious (3). Secondly, little is known about the identity of the ion-channel and its gating mechanism(s).

Due to certain characteristic features of the nitrosamines such as pH dependency, a hydrophobic/hydrophilic balance and a photolabile functional group allows one to gain considerable insight into the receptor-channel complex. With this in mind the present study was designed to provide a means for isolating and purifying the receptor with specific label at the active site.

The question remains whether the "fish receptor", from *Torpedo fuscomaculata*, is a model for the nAChR from other species and higher vertebrates. The answer is "yes". This is not surprising since electroplaque cells phylogenetically evolved from striated muscle cells (3). In an adult electromotor or neuromuscular junction, the distribution of the acetylcholine receptor is largely restricted to the cytoplasmic membrane which underlies the nerve ending. In this region, the receptor density reaches a value of about 15 000 to 20 000 molecules /μm^2 (2). This high density facilitates the purification of the receptor.

The lipid environment of the receptor exerts a critical, although permissive action on the regulatory properties of the receptor. Whereas solubilisation, which releases the receptor from its membrane components, is suggested to alter regulatory properties, receptor molecules have been shown to retain their integrity and ability to recognise agonists and antagonists. This suggests conformational changes of the receptor to be reversible.

Due to serious problems created by proteolysis, maximum precautions should have been taken i.e., a "cocktail" of protease inhibitors in all of the solutions used. Despite extensive degradation in receptor preparations ligand-receptor recognition still occurred suggesting that the functional integrity of the receptor remained intact.

Problems in accurately determining specific radioactivities of labelled preparations (2) were circumvented by employing the specific antagonistic properties of d-Tubocurarine. An accurate assessment of purity was achieved even though some non-specific interactions in crude-receptor preparations occurred. Finally, the values of specific activity are based on protein determinations made with soluble standard, serum albumen, which may not behave appropriately in the modified Folin-lowry reaction (2). The protein content of the crude-receptor fractions were found to be low in comparison to other reported findings, resulting in lower specific activities.

Recovery of active receptor through use of an easily identifiable physical parameter allowed for an improved purification by chromatofocusing over that of affinity chromatography. Even though the α-neurotoxins exhibit an extremely high affinity and specificity for the receptor, the majority of receptor is lost in an affinity chromatographic system.

It has been shown that ion-exchange resins, such as commercial Sepharose, are an improvement on Sephadex resins, allowing for higher recoveries in smaller volumes. They are however,not sufficiently characteristic allowing for recovery of receptor associated with tightly bound non-specific proteins.

The nAChR has been shown to consist of four different subunits constituting a receptor monomer of approximately 230 kDa.These monomers are linked through δ-δ subunits to form a dimeric molecule which is integrated in the post-synaptic membrane.

A physiological response i.e., contraction of rectus abdominis muscle, has shown nitrosamine compounds to be true agonists of the nAChR. The fact that they interact weakly with the receptor and are non-specific, due to a transient quaternary nitrogen, precludes them as photoaffinity ligands of the nAChR.

Structural-functional studies of receptor-ligand interactions indicate specific geometrical requirements for receptor activation. Since the distance between the quaternary nitrogen atom and negative group of the nitrosamines is not optimal for nicotinic receptor activation, weak interactions occur. For the case of DPN however, the possibility exists that the distance between the nitrogen atom and electron-dense benzene ring is

more optimal allowing for a stronger interaction with the receptor.

It has been shown that desensitisation of the receptor occurs in the presence of high concentrations of agonist and involves the same molecular transition as receptor activation. The fact that the response (i.e. muscle contraction) is delayed in the presence of the nitrosamines can be attributed to the accessibility of these ligands to the active site.

In conclusion, it remains an open question as to whether all of these effects of neurotransmitters and ligands on voltage-sensitive ion-conductances observed *in vitro* should necessarily be considered simulations of normally functioning synaptic interactions observed *in vivo* (120). Neurotransmitters and their receptors are therefore likely to remain a productive focus of future research.

