

University of Bath



**PHD**

**Studies on the nicotinic acetylcholine receptor of human muscle.**

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STUDIES ON THE NICOTINIC ACETYLCHOLINE  
RECEPTOR OF HUMAN MUSCLE

submitted by F. Anne Stephenson  
for the degree of Ph.D. of the  
University of Bath, 1980

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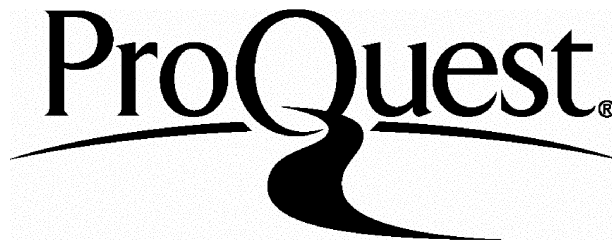
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### SUMMARY

1. A radioimmunoassay was established for the measurement of anti-(acetylcholine receptor) antibodies in the serum of myasthenic patients. It was found that 87% of myasthenic serum samples contained elevated levels of circulating anti-(acetylcholine receptor) antibody but that there was no direct correlation between the antibody titre and disease severity. Also, the anti-(acetylcholine receptor) antibody titre was measured in a series of patients that underwent a course of plasma exchange. The antibody titre was found to decrease following plasma exchange and the majority of patients obtained complete clinical remission for periods of up to 18 months following the completion of exchanges.

2. The nicotinic acetylcholine receptor protein has been purified from human skeletal muscle by a procedure involving extraction in non-ionic detergent followed by affinity purification on immobilised  $\alpha$  toxin. Purified receptor preparations had specific activities of 0.5-3.5  $\mu\text{mol}$   $\alpha$  bungarotoxin binding sites per gram protein and sedimented as a single  $^{125}\text{I}$ - $\alpha$  bungarotoxin-binding species in sucrose density gradient centrifugation with  $S_{20,w} = 9.5$ . The purified protein focussed as a single sharp band at pH 6.6 when labelled directly with  $^{125}\text{I}$  and at pH 5.1 when complexed to  $^{125}\text{I}$ - $\alpha$  bungarotoxin. Polyacrylamide gel electrophoresis under non-denaturing conditions of receptor-toxin complex showed a single protein component of similar mobility to that of acetylcholine receptor from *Torpedo marmorata*, whilst polyacrylamide gel electrophoresis of the purified receptor under denaturing conditions showed two major protein bands with molecular weights 42000 and 66000 respectively with the occasional appear-

ance of minor components at 56000 and 85000. Only the 42000 subunit was labelled with the affinity reagent, [ $^3\text{H}$ ]-MBTA.

The purified receptor bound  $^{125}\text{I}$ - $\alpha$  bungarotoxin and d-tubocurarine with  $K_D$  values of 0.5nM and 0.25 $\mu$ M respectively. It behaved similarly to impurified detergent-extracted human receptor in the radioimmunoassay for anti-(human acetylcholine receptor) antibodies and when injected into rabbits caused increased levels of the latter antibodies but did not cause experimental autoimmune myasthenia gravis.

## INTRODUCTION

"Toutes les substances qui tuent rapidement agissent en général sur les grands systèmes, système sanguin, système nerveux ou musculaire. Les effets peuvent être bien distincts pour deux substances qui agissent sur le même système. Cette localisation des actions toxiques nous permettra d'en suivre le mécanisme jusque dans les organes; elle met aux mains du physiologiste expérimentateur de véritables réactifs de la vie."

Claude Bernard (1857)



1.

In the latter part of the nineteenth century, Ramón y Cajal (1888) challenged the then generally accepted reticular theory of the structural organisation of the nervous system and proposed that it was not a continuous network but was composed of discrete units, the nerve cells or neurones, which communicated with each other only via specialised contacts. The histological findings of Ramón y Cajal were complemented by Sherrington (1925) whose electrophysiological studies established the electrical discontinuity of the nervous system. Sherrington used the term "synapse" to describe the specialised contacts seen by Ramón y Cajal and it was rapidly accepted that the synapse was perhaps the key regulatory component of the nervous system.

The realisation that the nervous system was electrically discontinuous implied the presence of some additional system of communication located at the synapse and led T.R. Elliot (1904) and W.E. Dixon (1906) to put forward the idea of neurohumoral transmission in which a chemical messenger is released at the synapse and conveys the message to adjacent cells. In 1921, Loewi demonstrated that transmission between the vagus nerve and the heart was mediated by a chemical messenger which he named "Vagustoff". In his experiment, he chronically stimulated the vagus nerve of an isolated frog heart. The bathing medium was added to a second heart and was found to mimic vagal stimulation. Loewi's pioneering experiment formed the basis of the neurotransmitter theory of synaptic transmission. It is now recognised that several criteria must be satisfied before a mediator can be established as a neurotransmitter (Werman 1966). These are:-

- (i) the mediator is synthesised and stored in the nerve terminal.
- (ii) the mediator is released on stimulation and the release is  $\text{Ca}^{2+}$  dependent.

2.

- (iii) the mediator produces a change in polarisation by an interaction at the post-synaptic site.
- (iv) the amount of mediator released is sufficient to initiate either depolarisation or hyperpolarisation.
- (v) there exists a method of removal of the mediator.

The neuromuscular junction is one of the better understood systems with regard to the organisation of the elements of neurotransmission. Its function is to transfer the propagated electrical impulse from the motor nerve endings to the muscle fibres leading ultimately to muscle contraction. In the majority of vertebrate muscles, each fibre is innervated by a single nerve and the area of contact between the two is termed the neuromuscular junction or motor end-plate. The muscle fibre has a resting potential of  $-90\text{mV}$  which is reversed upon stimulation by changes in the permeability properties of the muscle membrane to  $\text{Na}^+$  and  $\text{K}^+$ . It is now well established that acetylcholine is the only neuroactive agent at the vertebrate motor end-plate and it has been shown that it satisfies all the criteria for a neurotransmitter. Thus motor nerve fibres are known to contain acetylcholine and the enzyme responsible for its synthesis, choline acetyltransferase. Moreover, nerve-muscle preparations contain acetylcholinesterase, the enzyme responsible for the hydrolysis of acetylcholine. The neurophysiological action of acetylcholine was discussed by Hunt and Taveau (1906) and by Dale (1914) and its identity with "Vagustoff" of the Loewi experiments was demonstrated.

Initial attempts to show release of acetylcholine on stimulation of an isolated nerve-muscle preparation were hindered by its rapid hydrolysis. However, Dale *et al.* (1936) included eserine, an

3.

acetylcholinesterase inhibitor, in their perfusion fluid and were then able to detect the appearance of acetylcholine after direct nerve stimulation. Additionally they showed that the release of the neurotransmitter was caused by impulses in the motor axons as direct electrical stimulation of chronically denervated muscle released no acetylcholine. Stimulation of the motor nerve in the presence of curare, a substance known since 1840 (Bernard) to paralyse the muscles, released the normal amount of acetylcholine but no activation of the muscle fibres was seen. Also, stimulation in the absence of  $\text{Ca}^{2+}$  or in the presence of high  $\text{Mg}^{2+}$  concentration did not release acetylcholine. Measurements have been made of the amount of acetylcholine released on stimulation and a value of  $5 \times 10^6$  molecules per impulse per end-plate has been reported (Katz 1966) whereas it was found that  $10^7$  to  $5 \times 10^8$  molecules per impulse per end-plate were required to evoke a contraction of the muscle fibre (Katz 1966). The apparent discrepancy between the two values is probably accounted for by hydrolysis of acetylcholine in the perfusion fluid.

The pharmacological action of acetylcholine was demonstrated by the iontophoretic application of acetylcholine to isolated muscle fibres. It was shown that very low concentrations of acetylcholine were sufficient to depolarise the membrane and that iontophoretic application was effective in the region of the end-plate only. The excitatory action of the iontophoretically applied acetylcholine was apparent in the presence and absence of the motor axon. Its action was potentiated by the presence of anticholinesterases but reduced in the presence of curare and other quaternary ammonium compounds.

4.

Thus acetylcholine satisfies all the criteria set out for a mediator to be classified as a neurotransmitter at the skeletal neuromuscular junction. FIG. 1 is a schematic representation of the events at the neuromuscular junction during stimulation. The nerve impulse travels down the nerve fibre to the nerve terminal where, upon the entry of  $\text{Ca}^{2+}$ , acetylcholine is released. It is now generally accepted that the neurotransmitter is released in discrete quanta and it has been suggested that each quantal package is the content of a synaptic vesicle (del Castillo and Katz 1955). The release mechanism is thought to be by exocytosis and to involve the fusion of the membrane of the synaptic vesicles with the pre-synaptic membrane. The acetylcholine released then diffuses across the synaptic cleft and in fact a synaptic delay of up to  $\mu\text{s}$  can be recorded which represents the time between the arrival of the nerve impulse at the synapse and the appearance of a depolarisation in the muscle fibre. The interaction of acetylcholine with the muscle membrane alters the permeability of the membrane to ions and this in turn leads to the depolarisation of the muscle fibre. The acetylcholine is then rapidly hydrolysed by acetylcholinesterase and the choline produced is taken up by a specific transport mechanism into the pre-synaptic terminal and may be used in re-synthesis of acetylcholine. Depolarisation of the muscle membrane spreads rapidly into the T system of each muscle fibre and to the terminal cisternae. Depolarisation of the terminal cisternae alters their permeability properties to  $\text{Ca}^{2+}$  which is released inside the muscle cell. Binding of  $\text{Ca}^{2+}$  to the troponin system results in the activation of the actomyosin complex which leads to muscle contraction (review Perry 1979).

5.

The nature of the interaction of acetylcholine with the muscle membrane was not known until comparatively recently but as early as 1907, Langley on the basis of pharmacological studies, proposed that there is a "receptive substance" present in muscle which receives the nerve stimulus. Dale (1914) extended these observations in physiological studies of a series of choline derivatives and showed the presence of two distinct types of activity - a "muscarinic" action essentially mimicking the effects of parasympathetic stimulation and the alkaloid muscarine, and a "nicotinic" action possessed by nicotine and exerted at skeletal muscle and autonomic ganglia. Further study by Dale of the nicotine-responsive nerve-muscle junction showed that certain agents, "antagonists" reduced the response to acetylcholine whereas another group, "agonists", potentiated the response. In the case of both agonists and antagonists, there was found to be a strict structural requirement for a quaternary ammonium ion.

Thus the physiology and the pharmacology of the neuromuscular junction were quite well documented by the end of the 1930's but the receptor for acetylcholine was still an unknown quantity. However the fortuitous combination of two apparently unrelated factors have enabled the acetylcholine receptor to be recognised as a discrete unit and to be purified and characterised biochemically. These factors are firstly, the serendipitous discovery of the curarising mode of action of the elapid snake venoms and secondly, the abundant source of cholinergic receptor in the electric organ of the electric fishes.

FIG. 1 Schematic representation of the organisation of the vertebrate neuromuscular junction. (After Katz 1966)

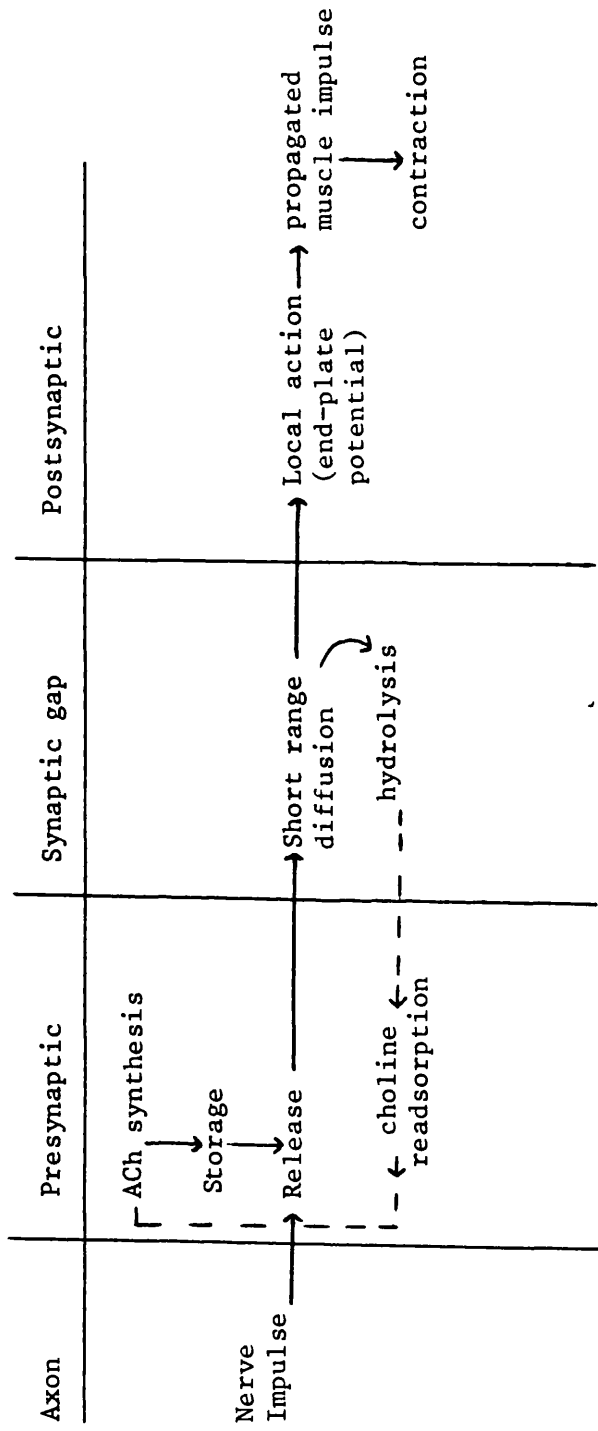
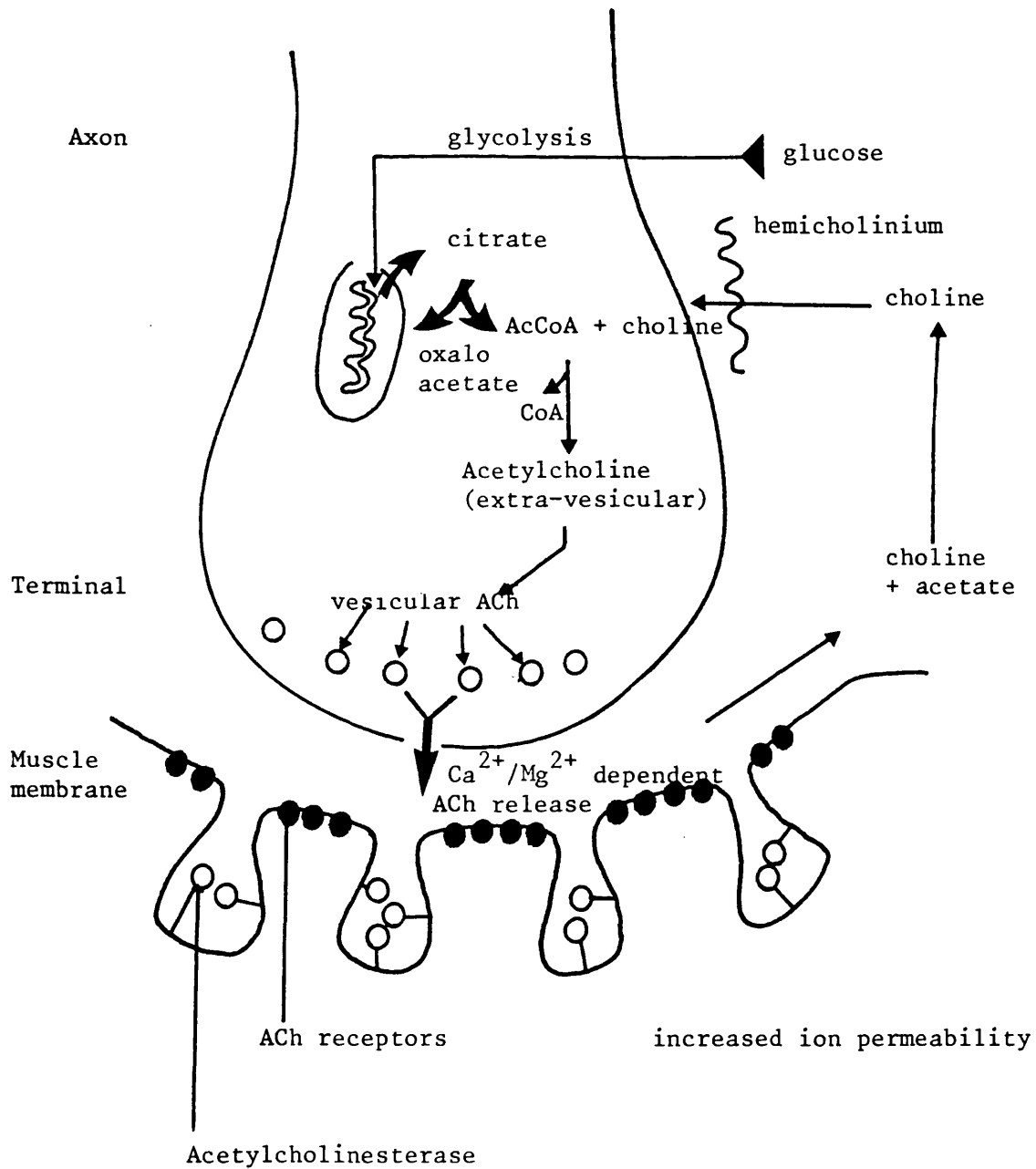


FIG. 2. The organisation of the cholinergic terminal at the vertebrate neuromuscular junction



The  $\alpha$  neurotoxins as probes for the acetylcholine receptor

It has been known for a long time that snakes from the elapid and hydrophobid families kill their prey by paralysing the respiratory muscles. In the late 1950's Lee (for review see Lee and Chang 1966) was able to explain the action of the venom in terms of its blockage of neuromuscular transmission. He showed that very low concentrations of the venom were capable of producing an anti-depolarising block at the nerve-muscle junction, very similar in action to that of acetylcholine antagonists, particularly curare. It did not affect the action potential of either the nerve or the muscle, the release of acetylcholine or its hydrolysis by acetylcholinesterase. Moreover the inhibitory action could be reversed by cholinergic effectors. Analysis of the crude venom showed that a single polypeptide, now called  $\alpha$  toxin, was responsible for the neuromuscular block, which as indicated by the above evidence results from specific binding of the  $\alpha$  toxin to the acetylcholine receptor.

Two types of  $\alpha$  neurotoxin have been isolated. Type I produce a reversible neuromuscular blockade whereas type II blockade is nearly irreversible. Both types of  $\alpha$  toxins are compact, basic polypeptides. Type I, for example  $\alpha$  toxin of *Naja naja siamensis* have 60-62 amino acid residues (Cooper and Reich 1972) and type II have 71-74 residues, for example  $\alpha$  bungarotoxin (from *Bungarus multicinctus*) has a molecular weight of 8000, 74 amino acid residues 5 disulphide bridges and an isoelectric point pI 9 (Dolly 1979).

The specific binding of the  $\alpha$  toxins to the neuromuscular acetylcholine receptor makes them ideal probes for the latter and for this purpose it is possible to radiolabel the  $\alpha$  toxins with the



retention of their biological activity. Several methods have been employed including incorporation of  $^{131}\text{I}$  or  $^{125}\text{I}$  into the tyrosine residue (Miledi *et al* 1971; Berg *et al* 1972); partial methylation ( $^{14}\text{CH}_3$ ) of amino groups (Biesecker 1973) and tritiation by incorporation of an acetyl (Dolly and Barnard 1974) or propionyl group (Barnard *et al* 1979).

The radiolabelled  $\alpha$  toxins have proved invaluable in that they are used to identify the receptor protein both in solution and in fragments of cell membrane where electrophysiological measurements of receptor function are not possible. They form the basis of the assays for the detection of acetylcholine receptor and have been used in autoradiographic studies to examine the density and distribution of the receptor in intact cell membranes. In such studies it has been found that in innervated muscle the receptor is concentrated within the motor end-plate. Further, the structure of the muscle sarcolemma in the end-plate region is highly convoluted and the receptor has highest density at the outermost tips of the fold (review see Dolly 1979). In denervated muscle, the acetylcholine receptors have been shown to proliferate over the whole membrane surface (Ginetzinsky and Sharmarina 1943, Axelsson and Thesleff 1959, Miledi 1960) and this phenomenon has been exploited in the purification of the receptor from skeletal muscle.

The  $\alpha$  toxins are of major use not only in the detection of acetylcholine receptors but also in its purification when the critical step is commonly affinity chromatography on a column of  $\alpha$  toxin. These methods will be fully discussed later.

The electric organs of the electric fish

The electric organs of the electric fishes consist of arrays of large flat cells called electroplaques, each of which is capable of producing a change in potential on receiving a nerve impulse. The potential generated by each electroplaque is of the order of 0.14V and as the cells are arranged in series, the discharge produced is the summation of the evoked potentials. The two most studied fish, *Electrophorus electricus* and *Torpedo marmorata* generate potentials of 600V and 40-60V respectively.

The electroplaque of both species is a giant syncytium containing several thousand nuclei. It is a highly asymmetric cell which receives nerve terminals on only one of its faces: the caudal one in *Electrophorus*, the ventral in *Torpedo*. The plasma membrane on both surfaces shows a remarkable increase of surface as a consequence of the proliferation of invaginations and villousities. In *Electrophorus*, nerve endings establish  $10^5$ - $10^6$  specific contacts with one electroplaque but cover only 1.4-2% of the surface of the membrane on the innervated face. In *Torpedo*, innervation is much denser and the innervated membrane less convoluted so that the subsynaptic areas occupy up to 50% of the total surface of the innervated membrane. Thus electric organs from *Torpedo* are expected to be much richer in receptor than those of *Electrophorus* (Changeux 1975).

In 1877, Du Bois-Reymond realised that the electrical discharge produced in the electric organs was generated in the same way as that at the nerve-muscle junction. Embryologically the electric organ develops from the same tissue as does skeletal muscle. However the contractile elements are absent from the electroplaques

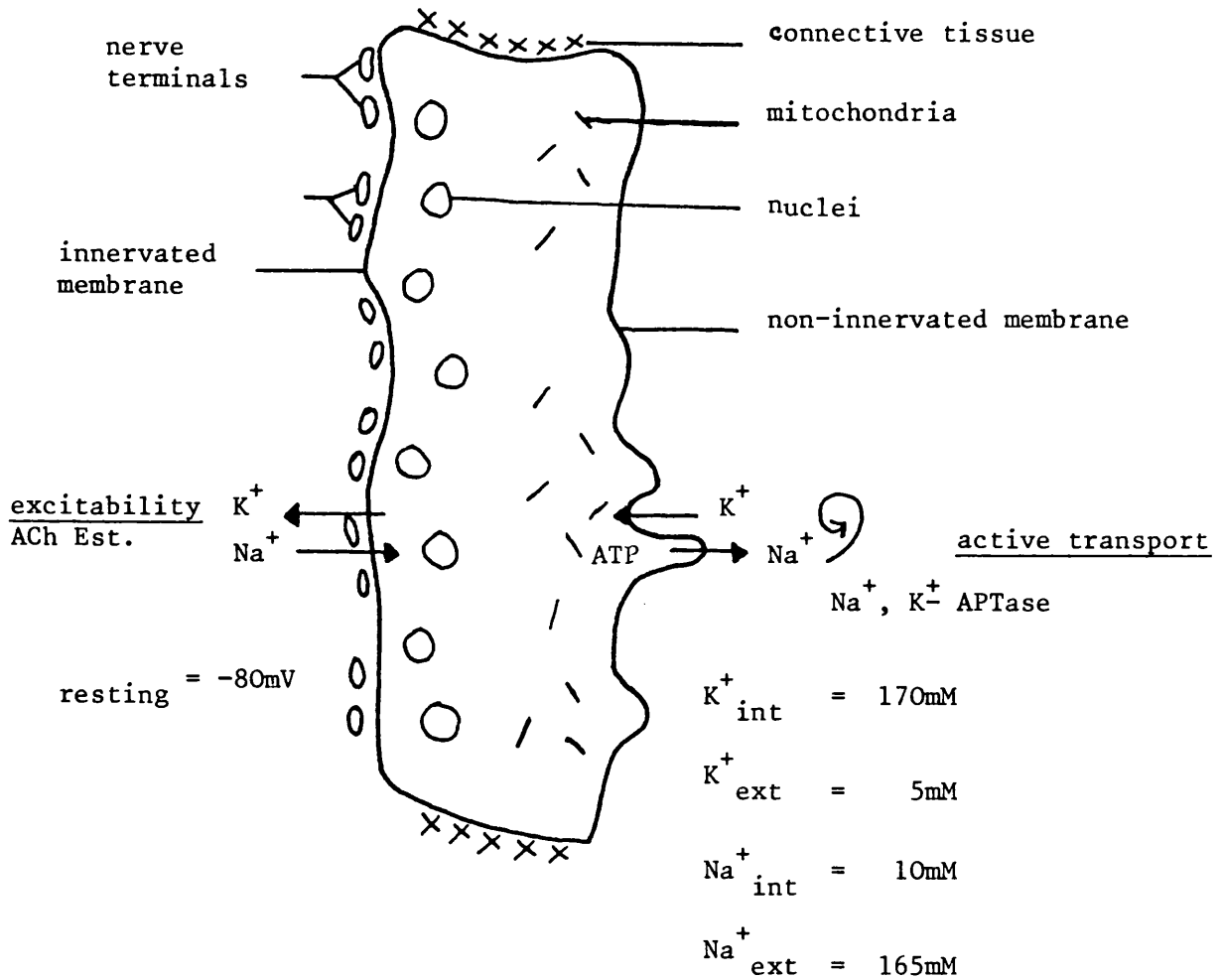


FIG. 3. The schematic representation of an isolated electroplaque. (After Changeux 1975)

which nevertheless respond to acetylcholine in a similar way to muscle cells. In the electric organ of *Electrophorus*, the response to acetylcholine is the generation of an action potential analogous to that seen in a muscle cell. However the electroplaque of *Torpedo* is unable to generate an action potential and the discharge of the cell derives from the summation of individual excitatory end-plate potentials. In both types of electroplaques the net influx of current then passes through the non-innervated face of the cell to complete the circuit (Lester 1977).

The electric organs of these fish are an ideal model for the study of neurotransmission at the neuromuscular junction as they possess similar excitable properties (acetylcholine is the neurohumoral activator) and the organisation of the myoneural elements is such that all mediators involved in the transduction process are present in abnormally high concentrations. Extensive investigations of the electric organs gave more information about the nature of the acetylcholine receptor and resulted ultimately in its purification.

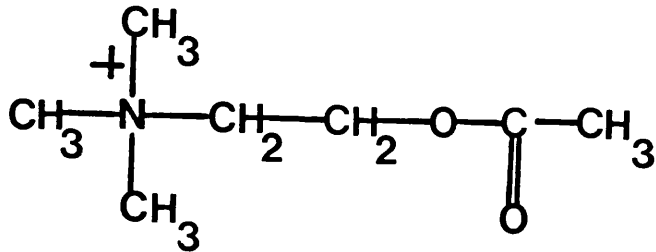
#### The pharmacological properties of the isolated electroplaque

Schoffeniels and Nachmansohn (1957) developed a system whereby an isolated electroplaque from *Electrophorus electricus* is mounted in a chamber such that the innervated membrane is bathed in one physiological solution and the non-innervated membrane in a second. A micro-electrode is inserted into the interior of the cell and a second micro-electrode is placed adjacent to the innervated face, external to the cell, such that the membrane potential and conductance can be measured. Pharmacological agents were then introduced into the physiological solution bathing the innervated face and the change in potential and

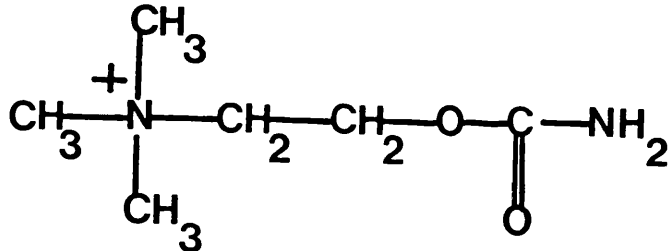
conductance recorded. The use of an isolated electroplaque overcame problems such as accessibility of the cholinergic agents to the innervated face and enabled low concentrations to be employed.

The pharmacological properties observed (Nachmansohn 1959) were similar to those seen at the nerve-muscle junction by Dale et al (1936). Agonist activity was found to be subject to strict structural limitations, requiring the presence of a trimethylammonium group and a carbonyl oxygen at a 59nm radial distance from the quaternary ammonium. Concentration-effect curves for agonist response showed saturation in that in the presence of excess agonist a maximal response was observed. The shape of the concentration-effect curves was slightly sigmoid and determination of the gradient of Hill plots showed the presence of co-operativity. The effect of an agonist was shown to reverse the blocking effect of an antagonist and *vice-versa* rather like the competitive interaction between substrate and inhibitor at an enzyme catalytic site. It was Nachmansohn (1959) who first noted the analogy between the binding of acetylcholine to its receptor and that of an enzyme catalytic site and proposed that the receptor was a protein.

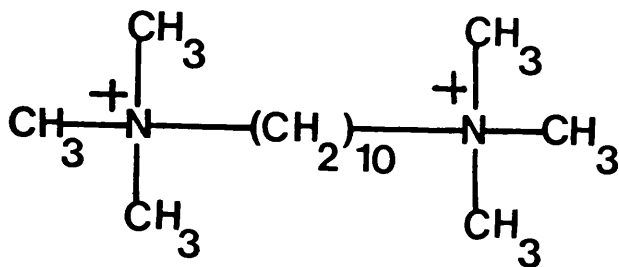
Studies with the isolated electroplaque distinguished the receptor site from the active site of acetylcholinesterase known to be present at high concentration on the innervated face (Marnay 1937). Exposure of the innervated membrane to agonists depolarised the membrane but did not inhibit acetylcholinesterase activity. Conversely high concentrations of anti-acetylcholinesterase did not inhibit the response to direct stimulation which was accordingly shown not to result from inhibition of the active site of acetylcholinesterase.

FIG. 4. Cholinergic effectors (A)Agonists

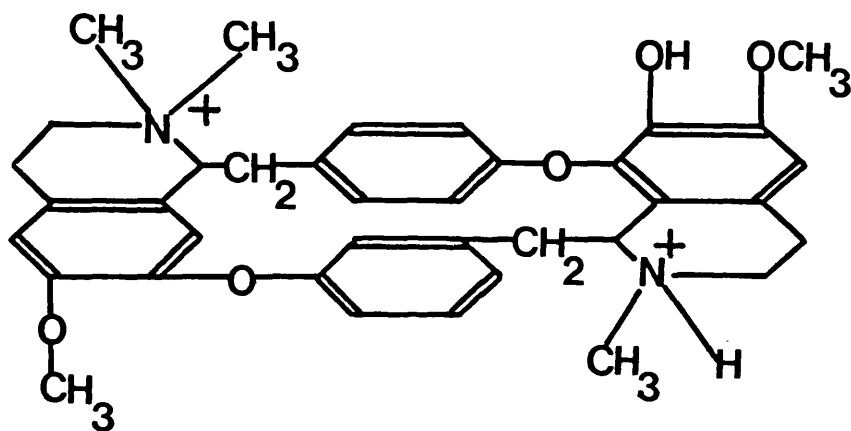
Acetylcholine



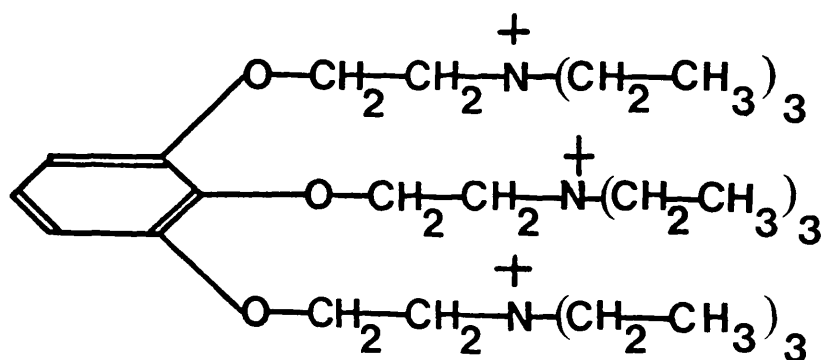
Carbamoylcholine



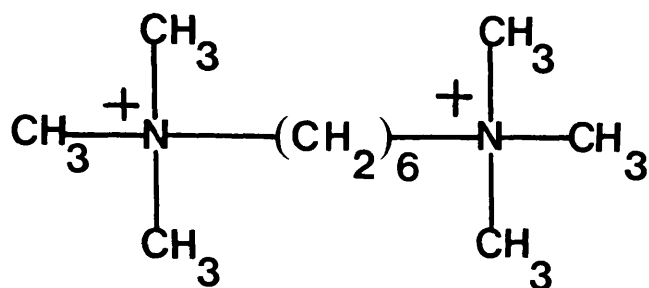
Decamethonium

FIG. 5. Cholinergic effectors (B)Antagonists

d-tubocurarine



Gallamine triethiodide



Hexamethonium

The response of the isolated electroplaque to acetylcholine is affected by sulphhydryl reagents. This was first investigated by Karlin and Bartels (1966) when they showed that pre-exposure of an isolated electroplaque to p-chloromercuribenzoate, a sulphhydryl reagent, reduced the response to both acetylcholine and carbamoylcholine the effect being reversed by treatment with thiols. Also, pre-exposure to the disulphide reducing agent, 1,4-dithiothreitol, produced a similar effect and interestingly altered the activity of hexamethonium from antagonist to agonist (Karlin and Winnick 1968) and enhanced the effect of decamethonium (Podleski *et al* 1969).