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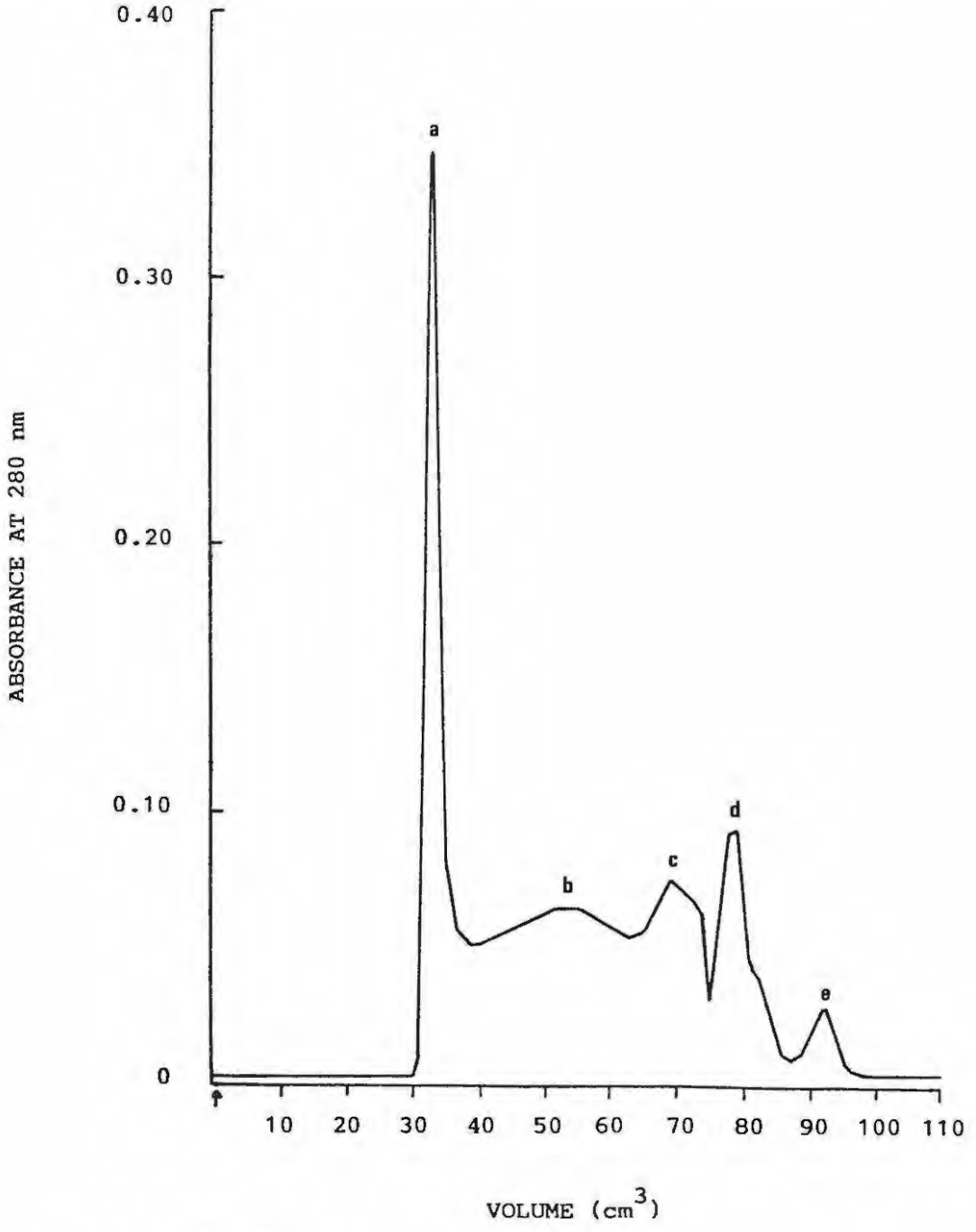
APPENDIX 1