Evidence from the work with isolated electroplaques supported the idea that the receptor is a protein with a site of action distinct from that of acetylcholinesterase and with a disulphide bridge in the vicinity of the acetylcholine binding site which has importance in the recognition of cholinomimetic agents.

#### Isolation of the acetylcholine receptor

Chagas and Ehrenpreis (review Hasson-Voloch 1968) made the first reports of purification of an acetylcholine receptor. Their receptors were obtained from *Electrophorus electricus* and were characterised by the binding of the radioactive antagonists, gallamine triethiodide and curare. However, the "receptor-ligand" complex isolated was readily dissociated by 0.02MNaCl although it was known that intact electroplaques would bind curare in the presence of 0.18MKCl. Subsequent work showed that the binding properties of these putative receptors could be attributed to non-specific electrostatic interactions.



A similar approach to purification was employed by De Robertis (review De Robertis 1971) who used radiolabelled d-tubocurarine to follow the receptor for acetylcholine. He showed that the specific binding of the cholinergic effector was localised to nerve-ending membranes in rat cerebral cortex (Azcurra and De Robertis 1967; De Robertis *et al* 1969). The binding component was extractable in a chloroform-methanol mixture and the extract shown to contain lipid and proteolipid only. Column chromatography of the organic extract showed the radioactivity to be bound to a proteolipid species. De Robertis obtained similar results using the electric organ of *Electrophorus electricus* and obtained a molecular weight of 40000 for the isolated proteolipid.

Simultaneously Changeux was studying the electric organ of *Electrophorus electricus*. The receptor was at this time thought to be a protein and evidence was accumulating to show that the receptor and acetylcholinesterase were discrete entities. Thus autoradiographic studies of mouse diaphragm suggested that receptor and acetylcholinesterase were two different macromolecules (Waser 1967) while Axelsson and Thesleff (1959) and Miledi (1960) showed that the two entities had opposite behaviours after denervation. Acetylcholinesterase tended to disappear but iontophoretic studies on the muscle membrane showed the receptor to have a wider distribution. There was also found to be a difference between the apparent dissociation constants of acetylcholine with receptor on the one hand and with acetylcholinesterase on the other (Karlin 1967). Moreover incubation of membranes from *Electrophorus electricus* with 1M NaCl solubilised all acetylcholinesterase activity whereas the binding properties of cholinergic effectors remained unchanged (Silman and Karlin 1967).

Changeux *et al* (1969) isolated a membrane fragment from *Electrophorus electricus* that was rich in acetylcholinesterase activity. In view of the observations of Silman and Karlin (1967) which suggested that the receptor was an integral membrane protein, Changeux *et al* (1970) used the ionic detergent sodium deoxycholate to solubilise a protein from the electric organ of *Electrophorus electricus*. They showed agreement between the dissociation constants ( $K_D$ ) for the binding of agonists and antagonists to the acetylcholinesterase-rich electroplaque membrane fragments, the isolated electroplaque and to the detergent solubilised receptor. It was also demonstrated that  $\alpha$  bungarotoxin, which blocked the response of isolated electroplaques to bath-applied carbamoylcholine also inhibited the binding of decamethonium to detergent-soluble protein (Changeux *et al* 1970a; Changeux *et al.* 1970b).

Miledi *et al* (1971) solubilised a protein- $^{131}\text{I}$ - $\alpha$  bungarotoxin complex from membranes of *Torpedo marmorata* that had been primarily exposed to  $^{131}\text{I}$ - $\alpha$  bungarotoxin. Gel chromatography of the radiolabelled material showed the radioactivity to be eluted separately from acetylcholinesterase. Meunier *et al* (1971a) repeated the experiment with *Electrophorus electricus* and obtained comparable results. Thus the acetylcholine receptor was isolated and shown to be distinct from acetylcholinesterase.

#### The assay for acetylcholine receptors

It was necessary to develop a quantitative, reproducible method for the detection of the isolated receptor. The assay method should satisfy several strict criteria if the measurements obtained are to be a true representation of the ligand-receptor interaction

*in vivo*. The conditions in brief outline are that:-

- (i) the ligand used as a probe must be fully active biologically so that it can mimic the activity of the parent compound at the receptor site.
- (ii) the binding observed must exhibit absolute structural and steric specificity which must relate to the known biological activity of the parent ligand, its structural agonists and antagonists.
- (iii) the binding should demonstrate saturability within a concentration range that can be meaningfully related to that of agonists which elicit the known biological response in intact biological systems.
- (iv) the binding interactions should reflect an affinity in keeping with the sensitivity of the tissue to the physiologically active concentration of the ligand.
- (v) the presence of binding should be restricted to tissue known to be physiologically sensitive to the ligand.

Initially [ $^{14}\text{C}$ ] decamethonium (Changeux *et al* 1971) or [ $^3\text{H}$ ] acetylcholine (Eldefrawi and Eldefrawi 1973a) were used as the ligand and bound ligand was separated from free ligand by equilibrium dialysis. Radiolabelled  $\alpha$  bungarotoxin has now become almost universal in assays for the detection of acetylcholine receptor because of its high sensitivity made possible by radiolabelling to specific activities of up to 675Ci/mmol (this thesis) and because of the virtually irreversible nature of its binding ( $K_D$   $10^{-10}\text{M}$ -  $10^{-12}\text{M}$  (Dolly 1979; Vincent and Newsom-Davis 1979)).

The most convenient method for the separation of bound  $\alpha$  bungarotoxin from free ligand utilises the acidic properties of the receptor macromolecule which is retained on DEAE cellulose filter discs whereas basic, unbound  $\alpha$  bungarotoxin is not (Schmidt and Raftery 1973(a); Dolly and Barnard 1977). Other methods that have been used for the separation of bound and free ligand include differential ammonium sulphate precipitation of the receptor-toxin complex (Meunier *et al* 1972(a)); gel filtration through a column of Sephadex G50 (Biesecker 1973); ultracentrifugation of the receptor-toxin complex in a sucrose density gradient (Lindstrom and Patrick 1974) and retention of the complex on a Millipore filter after a fifty fold dilution of the detergent in the incubation mixture (Olsen *et al* 1972).

A novel approach was the development of an assay using a tritiated affinity ligand 4-(N-maleimido) benzyltri [ $^3\text{H}$ ] methyl-ammonium iodide (Karlin and Cowburn 1973), which is highly specific for only one sub-unit of the receptor. The assay developed is more laborious and less sensitive than conventional methods but has the advantage of high specificity and total irreversibility.

Solubilisation and purification of the acetylcholine receptor from electric fish

In view of the high concentration of acetylcholine receptors in the electroplaque membranes of electric fish, this source has been most used for the purification of receptor. The most successful method for solubilising the receptor from its membrane environment is the extraction of the receptor in either ionic or non-ionic detergent. High salt concentration, enzymic digestion with collagenase or lipase, extraction in distilled water or prolonged sonication are all equally ineffective (Changeux *et al* 1971; Olsen *et al* 1972). A comparison of the efficiency of extraction by the detergents showed sodium deoxycholate to be the most effective in solubilising [ $^{14}\text{C}$ ] decamethonium binding activity from membranes of *Electrophorus electricus*. Removal of detergent was found to be necessary in order to avoid artefactual results in binding studies however (Changeux *et al* 1971) and the non-ionic detergent Triton X100 which circumvents this problem is now more widely used.

Conventional purification procedures, for example ammonium sulphate precipitation, DEAE cellulose chromatography, hydroxylapatite chromatography, have been used to purify the acetylcholine receptor but have generally not resulted in complete purification of the active receptor. The most dramatic purification is achieved by using affinity chromatography. The first affinity column utilised  $\alpha$  toxin from *N. nigricollis* venom coupled to Sepharose (Meunier *et al* 1971(b)) and [ $^{14}\text{C}$ ] decamethonium binding activity was thereby absorbed from a detergent extract leaving acetylcholinesterase activity in the supernatant. The complex formed in the affinity separation was difficult to dissociate so the more reversible Type I  $\alpha$  toxins (see  $\alpha$  toxins as

probes for acetylcholine receptors, page 8 ) were subsequently employed as immobilised ligands, for example *N. naja* (Karlsson et al 1972; Lindstrom and Patrick 1974) and *Naja naja siamensis* (Klett et al 1973; Eldefrawi and Eldefrawi 1973(b)). Desorption from the affinity column was attained by the use of cholinergic agents, carbamoylcholine (Lindstrom and Patrick 1974); hexamethonium (Klett et al 1973) or gallamine triethiodide (Dolly and Barnard 1975).

Other ligands have been successfully applied in the purification of receptor protein by affinity chromatography, for example synthesised quaternary ammonium derivatives (Olsen et al 1972; for review, Fulpius 1976).

The eluting ligand is easily removed by dialysis (Karlsson et al 1972; Olsen et al 1972) or by gel filtration (Shorr et al 1978). Additional steps are taken to purify the protein to homogeneity: ultracentrifugation in a sucrose density gradient (Lindstrom and Patrick 1974); chromatography on DEAE cellulose (Klett et al 1973) or DEAE Sephadex (Dolly and Barnard 1977) or by preparative electrophoresis (Eldefrawi and Eldefrawi 1973(b)). The receptor protein from electric fish has been purified to specific activities in the range 2-12  $\mu\text{mol}$   $\alpha$  bungarotoxin binding sites per gram protein (for reviews see Changeux 1975; Fulpius 1976; Heidmann and Changeux 1978).

Recently Sobel et al (1977) and Elliott et al (1979) reported a new method for the large scale purification of acetylcholine receptor from the electric organ of *Torpedo marmorata*. Acetylcholine receptor-rich membrane fragments were prepared and suspended in buffer containing Triton X100. The mixture was centrifuged and fractions collected which yielded a purified receptor with a specific activity

of  $9\mu\text{mol } [^3\text{H}] \alpha$  toxin binding activity/g protein. This method is convenient using *Torpedo marmorata* electric organ only because of the high initial concentration of receptor and is also only applicable to fresh (i.e. unfrozen) tissue (unpublished observations, T. Barkas).

#### The purification of acetylcholine receptor from skeletal muscle

Progress in the purification of the acetylcholine receptor from skeletal muscle has been much slower than from the electric fish because of the extremely low proportion of the total muscle protein that constitutes the receptor. It was observed by both Axelsson and Thesleff (1959) and Miledi (1960) that surgical denervation of muscle results in a proliferation of receptors over the whole muscle membrane, as shown by the response of the muscle to iontophoretic application of acetylcholine. Miledi and Potter (1971) found that the increased sensitivity to acetylcholine along the muscle fibre of denervated muscle paralleled an increase in specific  $^{131}\text{I}-\alpha$  bungarotoxin binding sites. Table 1 is a comparison of the acetylcholine receptor content of different tissues including denervated and innervated muscle. It shows that the acetylcholine receptor concentration in denervated muscle is comparable to that in *Electrophorus electricus* whereas innervated muscle has only one tenth of that concentration. The acetylcholine receptor from skeletal muscle behaves the same as does receptor from the electric organs in that it can be solubilised by non-ionic detergents (Miledi and Potter 1971; Berg *et al* 1972) and when solubilised it responds to the same pharmacological agents. Its purification has been reported from several mammalian species: denervated cat and rat muscle (Dolly and Barnard 1977; Shorr *et al* 1978); denervated

Table 1

Species	Binding capacity pmol/g tissue	Reference
<u>Torpedo marmorata</u>	1090	Miledi et al (1971)(b)
<u>Torpedo marmorata</u>	1720	Eldefrawi and Eldefrawi (1973)(b)
<u>Torpedo californica</u>	750	Schmidt and Raftery (1973)(b)
<u>Electrophorus electricus</u>	20-30	Eldefrawi et al (1971)
<u>Electrophorus electricus</u>	53	Olsen et al (1972)
Denervated rat muscle	60	Miledi and Potter (1971)
Denervated rat muscle	23	Berg et al (1972)
Denervated cat and rat muscle	80	Dolly and Barnard (1975)
Innervated rat muscle	2.5	Berg et al (1972)
Innervated rat muscle	8.5	Almon et al (1974)(a)
Innervated rat muscle	3.8	Colquhoun et al (1974)
Innervated human muscle	0.7	This thesis



and innervated rat muscle (Brookes and Hall 1975 ; Froehner et al 1977(a)(b)); from a non-fusing muscle cell line (Boulter and Patrick 1977) and from rat and foetal calf muscle (Lindstrom et al 1979(a)). The methods employed were essentially as for purification of the receptor from the electric fish using affinity chromatography as the main tool.

#### Criteria of purity of the isolated acetylcholine receptor

Acetylcholine receptor has been purified from the electric organs of the electric fish to specific activities of 2-12  $\mu\text{mol}$   $\alpha$  bungarotoxin binding sites per gram protein (see "solubilisation and purification of the acetylcholine receptor from electric fish", page 21 ). Similarly, maximum specific activities reported from cat and rat denervated muscle are 10-12  $\mu\text{mol}$   $\alpha$  bungarotoxin binding sites per gram protein ( Shorr et al 1978; Barnard et al 1979) but these activities were achieved only after a second passage through the  $\alpha$  toxin affinity column or passage through a lectin column. Shorr et al (1978) point out that for their purified receptor protein of the sub-unit molecular weight 41000, the theoretical specific activity is 24  $\mu\text{mol}$ /gram protein and possible explanations for the experimental results are an in-activation of the receptor during isolation or two sub-units binding to one toxin molecule or possibly a charge heterogeneity in the sub-units, with only one type binding toxin (Sobel et al 1977).

Further reported purifications include receptor from the non-fusing mouse muscle cell line purified to a specific activity of 2.6  $\mu\text{mol}$   $\alpha$  bungarotoxin binding sites/gram protein (Boulter and Patrick 1977) from rat denervated muscle to a specific activity of 8-10  $\alpha$  bungarotoxin binding sites/gram protein and from rat innervated muscle,

0.19 $\mu$ mol  $\alpha$  bungarotoxin binding sites/gram protein (Brockes and Hall 1975). In all purified receptor preparations, acetylcholinesterase activity was not detectable.

Polyacrylamide gel electrophoresis of purified native receptor from cat and rat muscle gave a single band (Dolly and Barnard 1977) as did that from *Electrophorus electricus* (Meunier et al 1974). Additionally, purified receptor labelled with  $^3\text{H}$ - $\alpha$  bungarotoxin gave an identical single band when subjected to polyacrylamide gel electrophoresis (Meunier et al 1974).

Isoelectric focussing in polyacrylamide gels also showed homogeneity of purified receptor with only one stained band visible. Isoelectric points reported are: pI 4.7 for *Electrophorus electricus* (Biesecker 1973); *Torpedo marmorata* pI 4.8 (Eldefrawi and Eldefrawi 1973(b)); *Torpedo Californica* pI 4.9 (Heilbronn et al 1974) and denervated cat muscle pI 4.9 (Shorr et al 1978). Focussing of the radiolabelled toxin-receptor complex gave one peak of radioactivity which was shifted towards basic pH, for example *Electrophorus electricus* (Biesecker 1973) pI 5.2 and *Torpedo marmorata* pI 5.3 (Sobel et al 1977) and denervated cat, rat muscle pI 5.3 (Dolly and Barnard 1977). Brockes and Hall (1975) were able to separate junctional (innervated) and extrajunctional (denervated) receptors on the basis of their isoelectric points. Toxin labelled extrajunctional receptor focus at pH 5.3 and toxin labelled junctional receptor at pH 5.1. This has also been repeated by Sugiyama (1979) with acetylcholine receptor extracted from a skeletal muscle cell line.

The physical properties of the purified receptor

(i) Determination of molecular weight

Determination of the molecular weight of the purified receptor in solution can only give approximate values because of the presence of bound detergent. Meunier *et al* (1972(b)) showed that Triton X100 can contribute up to 21% (160-170 molecules) of the total mass of the receptor-toxin complex in solution and similarly Karlin *et al* (1979) reported that the receptor binds 0.5g Triton X100 per gram protein. Values for the molecular weight of all species have been determined and range from 250000-370000 daltons (review see Changeux 1975; Fulpius 1976). Methods employed to obtain a value for the molecular weight of the protein include cross-linking of the receptor-toxin complex by the use of glutaraldehyde or p-difluorodinitrobenzene followed by analysis by sodium dodecyl sulphate polyacrylamide gel electrophoresis to give a value of 260000 (Biesecker 1973), passage of cat or rat denervated receptor-toxin complex down a gel filtration column to give a value of 370000 (Dolly and Barnard 1977) and osmometrical measurements in *Torpedo californica* receptor giving a value of 270000 (Martinez-Carrion *et al* (1975). Sedimentation of receptor or receptor toxin complex in a sucrose density gradient showed that the protein sedimented with a sedimentation coefficient  $s_{20,w}^{9S}$ . This was so for all species, *Torpedo marmorata* (Sobel *et al* 1978); *Electrophorus electricus* (Biesecker 1973); denervated cat and rat muscle (Dolly and Barnard 1977) and denervated rat muscle (Froehner *et al* 1977). A second species in both electric organ of *Torpedo californica* (McNamee *et al* 1975) and *Torpedo marmorata* (Barnard *et al* 1978) has been found with sedimentation coefficient  $s_{20,w}^{13S}$  and has been shown to be a dimeric form of the receptor, being

stabilised in the presence of N-ethyl maleimide (Barnard *et al* 1978). Calculation of the molecular weights of the monomer and dimers of *Torpedo californica* has given values of 323000 and 470000 (Meunier *et al* 1972(b)) in the presence of different detergents and perhaps truer values of 250000 and 500000 (Reynolds and Karlin 1978). The ratio of the Stokes radius to the minimum possible radius for the protein-detergent complex falls outside the range for globular proteins (Meunier *et al* 1972(b)) and indeed neutron scattering measurements of the monomer and dimer in detergent solution are consistent with the protein being an oblate ellipsoid with axial ratio of 4:1 (Karlin *et al* 1979).

(ii) The subunit composition of the purified receptor

The receptor has been shown to have an oligomeric structure as, in the presence of sodium dodecyl sulphate, it splits into subunits of low molecular weight (Karlin *et al* 1971; Meunier *et al* 1971(a), 1972(a); Raftery *et al* 1971). Analysis of the subunits by sodium dodecyl sulphate polyacrylamide gel electrophoresis under denaturing conditions has shown that receptor purified from *Torpedo marmorata* and *Torpedo californica* has four subunits with molecular weights 38-40000( $\alpha$ ); 50000 ( $\beta$ ); 57000 ( $\gamma$ ); 64000 ( $\delta$ ) present in the ratio of 2:1:1:1 (Barnard *et al* 1979; Lindstrom *et al* 1979(b); Raftery *et al* 1979; review see Heidmann and Changeux 1978). Saitoh *et al* (1979) on the other hand have reported that receptor purified from *Torpedo marmorata* had only one major subunit of 40000 and a minor subunit of 43000 which could be eliminated with alkaline treatment of receptor-rich membrane fragments. *Electrophorus electricus* has a subunit structure 38-40000; 50000, 57000 (Lindstrom *et al* 1979(a)).

For mammalian receptor, Lindstrom *et al* (1979(a)) using denervated rat and foetal calf muscle found four subunits corresponding to the  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$  chains of *Torpedo*. Boulter and Patrick (1977) demonstrated the presence of four subunits of molecular weights 72000; 65000; 53000 and 44000 in purified receptor of a non-fusing muscle cell line and Froehner *et al* (1977(a)) using denervated rat muscle found two major polypeptides with molecular weights 45000; 51000 and four minor chains of 49000; 56000; 62000; 110000 daltons. Barnard *et al* (1979) have identified purified receptor from denervated cat and rat muscle as having only one major subunit with molecular weight 43-44000.

(iii) Affinity labelling of the acetylcholine receptor protein

The affinity ligands were developed to obtain information about the active site of the receptor protein. The ligands form a covalent bond at the active site of the specific protein. They can be readily radiolabelled and are designed as transmitter analogues. Electrophysiological studies with an isolated electroplaque showed that both p-(trimethylammonium) benzediazonium fluoroborate (TDF) (Changeux *et al* 1967) and 4-(N-maleimido) phenyltrimethylammonium iodide (MPTA) (Karlin and Winnick 1968) reduced acetylcholine sensitivity. MPTA and 4-(N-maleimido) benzyltrimethylammonium iodide (MBTA) are the most useful affinity agents in that they are not so reactive as diazonium derivatives but have a forty fold faster rate of reaction with the reduced disulphide bond at the acetylcholine binding site than other membrane sites (Karlin and Winnick 1968).

Reiter *et al* (1972) first reported that only a peptide with molecular weight 42000 was labelled with the affinity ligand

[<sup>3</sup>H]-MBTA on exposure of an isolated electroplaque to the reagent. Further studies with receptor purified from the electric fish confirmed the initial observation that only the 42000 subunit is labelled (review see Karlin 1974; Heidmann and Changeux 1978).

In mammalian systems, Lyddiat *et al* (1979) labelled the 43-44000 subunit of denervated cat muscle with the affinity agent bromoacetylcholine (Silman *et al* 1969). Nathanson and Hall (1979) found that two polypeptides 45000, 51000 were labelled with [<sup>3</sup>H]-MBTA from purified receptor of both innervated and denervated rat muscle (also Froehner *et al* 1977(b)).

It is not possible to examine the subunits of the receptor labelled with  $\alpha$  bungarotoxin as the receptor-toxin complex is found to dissociate in the presence of sodium dodecyl sulphate (Berg *et al* 1972). Raftery *et al* (1979) covalently labelled purified receptor from *Torpedo californica* with a derivatised <sup>125</sup>I- $\alpha$  bungarotoxin containing a photo-labile group. After irradiation, the distribution of radioactivity after sodium dodecyl sulphate polyacrylamide gel electrophoresis showed that two subunits were labelled, 48000 (assumed to be a 40000 polypeptide associated with the 8000  $\alpha$  bungarotoxin) and 52000. Prior reduction of the receptor labelled the 65000 subunit. Similar experiments have been carried out with denervated rat receptor (Nathanson and Hall 1980) and the toxin derivative was found to label five polypeptides.

#### The chemical properties of the isolated receptor protein

Amino acid analysis of the purified receptor has confirmed the protein nature of the receptor and all amino acids associated with proteins were found (Changeux 1975). Devillers-Thiery *et al* (1979)

have reported the amino-terminal sequence of twenty amino acids for the 40000 subunit purified from the electric organ of *Torpedo marmorata*. Peptide mapping by proteolytic digestion of the isolated polypeptides of receptor showed no homology between chains (Froehner and Rafto 1979; Lindstrom et al 1979(b)).

The receptor protein also contains carbohydrate as shown by the interaction of the receptor with plant lectins (Meunier et al 1974) and this property has been utilised in the purification of mammalian muscle receptors (Shorr et al 1978; Brockes and Hall 1975). Polyacrylamide electrophoresis gels of purified receptor stain with reagents specific for carbohydrate (Shorr et al 1978; Lindstrom et al 1979(b)) and further, the carbohydrate composition of *Torpedo californica* has been analysed by gas-liquid chromatography (Lindstrom et al 1979; Mattson and Heilbronn 1975; Raftery et al 1976 (b)). Concanavalin A inhibits the binding of  $^{125}\text{I}$ - $\alpha$  bungarotoxin to purified receptor from cultured muscle cells (Boulter and Patrick 1977) and solubilised receptor from rat innervated and denervated muscle (Almon and Appel 1976). Removal of the carbohydrate from purified receptor of *Torpedo marmorata* has no effect on the antigenicity of the protein (Wonnacott et al 1980) but antibody fragments to the purified receptor inhibit concanavalin A binding (Wonnacott et al 1980).

Analysis of purified receptor for associated lipid has shown negative results (Changeux 1975) but Lindstrom et al 1979(b)) report the presence of certain fatty acids with selected detergent extraction procedures.

### Immunological properties of the acetylcholine receptor

The immunological characteristics of the receptor have important clinical implications and these will be detailed in the section, "the acetylcholine receptor and myasthenia gravis", (page 37).

### The binding properties of the acetylcholine receptor in its solubilised and membrane bound forms

It has been suggested that acetylcholine receptor in the post-synaptic membrane can oscillate between two conformations (Changeux *et al* 1976), an active form which would be associated with the binding of agonists and the opening of the ion channels and an inactive form which would be in the closed conformation and would be stabilised in the presence of antagonists.

Initial studies on the motion of proteins in excitable membrane fragments by nanosecond fluorescence polarisation spectroscopy, showed that proteins are strongly immobilised within the membrane phase (Wahl *et al* 1971). However, it is possible to show that the binding of an agonist alters the observed spectrum of light from fluorescent probes attached to the membrane-bound acetylcholine receptor and it can be concluded that the change in the spectrum is the result of a conformational change of the protein (Cohen and Changeux 1973; Bonner *et al* 1976). A third possible state of the membrane-bound acetylcholine receptor is one where the receptor is pharmacologically "desensitised". The phenomenon of desensitisation is known physiologically and is the inability of a muscle to respond to applied cholinergic effectors after a prolonged exposure to agonists. An explanation for desensitisation in molecular terms is that the prolonged exposure to agonists shifts the receptors towards a tightly binding conformation where the channel



is closed (for a more detailed discussion see Heidmann and Changeux 1978).

To compare the behaviour of the membrane-bound acetylcholine receptor with the purified protein it is necessary to study the binding of cholinergic effectors to both forms to see if the solubilisation and purification of the receptor results in a major change of binding properties. Also, in this way it may be possible to obtain more information about the molecular distinction between agonists and antagonists as outlined earlier.

The binding constants for cholinergic agents can be measured by two methods both involving the reaction of radiolabelled  $\alpha$  bungarotoxin and the presence of a competing cholinergic ligand. Firstly, determination of the protection constant,  $K_p$ , involves measurement of the retardation of the initial rate of association between toxin and receptor in the presence of competing ligands. Secondly, assuming cholinergic and toxin binding sites are mutually exclusive, the dissociation constant,  $K_D$ , of the cholinergic ligand can be determined from the apparent dissociation constant of  $\alpha$  bungarotoxin in the presence of ligand. Weber and Changeux (1974) determined the binding constants for cholinergic effectors to *Electrophorus electricus* receptor by both methods and showed that they were in agreement. Values determined for the receptor of several species in both membrane-bound and soluble form generally agree well (Dolly 1979 and Colquhoun 1979) despite evidence which suggests that solubilisation of the receptor by both sodium cholate (Meunier et al 1974) and Triton X100 (Fischbach and Lass 1978) alters the affinity of the receptor for cholinergic effectors and inhibits acetylcholine receptor function.

Binding studies on the solubilised receptor from rat diaphragm with  $\alpha$  bungarotoxin have shown that the reaction between ligand and receptor follows regular second order kinetics (Colquhoun and Rang 1976). However, both Brockes and Hall (1975) and Vincent and Newsom-Davis (1979) have shown that acetylcholine receptor -  $\alpha$  bungarotoxin complex from both rat muscle (innervated and denervated) and human muscle dissociated in a biphasic manner suggesting the presence of two classes of binding sites.

The dissociation constant (ca  $10^{-10}$  M) for  $\alpha$  bungarotoxin is several orders of magnitude lower than that for cholinergic effectors (for review of values see Dolly 1979) and is consistent with the known almost irreversible nature of the binding *in vivo*. A competitive relationship is observed between the binding of cholinergic effectors and the  $\alpha$  toxins to the receptor but the relative stoichiometry between the two groups of ligands is not clear. Values ranging between 1 and 2 are reported for the ratio of sites between  $\alpha$  toxins and the quaternary ligands and affinity agents (reviews see Fulpius 1976; Heidmann and Changeux 1978).

With the availability of the isolated receptor, it has been possible to obtain a complete picture of the receptor protein. The integration of the characterised protein back into the membrane may lead to a deeper understanding of the transduction of the chemical message into the depolarisation of the muscle membrane (Heidemann and Changeux 1978). This understanding has been greatly facilitated by the discovery of histrionicotoxin, an alkaloid from the Columbian tree frog, *Dendrobates histrionicus*, which reversibly blocks neurotransmission in muscle (Dolly et al 1977) and in *Electrophorus*

electroplaques (Kato and Changeux 1976) by inhibiting the ion-translocating activity of the acetylcholine receptor-channel complex without inhibiting the binding of acetylcholine to the acetylcholine receptor (Dolly 1979). Attempts to isolate the component that binds to histrionicotoxin, the ion conductance modulator, are still in their infancy, however, Sobel *et al* (1978) have reported the separation of the acetylcholine receptor binding subunit and a subunit of molecular weight 43000 that binds perhydro-histrionicotoxin. However, this has been disputed by other groups (Elliott *et al* 1979).

The acetylcholine receptor and myasthenia gravis

"Nevertheless those labouring with a want of spirits, will use these spirits for local motions as well as they can; in the morning they are able to walk firmly, to fling their arms about hither and thither or to take up any heavy thing; before noon the stock of spirits being spent, which had flowed into the muscles, they are scarcely able to move hand or foot. At this time, I have under my charge a prudent and honest woman who for many years has been subject to this sort of spurious palsy, not only in her members, but also in her tongue. She can for some time speak hastily or eagerly, she is not able to speak a word, but becomes suddenly as mute as a fish, nor can she recover the use of her voice for an hour or two."

Thomas Willis 1672

Myasthenia gravis is a neuromuscular disorder which pursues a remitting and relapsing chronic course and is principally characterised by a weakness of muscle (Simpson 1978(a)(b)). The prevalence of myasthenia gravis ranges between 1 in 10000 to 1 in 50000 and is found to be twice as common in females with the most common age of onset for myasthenia without thymoma of twenty years (Simpson 1978(c)). The first observed symptom of myasthenia gravis is often ptosis followed by diplopia due to paralysis of one or more of the external ocular muscles. The symptoms characteristically appear in the evening when the affected person is tired but often disappear with rest. Involvement of other facial muscles may lead to difficulty in swallowing and articulation and also to a characteristic snarling look when smiling because of weakness of the muscles controlling the mouth. In the generalised case, the fatiguability spreads to the limbs and the inter-costal muscles, which often leads to attacks of dyspnoea and can prove fatal. Myasthenia gravis is a chronic disease which sometimes shows spontaneous remission. However, the usual course of the disease is progressive and eventually the patient becomes bed-ridden and emaciated because of the difficulty in chewing and swallowing. Death results from bronchopneumonia, cardiac failure or from attacks of dyspnoea which become more frequent in the final stages.

The disease was first described in 1672 by Thomas Willis. In 1895, Jolly showed that the muscle strength of a myasthenic patient was normal initially but declined rapidly and he coined the name "myasthenia gravis". He also predicted the beneficial effects of anticholinesterases but they were not used for therapy until by Mary Walker in 1934. At this time it was also noted that myasthenia

gravis had a strong resemblance to curare poisoning and this observation with the other supportive evidence helped in pin-pointing the area of defect to the neuromuscular junction.

A defect in neuromuscular transmission can be either pre-synaptic, intra-synaptic or post-synaptic and several hypotheses have been proposed which take account of all three sites. Possible areas of defects and mechanisms are:-

Pre-synaptic site

- (i) A failure in impulse propagation in the axon terminal.
- (ii) An impaired synthesis of acetylcholine.
- (iii) A faulty packaging of acetylcholine in the synaptic vesicles.
- (iv) An impaired release of acetylcholine.
- (v) The release of "false" neurotransmitter.

Intra-synaptic site

- (i) An excess diffusion of acetylcholine away from the synaptic cleft.
- (ii) A diffusion barrier which prevents acetylcholine traversing the synaptic cleft.

Post-synaptic site

- (i) A combination of a circulating blocking agent with the acetylcholine receptor.
- (ii) A depletion of acetylcholine receptors.
- (iii) A desensitisation of acetylcholine receptors.
- (iv) An increased acetylcholinesterase activity.

The results of several different lines of investigation have located the post-synaptic membrane as the area of defect and the evidence is reviewed below.