Gel Chromatogram of Standard Molecular Weight Compounds on Sephacryl S-400.



a - blue Dextran

b - blue Dextran Dye

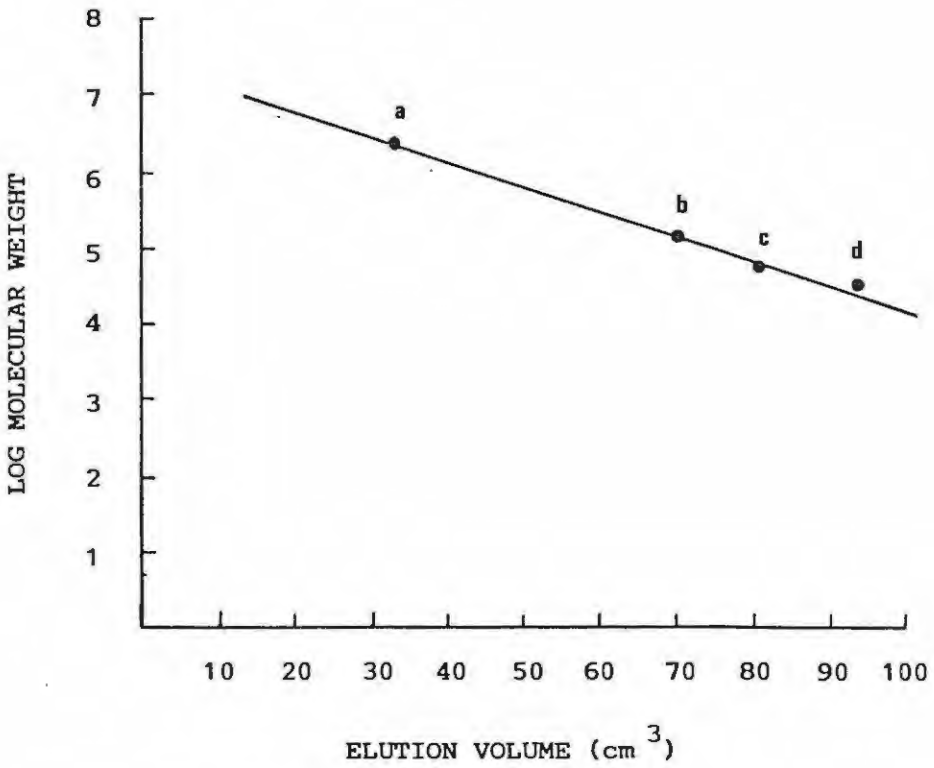
c - lactate Dehydrogenase

d - DNase

e - carboxypeptidase A

APPENDIX 2

Standard Curve for the Determination of Molecular Weight on gel chromatography



a - blue Dextran

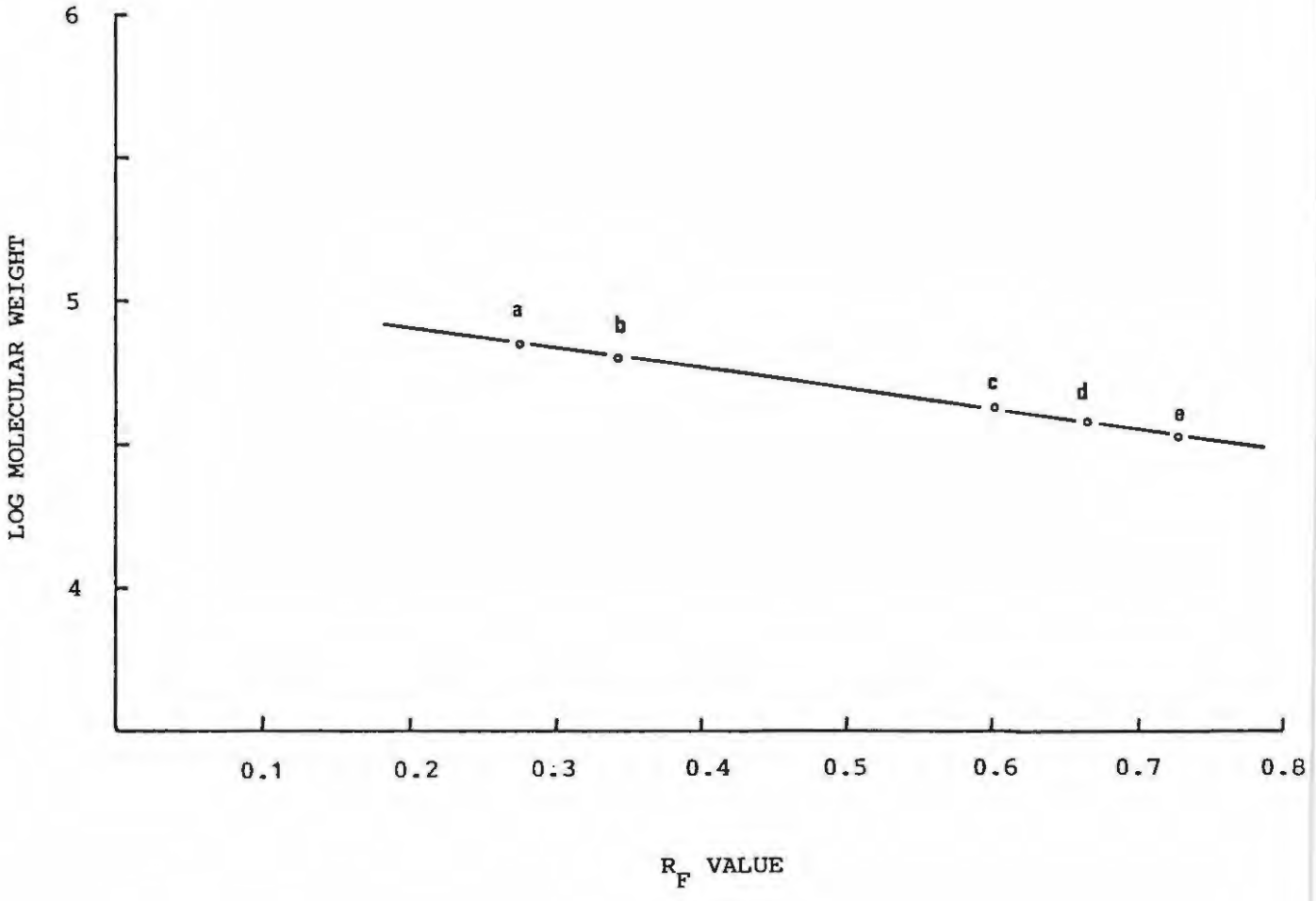
b - lactate Dehydrogenase

c - DNase

d - carboxypeptidase A