Neurophysiological studies of myasthenic patients have shown that the action potential generated by the nerve is normal. Intracellular recordings of the electrical activity of the muscle fibre however have shown that the miniature end-plate potentials are reduced in size (Elmqvist *et al* 1964) and that myasthenic motor end-plates *in vitro* are much less sensitive to iontophoretically applied acetylcholine than normal motor end-plates. Multi-electrode recordings from two or more muscle fibres from the same motor unit during a voluntary contraction, showed a greater time delay between the two impulses in myasthenic muscle fibres than in normal (Ekstedt and Stalberg 1967). This is attributed to the delay in onset of end-plate potentials resulting from the diminished size of the miniature end-plate potentials. Further, measurements of whole muscle to repetitive supramaximal stimuli of the nerve show that a significant decrement is observed in myasthenic patients (Desmedt 1966). The decremental response is consistent with the observed physical fatigability of the myasthenic and also with the experimental finding of reduced miniature end-plate potentials.

An investigation into the muscle fibres of myasthenic patients revealed that there were changes and these could be classified into three categories (Russell 1953). Firstly, an acute change where the muscle fibres undergo coagulative necrosis and become swollen losing their nuclei and myofibrils. There is a pronounced infiltration of inflammatory cells which are predominantly polymorphonuclear leucocytes and macrophages. Secondly, there is an observed formation of lymphorrhages, that is collections of small lymphocytes around single muscle fibres undergoing atrophy. The third category is one of simple atrophy where the nuclei remain at the periphery of the fibres; there is no loss of

cross-striations; there is no infiltration of inflammatory cells and the observed atrophy is confined to single or groups of fibres.

Morphological examination of the neuromuscular junction has revealed two abnormalities in myasthenic patients. One of these which is termed a dystrophic neuromuscular junction, involves the branching of the single motor neurone axon to form multiple end-plates on the same muscle fibre. In the second type, the dysplastic neuromuscular junction, the end-plates are lengthened on the surface of the fibre (Coers and Woolf 1959). Neither of these observations is specific to myasthenic muscle but it is interesting to note that it is possible to observe these changes without any microscopic evidence of degeneration of the muscle fibre and therefore is indicative of a primary lesion at the neuromuscular junction. A more detailed examination of the myasthenic neuromuscular junction under the electron microscope has shown a generalised simplification of structure. A widening of both the primary and secondary clefts is observed with a reduction in the number of secondary clefts (FIG 6 ) (Santa *et al* 1972). Also, Fambrough *et al* (1973) demonstrated a decrease in number of acetylcholine receptors at the motor end-plate and found no evidence to suggest that the receptors had become distributed over the whole muscle membrane surface as in denervation. In contrast, the structure of the axon terminal was retained with the normal number of synaptic vesicles present and twice the normal concentration of acetylcholine (Ito *et al* 1976). The axon terminal is seen to be reduced in size despite its retention of all other characteristics and its place is taken by an extension of the Schwann cell cytoplasm (Iwayama and Ohta 1972).



Pharmacological tests of myasthenic patients have shown that they are unusually susceptible to the effects of curare. They are resistant to the prompt depressant action of acetylcholine when injected intra arterially and are also resistant to the administration of decamethonium. Moreover their symptoms are alleviated by anti-acetylcholinesterases.

The overall evidence indicates the neuromuscular junction as the site of the lesion in myasthenia gravis and recent observations have indicated the presence of a humoral neuromuscular blocking agent in myasthenic sufferers which may be important in the initiation and pathogenesis of the disease.

It has been found that one in seven children born to myasthenic mothers show transient symptoms of myasthenia which disappear after a few weeks of life. In apparently normal neonates a decremental response has been observed to repetitive nerve stimulation and also a resistance to decamethonium. The suggestion is that a blocking agent, probably immunoglobulin G (IgG), crosses the placenta and is effective in the blocking of neurotransmission until the baby's own immune system begins to function.

In 1944, Wilson and Stoner applied serum from a myasthenic patient to a nerve-muscle preparation from frog, and demonstrated that it had a blocking effect following stimulation of the nerve. Similarly, Toyka (1975) injected immunoglobulins from myasthenic patients into mice and after 10-14 days observed a decremental response to repetitive nerve stimulation, reduced miniature end-plate potentials and a decrease in acetylcholine receptor concentration at the motor end-plate. Almon *et al* (1974(b)) and Bender *et al* (1975)

found that serum taken from myasthenic patients contains a factor which can inhibit binding of radiolabelled  $\alpha$  bungarotoxin to the acetylcholine receptors of rat diaphragm. Lindstrom et al (1976(a)) demonstrated the presence of circulating antibody to acetylcholine receptor in 90% of patients with myasthenia gravis.

Thus, it is established that there is a neuromuscular blocking agent in the sera of most myasthenic patients and it is now accepted that it is an antibody produced to the host acetylcholine receptor. Simpson (1960) first proposed that myasthenia gravis is an autoimmune disorder. He based his hypothesis on several observations which are included in the following summary of evidence supporting an autoimmune basis for the disease (McComas 1977).

- (i) Myasthenia gravis resembles systemic lupus erythematosus, a known autoimmune disorder, in that it is more common in females and has the highest incidence of onset in the third decade.
- (ii) Myasthenic patients often have other autoimmune disorders, for example rheumatoid arthritis, systemic lupus erythematosus and Sjogrens syndrome.
- (iii) the infiltration of lymphocytes within the myasthenic muscles is suggestive of an autoimmune disorder.
- (iv) there is a high incidence of thymic abnormalities i.e. thymic hyperplasia and thymoma, in myasthenics and it is known that the thymus has an important role in immune mechanisms.
- (v) it was reported in 1960 by Strauss that there are circulating antibodies to the A band region of striated muscle fibres in the sera of myasthenic patients. Later (1976(a)) Lindstrom detected antibodies to the acetylcholine receptor in myasthenic patients.

- (vi) the development of transient myasthenia in the infants of affected mothers is indicative of a neurohumoral agent traversing the placenta and it is known now that IgG can be transported from mother to baby via the placenta.
- (vii) immunosuppressant drugs are found to be beneficial in treatment of myasthenia.
- (viii) experimental models have been produced which resemble myasthenia gravis and are obtained by immunisation of animals with preparations of thymus (Goldstein model) or with purified acetylcholine receptor (EAMG).

#### Experimental autoimmune myasthenia gravis (EAMG)

The first model developed to mimic myasthenia gravis was the Goldstein model (Goldstein and Whittingham 1966) where animals were injected with a saline extract of thymus or striated muscle. Two weeks after immunisation a neuromuscular block was observed but was found to be short lived. A second model was that of Satyamurti *et al* (1975) and this model has now been superseded by that generally referred to as experimental autoimmune myasthenia gravis (EAMG).

EAMG was first induced in the rabbit by Patrick and Lindstrom (1977). The animals were immunised with acetylcholine receptor from *Electrophorus electricus* and showed a paralysis of the skeletal musculature within three weeks of the second injection. The rate of onset of paralysis was rapid and respiratory distress was evident within twenty four hours. The rabbits responded to an intravenous injection of antiacetylcholinesterases and antibodies to the immunogen were detected in the serum. Further, pre-exposure of the innervated face of an isolated electroplaque of *Electrophorus electricus* to the serum

from a rabbit with EAMG, blocked subsequent depolarisation with carbamoylcholine (Patrick *et al* 1973). Electrophysiological investigations showed that rabbits with EAMG exhibited a decremental response to repetitive nerve stimulation as seen with myasthenic patients. They also have reduced miniature end-plate potentials (Eldefrawi 1978) and the release of acetylcholine output at rest and during stimulation is normal (Kelley *et al* 1978).

EAMG has also been reported by Sugiyama *et al* (1973) with acetylcholine receptor purified from *Electrophorus electricus*. Similarly, EAMG has been induced in rabbits with purified acetylcholine receptor from *Torpedo marmorata* (Heilbronn and Mattson 1974); *Torpedo californica* (Aharanov *et al* 1977); a mouse muscle cell line (Boulter and Patrick 1977) and from foetal calf muscle (Lindstrom *et al* 1979(a)). Lindstrom *et al* (1978) have used the constituent polypeptide chains of both *Torpedo californica* and *Electrophorus electricus* successfully in the induction of EAMG.

EAMG has been reported in species other than rabbits, for example, mice, guinea pigs, frogs (review see Lindstrom 1979) and rhesus monkeys where the resemblance with myasthenia gravis is particularly striking as the affected monkeys show ptosis, facial diplegia and jaw paralysis (Tarrab-Hazdai *et al* 1975). Immunisation of Lewis rats with purified acetylcholine receptor results in a biphasic form of EAMG, including an acute and chronic phase. The acute phase is apparent 8-11 days after immunisation. It subsequently subsides and after thirty days a chronic phase is observed and it is this phase that most closely resembles EAMG.

The characteristic feature of acute EAMG is that of a massive phagocytic invasion which is so intense that a yellow colouration is seen at the motor end-plate. Rats with EAMG exhibit all the electrophysiological features observed in the naturally-occurring disease (Engel *et al* 1976; Lambert *et al* 1976) except that iontophoretic examination of the muscle fibres shows denervation effects which are not seen in myasthenia gravis.

Chronic EAMG again shows all the features characteristic of myasthenia gravis and there is no evidence of denervation such as proliferation of acetylcholine receptor over the whole muscle membrane surface. The weakness in the rat observed during chronic EAMG persists until death or for at least eighty days following initial immunisation (Lindstrom 1979).

EAMG can also be induced by passive transfer as in myasthenia gravis (Toyka 1975). This is done by injection of serum from rats with chronic EAMG containing anti-(acetylcholine receptor) antibodies. Passive EAMG is similar to the acute phase of the experimental disease (Lindstrom *et al* 1976(b)(c); Lennon *et al* 1978).

The initial and now seemingly naive theory of EAMG was that immunisation of an experimental animal with purified acetylcholine receptor stimulates an immune response with the production of antibodies directed against the immunogen. These antibodies are able to cross-react with host acetylcholine receptor thus resulting in a blockade of neuromuscular transmission by the prevention of the access of the released acetylcholine to the receptor protein.

Analysis of the serum of animals with EAMG has demonstrated both the presence of circulating acetylcholine receptor antibodies to the foreign immunogen (Patrick and Lindstrom 1973) and to the host acetylcholine receptor (Aharanov et al 1977). Additionally, Lindstrom (review 1979) has demonstrated the presence of antibody bound to acetylcholine receptor in the muscle membrane. The binding of antibody to the receptor *in vitro* results in a blockade of the depolarising response normally observed with the iontophoretic application of carbamylcholine to an isolated electroplaque (Patrick and Lindstrom 1973). It is also shown to decrease the acetylcholine sensitivity of the receptor (Bevan et al 1976) but this effect can be partially removed by the use of low temperature or inhibitors of energy metabolism (Bevan et al 1977; Heinemann et al 1977). Thirdly, rat muscle cells exposed to the specific antiserum show a decrease in mean conductance of activated acetylcholine receptor and a decrease in the mean open time of the ion channel (Heinemann et al 1977). The binding of antibody to receptor is also shown to increase the rate of destruction of receptor *in vivo*, a phenomenon known as antigenic modulation (Heinemann et al 1978; Reiness et al 1978).

In the acute phase of EAMG in the rat, the total acetylcholine receptor concentration is reduced by half but subsequently doubles probably because of a repair process and in a response to denervation.

Similarly, in the chronic phase, there is a low concentration of acetylcholine receptor which is explained in terms of the accelerated degradation of acetylcholine receptor by antigenic modulation.

Acetylcholine receptor in the muscle membrane is degraded by an internalisation process followed by a proteolytic digestion within the lysosomes. It has been possible to measure the turnover of receptor in cultured muscle cells by the loss of radiolabelled  $\alpha$  bungarotoxin binding sites from the cell surface or the release of  $^{125}\text{I}$ -tyrosine, the break-down product of the labelled toxin, into the culture medium.

In the presence of anti-(acetylcholine receptor) antibodies an accelerated degradation is observed (Heinemann *et al* 1977; Reiness *et al* 1978) which is inhibited by low temperature, inhibitors of energy metabolism (Heinemann *et al* 1977) and by inhibitors of lysosomal proteases (Merlie *et al* 1979). The modulation is independent of complement and there is no evidence to suggest that there is a compensating increase in the synthesis of receptor. However, it is possible that in cultured muscle cells, synthesis is already occurring at a maximal rate (Lindstrom 1979). Interestingly, newly synthesised and incorporated acetylcholine receptors are degraded at the normal rate following modulation (Lindstrom and Einarson 1979).

It is suggested that cross-linking of receptors by antibody is the initiating factor for the increased rate of degradation. In support of this are reports (Lindstrom and Einarson 1979; Drachman *et al* 1978) that  $\text{F(ab}')_2$  but not  $\text{F(ab)}$  fragments from anti-(acetylcholine receptor) IgG induce receptor modulation in cultured muscle cells. Secondly, acetylcholine receptor extracted from rats with chronic EAMG sediments in a sucrose density gradient with a sedimentation coefficient  $S_{20,w}$  greater than 18S suggesting the aggregation of several antibodies and receptor.

FIG. 6

## A Diagrammatic representation of a normal neuromuscular junction

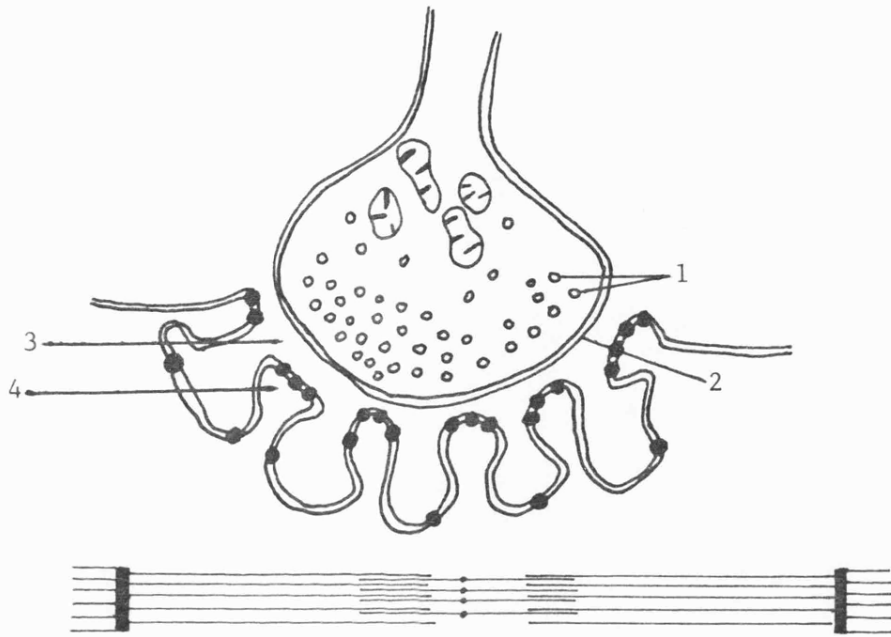
- (1) acetylcholine containing vesicles in the nerve ending
- (2) the presynaptic membrane of the nerve
- (3) the intersynaptic space across which acetylcholine must diffuse after release through specific regions in the presynaptic membrane
- (4) folds in the postsynaptic membrane of the muscle; the acetylcholine receptors are concentrated at the tips and are close to the sites of acetylcholine release

## B Diagrammatic representation of a neuromuscular junction in chronic EAMG and human myasthenia gravis

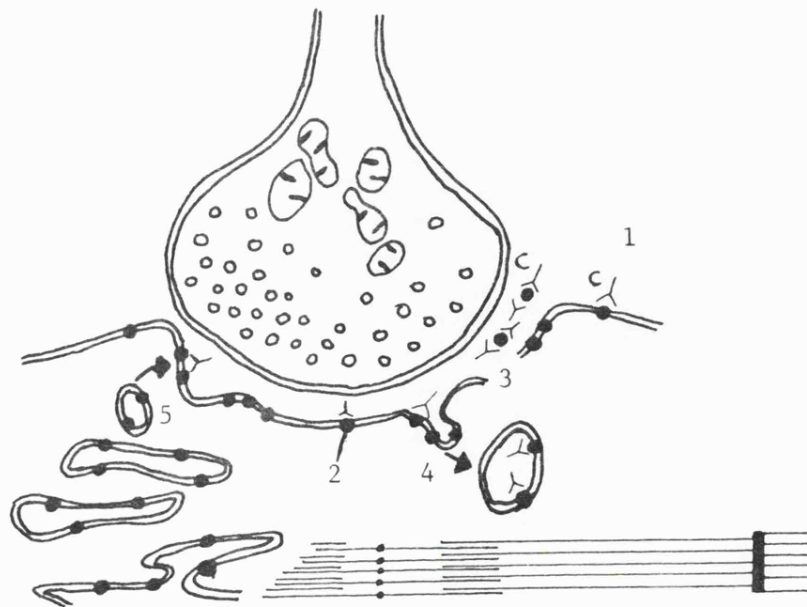
- (1) the folded structure of the postsynaptic membrane is highly simplified and contains a greatly reduced number of acetylcholine receptors
- (2) many of the acetylcholine receptors that remain have antibody bound to them
- (3) complement mediated focal destruction of the postsynaptic membrane causes loss of acetylcholine receptors through shedding of complexes of antibody, acetylcholine receptors and complement into the intersynaptic space
- (4) antigenic modulation of the acetylcholine receptor causes loss of receptors independent of complement. This may proceed through endocytosis of the receptors aggregated by antibodies
- (5) enhanced synthesis of the acetylcholine receptors may partially compensate for the loss of receptor from the membrane



A



B



After Lindstrom (1979)

EAMG is also affected by the complement system. Rats, depleted of their complement system by pre-treatment with cobra venom factor, did not develop EAMG when immunised with acetylcholine receptor (Lennon *et al* 1978). They did produce antibodies to the immunogen and it was shown that they were complexed with the muscle acetylcholine receptors but no clinical features of EAMG were apparent (Lennon *et al* 1978(b)). This observation contradicts the finding of Heinemann *et al* (1977) that modulation is independent of complement.

#### The autoimmune response in myasthenia gravis

The first report of the presence of an antibody to the acetylcholine receptor in the sera of myasthenic patients was made by Almon *et al* (1974). They showed that pre-incubation of solubilised acetylcholine receptor with some myasthenic sera, inhibited the binding of  $^{125}\text{I}$ - $\alpha$  bungarotoxin to the receptor. Subsequently, Aharonov *et al* (1975(a)) also detected anti-(acetylcholine receptor) antibodies in myasthenic sera using a micro-scale complement fixation assay and Bender *et al* (1975) obtained similar results using a staining technique to visualise the neuromuscular junction. Lindstrom *et al* (1976(a)) and Lindstrom (1977) devised a convenient assay for the detection of the specific antibodies. It consisted of labelling solubilised but unpurified acetylcholine receptor from human muscle with  $^{125}\text{I}$ - $\alpha$  bungarotoxin. Myasthenic serum is added and the receptor-antibody complex precipitated with a second antibody. Several groups have been successful in determining the anti-(acetylcholine receptor) antibody titre in myasthenic serum using this method with minor modifications (Monnier and Fulpius 1977; Lefvert *et al* 1978; Barkas *et al* 1979; Newsom-Davis *et al* 1978; Dwyer *et al* (1979).

In parallel with the studies described in the first section of this thesis, there have been attempts to correlate the concentration of circulating antibody with disease severity. The presence of circulating antibody is a good diagnostic test for myasthenia in that it is present in at least ninety percent of clinically diagnosed myasthenics (Lindstrom *et al* 1976(a); Monnier and Fulpius 1977). However, the level of antibody is found not to correlate with disease severity (Lindstrom *et al* 1976; Barkas *et al* 1979) but Lefvert *et al* (1978) and Carter *et al* (1980) found good correlation when the antibody titre was compared with an individual patient's progress. Patients with ocular myasthenia only have generally lower titres than more severe cases (Lindstrom *et al* 1976(a); Lefvert *et al* 1978). Acetylcholine receptor antibodies have been detected in neonatal myasthenics (Lefvert *et al* 1978) and also in the cerebrospinal fluid of some patients (Bergstrom *et al* 1978; Keesey *et al* 1978). They have also been detected in the serum of patients with rheumatoid arthritis who have been treated with D-penicillamine and who showed symptoms of myasthenia gravis (Vincent *et al* 1978; Argov *et al* 1980). These symptoms responded to anticholinesterases but disappeared on withdrawal of D-penicillamine.

Perhaps in retrospect, it is not surprising that the concentration of circulating antibody does not correlate with disease severity as such overall titres may not be representative of the concentration of antibody bound to receptor at the neuromuscular junction. It has been shown that the anti-(acetylcholine receptor) antibody population is not homogeneous. The heterogeneous population has been shown by the determination of antibody titres with different species of antigens (Lindstrom *et al* 1978(c); Weinberg and Hall 1979; Savage-Marengo *et al* 1980) and by the interaction of receptor and one antibody sub-

population in the inhibition of concanavalin A binding (Mittag *et al* 1978). One potentially important sub-population of anti-(acetylcholine receptor) antibodies is that directed against the acetylcholine binding site. This population is not detected in the radioimmunoassay described by Lindstrom (1977) in which the acetylcholine binding site is occupied by  $\alpha$  bungarotoxin. Zurn and Fulpius (1977) found that in a rabbit with EAMG, at the onset of paralysis, antibodies directed at the  $\alpha$  bungarotoxin binding site were four times higher than antibodies directed at the other antigenic determinants of the receptor. Vincent and Newsom-Davis (1979) have investigated the anti-( $\alpha$  bungarotoxin binding site) antibody in a series of myasthenic patients and found a wide range of values for the ratio of total antibody to antibody directed at the toxin binding site. It was found however, that patients in remission do not have a significant anti-(toxin site) antibody titre. Lennon and Lambert (1980) have recently been successful in the production of monoclonal rat antibodies reactive with muscle acetylcholine receptor. They found that the antibodies raised were capable of causing an impairment of neuromuscular transmission in mice, rats and guinea pigs and that these same antibodies bound to an extracellular antigenic determinant of the membrane bound acetylcholine receptor that was distinct from the binding site of cholinergic ligands. This evidence suggests that the sub-population of anti-(acetylcholine receptor) antibodies directed at the acetylcholine binding site is not the key factor in the neuromuscular blockade produced in myasthenia gravis.

Other biochemical investigations of myasthenia gravis

Other biochemical investigations of myasthenia gravis have revealed similar results to those found in EAMG. It has been demonstrated that the amount of acetylcholine receptor extracted by detergent solubilisation from muscle biopsies of myasthenics is less than in controls (Lindstrom and Lambert 1978); that IgG is located on the tips of the folds of the post-synaptic membrane (Engel *et al* 1977) and that extracted receptor is complexed with antibody (Lindstrom and Lambert 1978). Also, myasthenic serum causes antigenic modulation of rat muscle cells in culture (Drachman *et al* 1978). It has been found that sera from myasthenic patients have a wide range of haemolytic complement activity outside the values for normal subjects (Nastuk *et al* 1960) and that the C<sub>3</sub> component of the complement system is found on the post-synaptic membrane of myasthenics and has the same distribution as bound antibody and  $\alpha$  bungarotoxin binding sites (Sahashi *et al* 1978).

The role of the thymus gland has also been investigated in myasthenia gravis as thymic hyperplasia and thymoma are often associated with myasthenia. Aharonov *et al* (1975(b)) found an immunological cross-reaction between the acetylcholine receptor of *Electrophorus electricus* and extracts of calf thymus. The cross-reaction was demonstrated on both a cellular and humoral level. Engel *et al* (1977) and Uddgard and Heilbronn (1977) report similar evidence for the presence of acetylcholine receptors in the thymus of both myasthenic and normal patients. Experiments using normal and thymectomised CB-mice, show that after immunisation with purified acetylcholine receptor, the antibody response is thymus-dependent.

However, in these instances, clinical and electrophysiological signs of EAMG seldom occur (Heilbronn 1979). The implications of these observations are not clear but it has been suggested that they may be connected with a break in immunotolerance (Heilbronn and Stalberg 1978).

Lymphocyte-mediated immunological reactions have been demonstrated. Peripheral blood lymphocytes from myasthenic patients are stimulated when cultured *in vivo* with acetylcholine-receptor enriched electric eel extract (Abramsky *et al* 1975) or with purified *Torpedo* acetylcholine receptor (Richman *et al* 1976). Secondly, peripheral and thymic lymphocytes from patients have cytotoxic effects on muscle cells grown in tissue culture (Kott and Rule 1973).

A genetic element has also been shown to be involved with myasthenia gravis. Histocompatibility typing has shown a high incidence of HLA-8 amongst young patients with thymitis in contrast with the high incidence of HLA-2 in a group of older patients and in those with thymoma (Havard 1977). Naeim *et al* (1978) found similar results in that they found a high incidence of HLA-B8 in non-thymoma myasthenic patients.

#### Therapy of myasthenia gravis

Anti-acetylcholinesterase therapy is used for immediate treatment of myasthenia gravis however, it does not prevent the progressive chronic course of the disease. Myasthenic patients are also shown to respond to treatment with corticosteroids (review see Havard 1977). A positive response to corticosteroids is not accompanied by a decrease in anti-(acetylcholine receptor) antibody

(Bradley *et al* 1979).

A surgical approach to the long-term treatment of myasthenia gravis is thymectomy. Myasthenia gravis can be classified into three stages; an active one which is characterised by remissions and lasts for five to seven years, an inactive stage which is characterised by few spontaneous remissions and finally a burned-out stage when the responsiveness to anti-acetylcholinesterase drugs is markedly decreased (Fraser *et al* 1978). Thymectomy is found to be most beneficial if performed during the active stage and improvement in symptoms is usually seen from between two to three years post-operatively. In one series of patients, Fraser *et al* (1978) had a ninety two percent success rate when thymectomised myasthenic patients showed no or mild symptoms only. Patients with thymoma generally have higher anti-(acetylcholine receptor) antibody titres than thymectomised patients (Lindstrom *et al* 1976(a)). Similarly, individual patients show a reduction in anti-(acetylcholine receptor) antibody titre following thymectomy (Lefvert *et al* 1978).

An alternative approach is one of thoracic duct drainage and it was found that following this treatment, patients were able to have a seventy percent reduction in anti-acetylcholinesterase therapy (Tindall *et al* 1973). A similar treatment to thoracic duct drainage in that it is a fluid removal process is plasma exchange or plasmapheresis. In plasma exchange, the patient's plasma is exchanged for fresh donor plasma or plasma substitute. Usually several exchanges are made over a short time period and at the same time the patient is given immunosuppressive treatment. The advantages of plasma exchange are that it works quickly and the effects can be long-lasting when com-

bined with immunosuppression therapy (Behan *et al* 1979). However, there is some debate about the value of plasma exchange as Newsom-Davis and colleagues (Newsom-Davis *et al* 1979; Newsom-Davis and Vincent 1979) observe the same results with plasma exchange or immunosuppression alone. Plasma exchange results in the dramatic reduction of circulating anti-(acetylcholine receptor) antibody (Barkas *et al* 1979; Carter *et al* 1980; Newsom-Davis *et al* 1978).

Thirdly, a novel approach to the therapy of myasthenia gravis has been the design of a specific immunosuppressant agent by Fuchs *et al* 1978. They found that an irreversibly denatured acetylcholine receptor, formed by complete reduction and carboxymethylation of the receptor in guanidine hydrochloride (Bartfeld and Fuchs 1977), was effective in that it did not induce EAMG in rabbits; it was effective in preventing the onset of the disease following immunisation with native receptor from *Torpedo* and more importantly, in some cases administration of the denatured receptor was found to cure animals with EAMG (Fuchs *et al* 1978). The mechanism of the therapeutic effect of the reduced, carboxymethylated receptor is open to speculation but it seems likely that antibodies to the denatured receptor may not block the physiological function of the receptor but may compete with antibodies against the myasthenic determinants in the binding to self receptor (Fuchs *et al* 1978).

#### The origin of the autoimmune response in myasthenia gravis

The initiating factor of the autoimmune response in myasthenia gravis is not known. The evidence amassed suggests that the auto-antigen is the acetylcholine receptor. Sera from myasthenic patients react better with extra-junctional receptors than junctional receptors



from rat muscle (Weinberg and Hall 1979) indicating that perhaps the initiating antigen is other than normal junctional receptor. Secondly, antisera to denatured  $\alpha$  sub-units of *Torpedo californica* react better with extra-junctional receptors whereas anti-sera to native receptor of *Torpedo californica* does not distinguish between junctional and extra-junctional receptors (Lindstrom *et al* 1979(a)) suggesting that the determinant unique to the extra-junctional receptor may resemble denatured receptor protein, probably on the  $\alpha$  chain. It is not known whether the antibodies present in myasthenic serum are directed solely at antigenic determinants on the extracellular surface of the acetylcholine receptor or whether intracellular determinants are also important. If intracellular determinants are involved, then it would suggest that in myasthenia gravis, perhaps the initiating factor is the shedding of the receptor from the post-synaptic membrane, followed by the autoimmune reaction to, as it were, now foreign antigens.

It has also been suggested that the thymus gland is the source of the autoimmune response (Drachman *et al* 1979; Lindstrom 1979). The presence of acetylcholine receptors in the thymus has led to a feeling that the receptor-bearing cells may be vulnerable to attack by immunocompetent cells of the thymus. Some alteration in either these specific cells or the lymphocytes may serve to break tolerance and thereby initiate an autoimmune response. It has been suggested that a viral infection is one possible mechanism by which surface proteins including the acetylcholine receptor might be modified. Certainly Simpson (1960) reported that the onset of myasthenia gravis commonly followed an infection of the upper respiratory tract. He also found that myasthenia gravis was often precipitated by an emotional upset.

It is also possible that myasthenia gravis may be the result of a genetic predisposition to an altered immune response. The high incidence of HLA-B8 histocompatibility antigens is also found with other autoimmune disorders and therefore suggests that they are responsible for an increased susceptibility to autoimmune disorders rather than the initiation of an immune response specifically to the acetylcholine receptor (Lindstrom 1979).

Thus, whilst the pathogenesis of myasthenia gravis is now well-documented and more understood, the initiation is still a matter of speculation. The study of myasthenia gravis has far-reaching consequences in that as an established autoimmune disorder where the auto-antigen is known and well characterised, it may be useful in gaining a deeper understanding of other autoimmune disorders, for example in the insulin resistant form of diabetes where circulating auto-antibodies to the insulin receptor have been detected (Harrison *et al* 1978) and in Graves disease where anti-(TSH) antibodies have been demonstrated (Smith and Hall 1974). It has been suggested recently that since autoimmune disorders often occur concurrently in the same patient then there may be an altered regulation of immune system function (Waldmann *et al* 1978) which may be associated with altered suppressor-cell activity. Indeed, in a preliminary study, Mischak *et al* (1979) were able to show that mitogen-induced suppressor cell activity in myasthenia gravis patients is significantly lower than that seen in healthy or neurologic controls.

In this thesis, the initial aim was to set up a radioimmuno-assay for the detection of anti-(acetylcholine receptor) antibodies in the serum of myasthenic patients and to determine whether there existed any

correlation between the antibody titre and disease severity. As the work progressed, it was found that there was no simple correlation between circulating specific antibody and the severity of symptoms, an observation which has since also been reported by other groups (Lindstrom *et al* 1976; Monnier and Fulpius 1977). A possible explanation for the lack of correlation was that perhaps a specific sub-population of anti-(acetylcholine receptor) antibodies was the important parameter in the pathogenesis of the disease. At that time a possible candidate for such a sub-population was an antibody directed at the  $\alpha$  bungarotoxin binding site which is not detected in the established radioimmunoassay (FIG 7 page 69). A way to eliminate this discrepancy would be to have an antigen that was directly radio-labelled with no dependence on an external ligand. Already, in the laboratory, there was an established radioimmunoassay using purified  $^{125}\text{I}$ -acetylcholine receptor from *Torpedo marmorata* (Barkas *et al* 1978) as the antigen. Thus, it was decided to purify and characterise the nicotinic acetylcholine receptor of human muscle with a view to providing a well-characterised, pure antigen with which to investigate the antibody population of myasthenic patients. The purification of the receptor to homogeneity was not a simple process. An account of the many problems and of the results is given in the second part of the thesis.

..... **MATERIALS** .....

MATERIALS

$\alpha$  bungarotoxin was obtained from the Boehringer Corporation (Lewes, Sussex U.K.). *Naja naja siamensis* venom was from the Miami Serpentarium (Miami, Florida U.S.A.). All radiochemicals were supplied by the Radiochemical Centre (Amersham, Bucks. U.K.) unless otherwise stated. Benzoquinonium was a gift from Stirling Winthrop (Rensselaer, New York U.S.A.). Pancuronium bromide was a gift from Organon Laboratories (Glasgow U.K.). Gel filtration reagents were supplied by Pharmacia Ltd. (Hounslow U.K.). Ampholines pH 4-6; pH 3.5-10 were from LKB Ltd. (Croydon U.K.). The enzymobead radioiodination reagent was supplied by Bio-Rad Laboratories (Bromley U.K.). Soluene-350 and diphenyloxazole were supplied by Packard (Reading, Berks. U.K.). Adjuvants were supplied by Miles Laboratories Ltd. (Slough U.K.).