APPENDIX 3

Standard Curve for the Determination of Molecular Weight by SDS-PAGE



a – BSA

b – catalase

c – ovalbumen

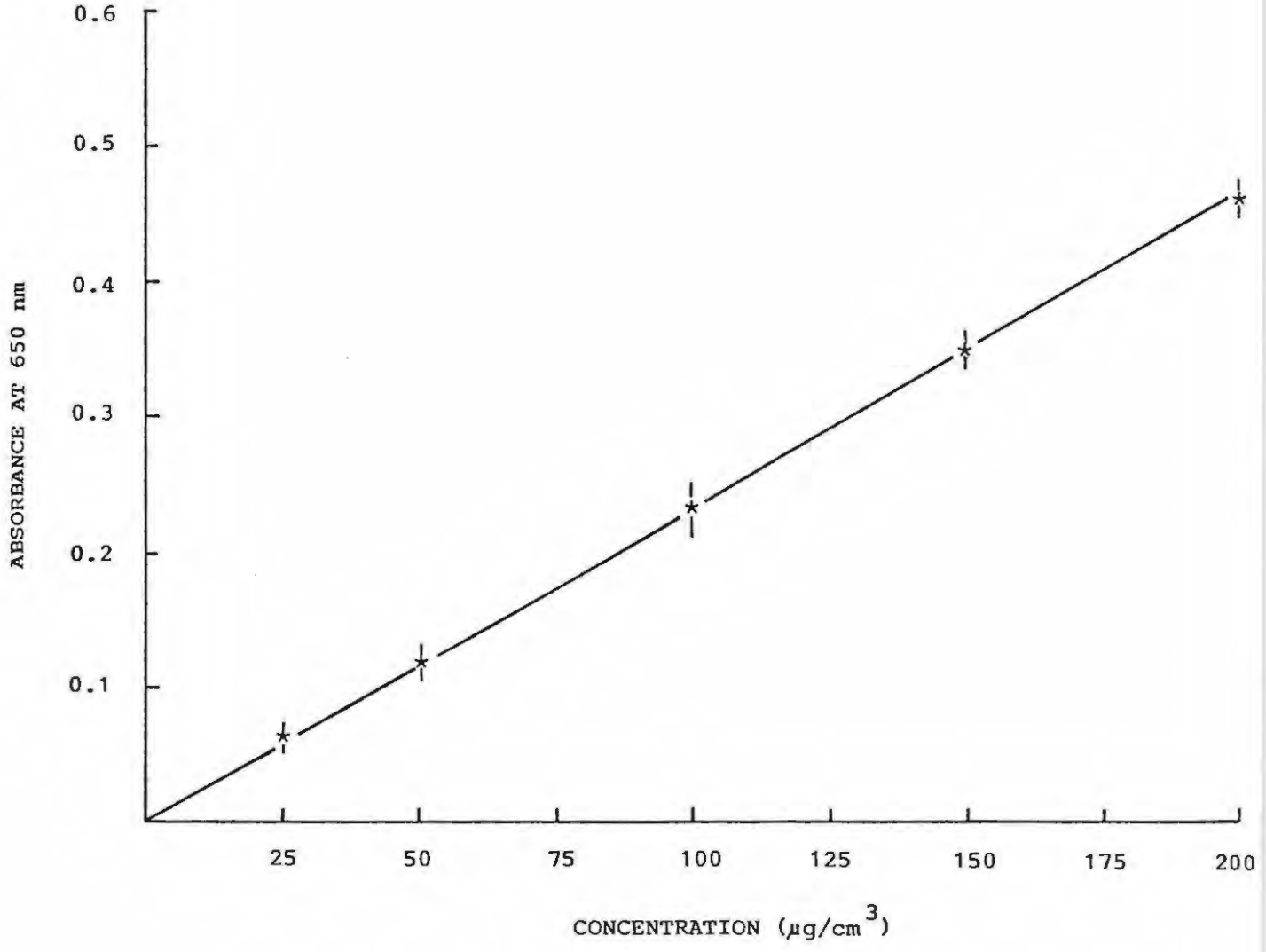
d – lactate dehydrogenase

e – carboxypeptidase A

APPENDIX 4

Standard Curve for the Determination of Protein by the modified Lowry Method (116).

Means of six readings and their S.E.M's.



APPENDIX 5

Calculation of volume of ligand to be added to the isolated organ chamber for the cumulative dose-response curves.

50µl of a stock solution of ligand (10^{-5} M/l) gives a final concentration of 10^{-8} M/l in the chamber.

To achieve a 10^{-7} M/l final concentration, 45µl* of stock solution (10^{-4} M/l) must be added.

*Calculated as follows :

$(1 \times 10^{-7}) - (1 \times 10^{-8})$

$= 9 \times 10^{-8}$

hence multiply : 9 x 50µl

= 450µl

hence divide : 450÷10

= 45µl