Amputated limb muscle and normal human serum were supplied by the Royal United Hospital (Bath U.K.), St. Martin's Hospital (Bath U.K.) and the Bristol Royal Infirmary (Bristol U.K.).

Myasthenic serum samples and clinical data were provided by Dr. P.O. Behan and colleagues of the Department of Neurology, Institute of Neurological Sciences (Glasgow U.K.).

All other materials were supplied by either Sigma Chemical Company (Kingston-upon-Thames U.K.) or B.D.H. Chemicals (Poole, Dorset U.K.).

Section A

Anti-(acetylcholine receptor) antibodies in patients with myasthenia  
gravis

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METHODS(1) The Preparation of High Specific Activity  $^{125}\text{I}$ - $\alpha$  bungarotoxin

$\alpha$ -Bungarotoxin was labelled with  $^{125}\text{I}$  by the method of Urbaniak *et al* (1973).

Carrier-free  $\text{Na}^{125}\text{I}$  (100mCi/ml) in dilute NaOH solution (10 $\mu$ l) was added to  $\alpha$  bungarotoxin (10 $\mu$ g) dissolved in 0.05M potassium phosphate buffer, pH 7.5 (20 $\mu$ l). Then, 0.5% (w/v) Chloramine T in 0.05M potassium phosphate buffer, pH 7.5 (10 $\mu$ l) and 0.05M potassium phosphate buffer, pH 7.5 (10 $\mu$ l) were added. The mixture was stirred for 1 min at 23 $^{\circ}$ C after which 0.016% (w/v) sodium metabisulphite in 0.05M potassium phosphate buffer, pH 7.5, (750 $\mu$ l) and 1% (w/v) potassium iodide in 0.05M potassium phosphate buffer, pH 7.5 (200 $\mu$ l) were added.  $^{125}\text{I}$ - $\alpha$  Bungarotoxin was separated from free  $^{125}\text{I}$  by passage down a Sephadex G25 column (25cm x 1cm) equilibrated with 0.01M potassium phosphate buffer, pH 7.4, containing 1% (w/v) bovine serum albumin and eluted with the same buffer. Fractions (1ml) were collected and samples (10 $\mu$ l) counted in an LKB 1280 Ultrogamma counter. Peak tubes were pooled and the specific activity of the  $^{125}\text{I}$ - $\alpha$  bungarotoxin was calculated, assuming 100% recovery of protein. The biological activity of the toxin and the amount of  $^{125}\text{I}$ -protein precipitated by trichloroacetic acid was determined (Methods page 62).  $^{125}\text{I}$ -toxin was stored at 4 $^{\circ}$ C in elution buffer for up to 4 weeks.

(2) The preparation of low specific activity  $^{125}\text{I}$ - $\alpha$  bungarotoxin

Carrier-free  $\text{Na}^{125}\text{I}$  (100mCi/ml) in dilute NaOH solution (10 $\mu$ l) was added to  $\alpha$  bungarotoxin (1mg) dissolved in 0.05M potassium phosphate buffer, pH 7.5 (1ml). Then, 0.02% (w/v) chloramine T in

0.05M potassium phosphate buffer, pH 7.0 (125 $\mu$ l) was added and the mixture stirred at 4 $^{\circ}$ C for 5 min after which 0.02% (w/v) sodium metabisulphite in 0.05M potassium phosphate buffer, pH 7.5 (125 $\mu$ l) was added. The  $^{125}\text{I}$ - $\alpha$  bungarotoxin was then subjected to chromatography on Sephadex G25 as described above. The specific activity, biological activity and  $^{125}\text{I}$ -protein precipitated by trichloroacetic acid were determined. Labelled toxin was stored at 4 $^{\circ}$ C in elution buffer for up to 4 weeks.

### (3) Iodination of $\alpha$ bungarotoxin by the lactoperoxidase method

Enzymobead radioiodination reagent was rehydrated with distilled water (0.5ml) for 1h at 23 $^{\circ}$ C and then used immediately or stored at -20 $^{\circ}$ C.

2% (w/v) Aqueous  $\alpha$ -D-glucose solution was kept at 4 $^{\circ}$ C overnight to mutarotate to give a solution containing  $\beta$ -D-glucose. The following reagents were mixed; 0.2M potassium phosphate buffer, pH 7.2, (50 $\mu$ l);  $\alpha$  bungarotoxin (25 $\mu$ g) dissolved in 0.05M potassium phosphate buffer, pH 7.5 (25 $\mu$ l); the hydrated enzymobead radioiodination reagent (50 $\mu$ l); carrier-free Na $^{125}\text{I}$  (100mCi/ml) in dilute NaOH solution (10 $\mu$ l) and the  $\beta$ -D glucose-containing solution prepared as above (25 $\mu$ l). The reactants were stirred for 20 min at 23 $^{\circ}$ C and then centrifuged for 10 min at 1000g. The supernatant was decanted and the  $^{125}\text{I}$ - $\alpha$  bungarotoxin chromatographed on Sephadex G25 as above. The specific activity, biological activity and  $^{125}\text{I}$ -protein precipitated by trichloroacetic acid were determined and the labelled toxin stored at 4 $^{\circ}$ C in elution buffer for not more than 4 weeks.



(4) N-[propionyl-<sup>3</sup>H] propionylated  $\alpha$  bungarotoxin

N-[propionyl-<sup>3</sup>H] propionylated  $\alpha$  bungarotoxin (65Ci/mmol)

was purchased from the Radiochemical Centre, Amersham.

The biological activity of the tritiated toxin was determined and the <sup>3</sup>H-protein precipitated with 6% (w/v) trichloroacetic acid (Methods page 62).

(5) The determination of the biological activity of radiolabelled  $\alpha$  bungarotoxin

The biological activity of the labelled  $\alpha$  bungarotoxins was determined by measuring the amount of <sup>125</sup>I- $\alpha$  bungarotoxin bound to an excess of purified acetylcholine receptor from *Torpedo marmorata*. The method of assay was modified from that of Schmidt and Raftery (1973) (for details see Methods page 64). When using N-[propionyl-<sup>3</sup>H] propionylated  $\alpha$  bungarotoxin, the method of assay was as above except that for the measurement of radioactivity, the DE81 filter discs were dried under an infra-red lamp and then added to scintillant (10ml) which contained 10% (v/v) Soluene 350, 0.6% (w/v) diphenyloxazole, in toluene. Aqueous samples were counted in 0.3% (w/v) diphenyloxazole, in 25% (v/v) Triton X100, 75% (v/v) Xylene (Dolly and Barnard 1977). Samples were counted in a Packard scintillation spectrometer.

(6) Determination of the <sup>125</sup>I-protein precipitated by trichloroacetic acid

The radiolabelled  $\alpha$  bungarotoxins were diluted 1 in 100 with 0.01M potassium phosphate buffer, pH 7.4, containing 1% (w/v) bovine serum albumin. Quadruplet samples of the diluted toxin (50 $\mu$ l) were

placed on ice and 12% (w/v) cold trichloroacetic acid (50 $\mu$ l) added. After 30 min the precipitate was collected on a Whatman GFC filter washed with 6% (w/v) cold trichloroacetic acid (5ml) and counted in an LKB 1280 Ultrogamma counter. Acid precipitable counts were expressed as a percentage of the total counts added.

(7) The effect of iodination on the biological activity of the high specific activity  $^{125}\text{I}$ - $\alpha$  bungarotoxin

To determine the effect of the substitution of an atom of  $^{125}\text{I}$  into the  $\alpha$  bungarotoxin molecule, an isotope dilution experiment was carried out.

$^{125}\text{I}$ - $\alpha$  Bungarotoxin (0.16pmol) was incubated with purified acetylcholine receptor from *Torpedo marmorata* (82fmol) and the  $^{125}\text{I}$ - $\alpha$  bungarotoxin binding to the receptor was measured. Part of the  $^{125}\text{I}$ - $\alpha$  bungarotoxin in the assay was then replaced by known, increasing amounts of unlabelled  $\alpha$  bungarotoxin and the samples processed as usual (Methods page 64).

(8) The assay of activity of acetylcholine receptor

Two methods have been used for the assay of acetylcholine receptor.

(i) For detergent extracts, an ammonium sulphate precipitation assay adapted from the method of Meunier *et al* (1972(a)) was used.  $^{125}\text{I}$ - $\alpha$  bungarotoxin (0.5-1.5nM) was added to acetylcholine receptor-containing samples (100 $\mu$ l). Saturated ammonium sulphate solution (75 $\mu$ l) was added to give a 30% (v/v) final concentration. The samples were left for 16h at 4 $^{\circ}$ C and then filtered on Whatman GFC filter discs. The filter discs

were washed with 30% (v/v) ammonium sulphate solution (3ml) and counted in an LKB 1280 Ultrogamma counter. The specific binding was measured by carrying out all the assays in the presence and absence of 0.1mM d-tubocurarine and the  $^{125}\text{I}$ - $\alpha$  bungarotoxin that remains bound in the presence of this concentration of competing ligand is defined as non-specifically bound. Thus specific binding is defined as that portion of the total radioactivity, i.e.  $^{125}\text{I}$ - $\alpha$  bungarotoxin, bound that is displaced by this concentration of competing ligand (Hollenberg and Cuatrecasas 1979). Receptor activity is expressed as molarity of  $\alpha$  bungarotoxin binding sites.

(ii) Assay of purified acetylcholine receptor was by filtration on DEAE cellulose. The method used was essentially that of Schmidt and Raftery (1973a).

Acetylcholine receptor (100 $\mu$ l) and 0.5-1.5nM  $^{125}\text{I}$ - $\alpha$  bungarotoxin (50 $\mu$ l) were incubated for 1.5h at 23 $^{\circ}\text{C}$ . Termination of the assay was by addition of 0.01M potassium phosphate buffer, pH 7.4, containing 1% (v/v) Triton X100 and 0.1% (w/v) bovine serum albumin (1ml). The sample was filtered through two DE81 cellulose filter discs (2cm diameter) and the discs were washed with the above buffer (5ml). Blanks contained buffer only or 0.1mM d-tubocurarine. The washed discs were counted in an LKB 1280 Ultrogamma counter and the specific activity was calculated and expressed as above.

(9). Preparation of human immunoglobulin G (IgG)

IgG was prepared by the method of Stevenson and Dorrington (1970). A solution of saturated ammonium sulphate was prepared by dissolving ammonium sulphate (500g) in distilled water (500ml) at 50 $^{\circ}\text{C}$ . The solution was left to stand overnight at 23 $^{\circ}\text{C}$  and then the super-

natant was adjusted to pH 7.2 with dilute  $H_2SO_4$  (Heide and Schwick 1978).

Saturated ammonium sulphate (6ml) was added drop-wise to normal human serum (10ml) with stirring at  $23^{\circ}C$ . The solution was stirred for a further 30 min at  $23^{\circ}C$  and centrifuged at 5000g for 20 min. The precipitate was dissolved in 0.03M potassium phosphate buffer, pH 7.3 (10ml) and dialysed overnight at  $4^{\circ}C$  against 0.03M potassium phosphate buffer, pH 7.3 (1l). The diffusate was applied to a column (14.5cm x 2.5cm) of DE52 cellulose equilibrated with 0.03M potassium phosphate buffer, pH 7.3. The column was eluted with the same buffer and fractions (5ml) were collected. The optical density of the fractions at 280nm was measured and the peak fractions pooled. The purified IgG was dialysed against 0.01M potassium phosphate buffer, pH 7.4, containing 0.15 M NaCl and stored at  $-20^{\circ}C$ .

(10). Preparation of anti-(human IgG) antibodies

Human IgG (1.5ml, 1mg/ml) was emulsified with Freund's complete adjuvant (1.5ml) and injected intramuscularly at one site into the hind leg of a goat. After three weeks, the procedure was repeated exactly except that Freund's incomplete adjuvant was used. The serum of the goat was assayed for the presence of anti-(human IgG) antibodies (Methods page 65) and injections were continued at three-weekly intervals until an adequate titre was obtained (Methods page 66).

(11) Assay of anti-(human IgG) antibodies

The presence of anti-(human IgG) antibodies in goat serum was measured by two methods.

(i) Firstly a double immunodiffusion method derived from that of

Ouchterlony (1961) was used. A 1% (w/v) agar solution in 0.01M potassium phosphate buffer, pH 7.4, containing 0.15M NaCl and 0.1% (v/v) Triton X100 was prepared by heating the components in a boiling water bath. The solution was then poured onto a level glass plate which was equilibrated at 50°C. A series of holes was made in the gel using a Pasteur pipette attached to a vacuum line, such that there was one central hole with six surrounding holes in a circular formation. Serial two fold dilutions of normal human serum from neat serum to 1 in 256 were prepared using 0.01M potassium phosphate buffer, pH 7.4, containing 0.15M NaCl. Each dilution of normal serum (20µl) was then applied to one of the outer wells. The goat anti-(human IgG) serum (20µl) was applied to the central hole. The plate was left in a humid atmosphere for two days and then examined for the formation of precipitin lines. Formation of such a line against a 1 in 16 or 1 in 32 diluted normal serum was considered to be evidence of adequate anti-(human IgG) antibody titre.

(ii) Secondly, increasing concentrations of goat anti-(human IgG) antiserum were added to the radioimmunoassay for anti-(acetylcholine receptor) antibodies (Methods page 66) until the formation of an immune precipitate reached a plateau value. This value of goat anti-(human IgG) serum (of the order of 100µl) was then taken as that needed to precipitate human serum (5µl).

(12). The radioimmunoassay to determine anti-(acetylcholine receptor) antibodies

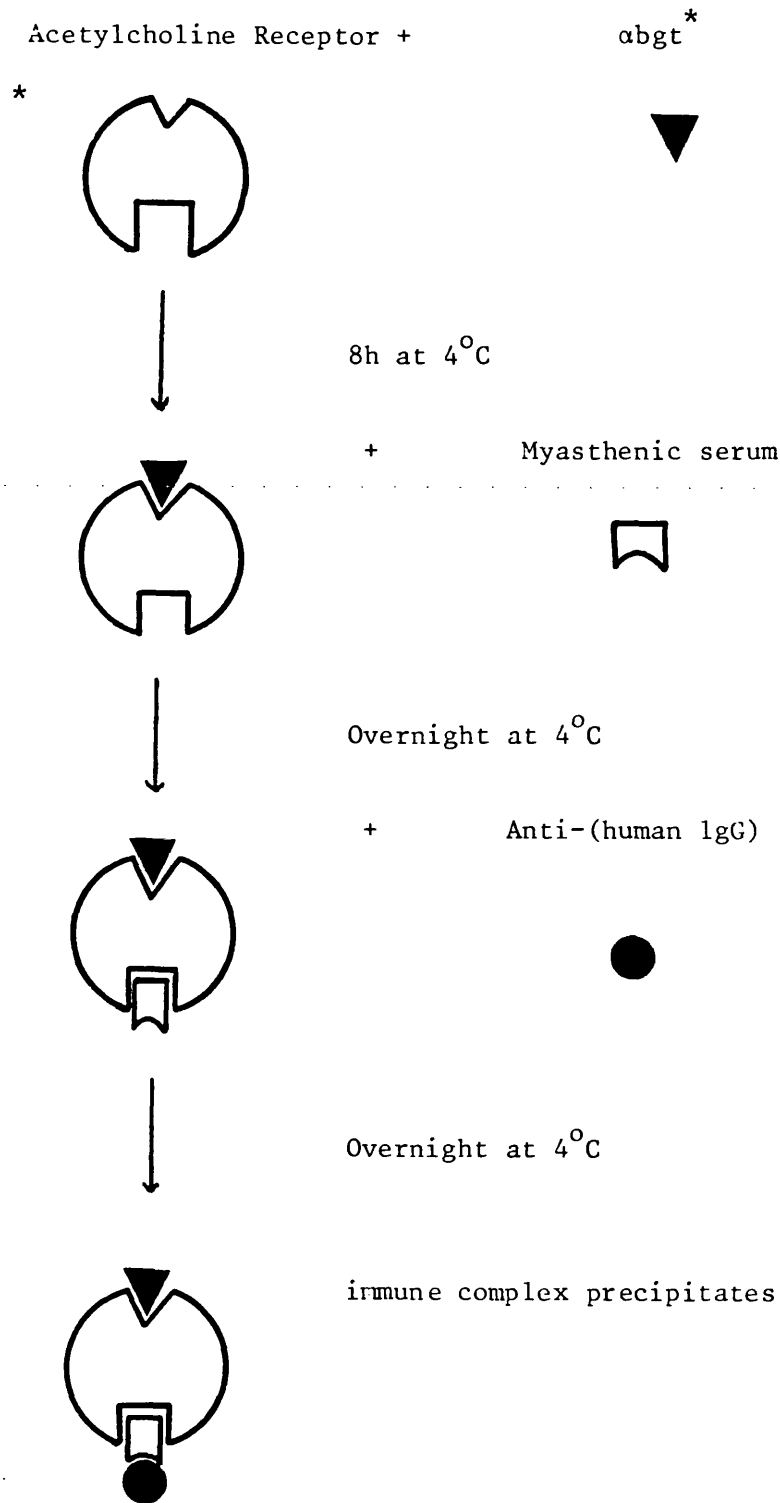
The radioimmunoassay for the detection of anti-(acetylcholine receptor) antibodies was a modification of the method of Lindstrom (1977).

Human skeletal muscle (from limb amputation) was either stored at  $-80^{\circ}\text{C}$  or used immediately. Muscle (300g) was chopped and homogenised in 0.01M potassium phosphate buffer, pH 7.4, (1.2l) containing 0.1M NaCl and 0.01M  $\text{NaN}_3$ , in a Waring blender for 1 min at  $4^{\circ}\text{C}$ . The homogenate was centrifuged at 20000g for 60 min at  $4^{\circ}\text{C}$  and the pellet was extracted in 0.01M potassium phosphate buffer (600ml), pH 7.4, containing 0.1M NaCl, 0.01M  $\text{NaN}_3$  and 2% (v/v) Triton X100, for 4h at  $23^{\circ}\text{C}$ . The extract was centrifuged at 20000g for 60 min at  $4^{\circ}\text{C}$  and the resulting supernatant was filtered through glass wool to remove lipid particles and stored at  $4^{\circ}\text{C}$ . The supernatant contains solubilised acetylcholine receptor which is used as the antigen in the radioimmunoassay.

Solubilised acetylcholine receptor from human muscle ( $1.5 - 2 \times 10^{-10}\text{M}$ ) was incubated with  $^{125}\text{I}$ - $\alpha$  bungarotoxin ( $3 - 6 \times 10^{-10}\text{M}$ ) for 8h at  $4^{\circ}\text{C}$ . Serum (5 $\mu\text{l}$ ) was added and the mixture was kept at  $4^{\circ}\text{C}$  overnight. Goat anti-(human IgG) serum (100 $\mu\text{l}$ ) was added and a precipitate was allowed to form overnight at  $4^{\circ}\text{C}$ . The samples were then centrifuged at 3000g for 10 min, the supernatant was discarded and the pellet was washed with 0.01M potassium phosphate buffer (1ml), pH 7.4, containing 0.15M NaCl. The pellet was counted in an LKB 1280 Ultrogamma counter. Control samples were preincubated with 0.1mM d-tubocurarine before addition of  $^{125}\text{I}$ - $\alpha$  bungarotoxin and the resulting counts were subtracted from test values to give specifically bound  $^{125}\text{I}$ - $\alpha$  bungarotoxin. Titres were expressed as moles  $^{125}\text{I}$ - $\alpha$  bungarotoxin binding sites precipitated per litre of serum. Sera with titres greater than  $30 \times 10^{-10}\text{M}$  were diluted five and ten times with normal human serum and the assay repeated until a plateau value for the titre was obtained. FIG. 7 is a diagrammatic representation of the radioimmunoassay.

The  $^{125}\text{I}$ - $\alpha$  bungarotoxin in the radioimmunoassay is present in a concentration close to the dissociation constant for the reaction between the labelled toxin and the acetylcholine receptor. (Results, page 166). An experiment was carried out in which a radioimmunoassay was done with two myasthenic sera of known titre with increasing  $^{125}\text{I}$ - $\alpha$  bungarotoxin concentrations. A correction factor to the antibody titre determined by the usual radioimmunoassay was determined and routinely used in all subsequent assays.

FIG. 7. A diagrammatic representation of the radioimmunoassay for anti(acetylcholine receptor) antibodies



\* Macromolecules are not drawn to scale



EXPERIMENTAL AND RESULTS

All data are presented as mean  $\pm$  S.E.M. (number of determinations).

1. Iodinated  $\alpha$  Bungarotoxin(i) The properties of high specific activity  $^{125}\text{I}$ - $\alpha$  bungarotoxin

$^{125}\text{I}$ - $\alpha$  bungarotoxin was labelled to a specific activity of  $675 \pm 27$  Ci/mmol (7) with  $87 \pm 1.42\%$  (7) incorporation of the total  $^{125}\text{I}$  added to the reaction vessel (FIG. 8 page 73). The toxin had a biological activity of  $49.6 \pm 3.67\%$  (7) (range is 35% - 60%) by titration with excess acetylcholine receptor purified from *Torpedo marmorata* (FIG.9 page 74), where the biological activity is defined as:-

$$\text{Biological activity} = \frac{^{125}\text{I bound}}{\text{Total } ^{125}\text{I added}} \times 100 \quad \%$$

A filter blank which contained buffer and  $^{125}\text{I}$ - $\alpha$  bungarotoxin only (page 64) contained  $1.8 \pm 0.2\%$  (7) of the total radioactivity added to the assay sample.

It was found that  $69 \pm 3\%$  (7) of the total radioactivity was precipitated by 6% (w/v) trichloroacetic acid.

An experiment was carried out to determine the effect of iodine substitution on the activity of the toxin (page 63) where, in an assay for the measurement of acetylcholine receptor from *Torpedo marmorata*,  $^{125}\text{I}$ - $\alpha$  bungarotoxin was replaced by increasing, known amounts of non-labelled  $\alpha$  bungarotoxin. The experimental results, that is the  $^{125}\text{I}$ - $\alpha$  bungarotoxin bound, were plotted against the theoretical results which were calculated from the displacement of

radiolabelled ligand with cold ligand (FIG.10 page 75). The plot gave a straight line with gradient = 0.95 with a linear correlation coefficient of 0.98.

Storage of  $^{125}\text{I}$ - $\alpha$  bungarotoxin for periods of more than three weeks at  $4^{\circ}\text{C}$  showed that the biological activity decreased by up to 50% and the radioactivity precipitated by 6% (w/v) trichloroacetic acid decreased by 20%. Thus, it was customary to prepare the  $^{125}\text{I}$ - $\alpha$  bungarotoxin every three weeks.

(ii) The properties of low specific activity  $^{125}\text{I}$ - $\alpha$  bungarotoxin

In the preparation of the low specific activity  $^{125}\text{I}$ - $\alpha$  bungarotoxin (page 60), the conditions were chosen such that they were milder than for the high specific activity  $^{125}\text{I}$ - $\alpha$  bungarotoxin. The concentration of the oxidising agent, Chloramine T, was decreased; the amount of  $\alpha$  bungarotoxin concentration was increased to 1mg and the reaction was carried out at  $4^{\circ}\text{C}$  (page 60). The  $^{125}\text{I}$ - $\alpha$  bungarotoxin was labelled to a specific activity of 3.3Ci/mmol with 41% incorporation of the total  $^{125}\text{I}$  added to the reaction vessel. The biological activity was 75%; the filter blank was 2.7% of the total radioactivity and 60% of the radioactivity was precipitated with 6% (w/v) trichloroacetic acid.

(iii) The properties of  $^{125}\text{I}$ - $\alpha$  bungarotoxin labelled by the lactoperoxidase method

This mild, enzymic radio-iodination procedure labelled  $^{125}\text{I}$ - $\alpha$  bungarotoxin to a specific activity of 324 Ci/mmol with 78% incorporation of total  $^{125}\text{I}$  added to the reaction vessel. The biological activity was 71%; the filter blank was 2.3% of the total radioactivity and 60% of the radioactivity was precipitated with 6% (w/v) trichloroacetic acid.

(iv) A comparison of the activity of the radiolabelled toxins

It was necessary to compare the activity of the high specific activity  $^{125}\text{I}$ - $\alpha$  bungarotoxins directly with that of other labelled  $\alpha$  bungarotoxins of higher biological activity.

For each  $^{125}\text{I}$ - $\alpha$  bungarotoxin preparation, the acetylcholine receptor concentration of a preparation from *Torpedo marmorata* was measured in a DEAE cellulose filtration assay (page 64). For each toxin, the concentration of purified acetylcholine receptor was chosen such that one was on the linear part of the curve in FIG. 9 page 74. Table 2 is a comparison of some of the results obtained. The results in the first column are the experimentally determined values for the acetylcholine receptor concentration. These values are then corrected for 100% biological activity of  $^{125}\text{I}$ - $\alpha$  bungarotoxin and the new value compared with the experimentally determined acetylcholine receptor concentration using N-[propionyl -  $^3\text{H}$ ] propionylated  $\alpha$  bungarotoxin (cf correction factor, column 4).

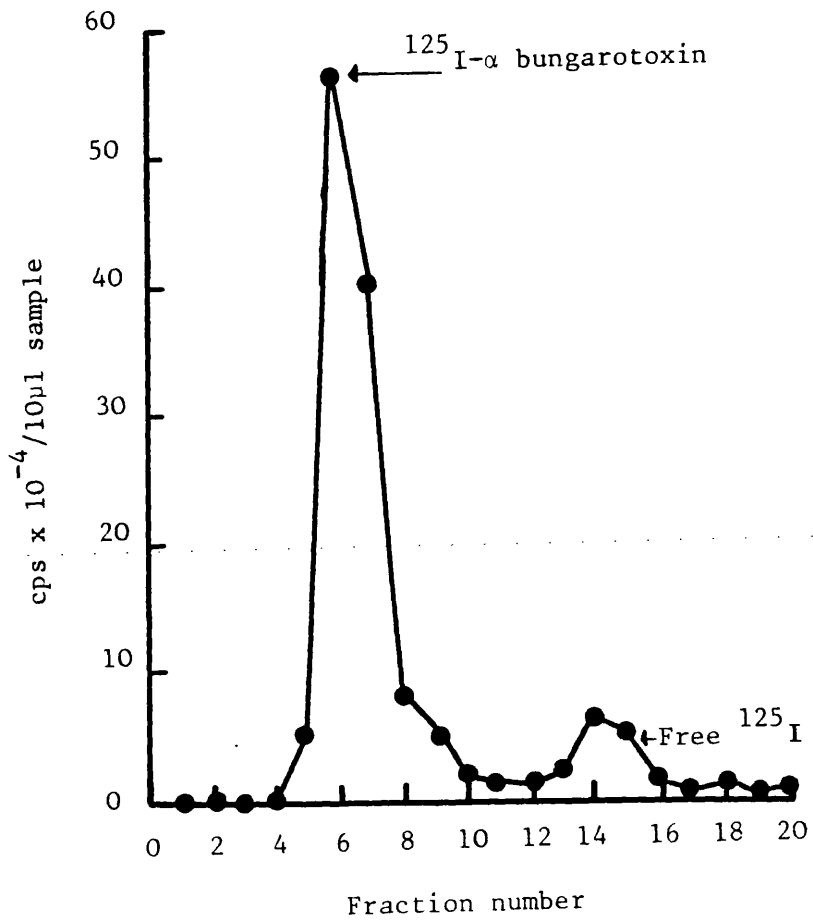


FIG. 8. Gel filtration of  $^{125}\text{I}-\alpha$  bungarotoxin on Sephadex G25

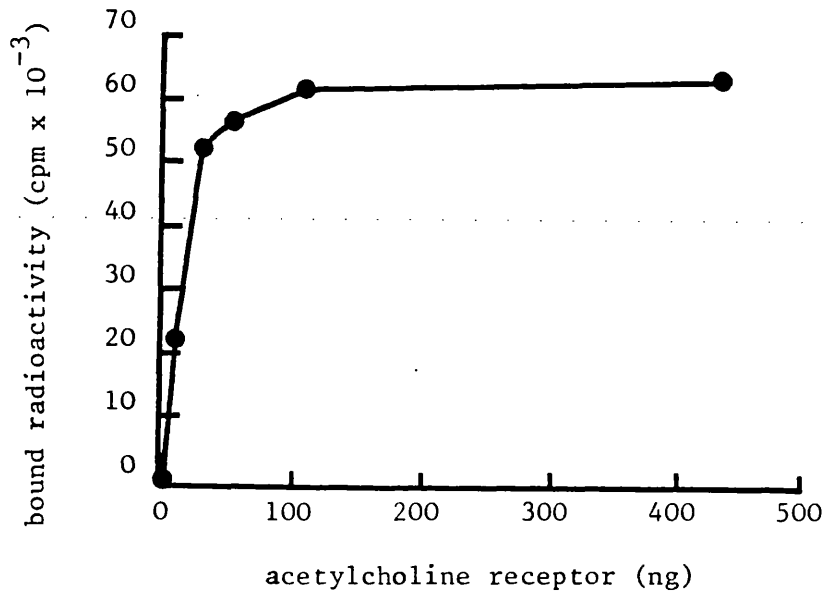


FIG. 9. Titration of  $^{125}\text{I}$ - $\alpha$  bungarotoxin (1nM, 130000cpm) with excess acetylcholine receptor purified from *Torpedo marmorata*

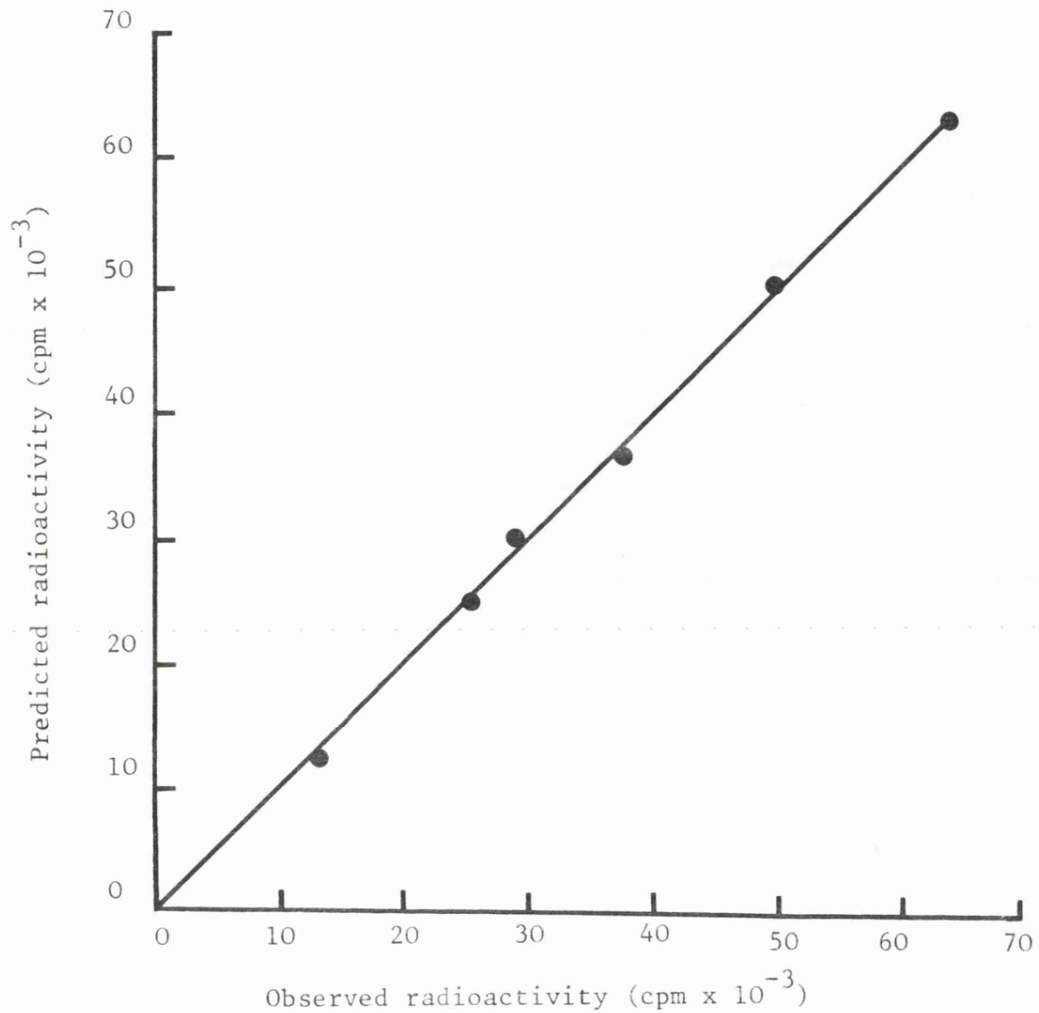


FIG. 10. The effect of iodine substitution on the biological activity of  $^{125}\text{I}$ - $\alpha$  bungarotoxin

The activity of a preparation of acetylcholine receptor purified from *Torpedo marmorata* was measured using  $^{125}\text{I}$ - $\alpha$  bungarotoxin as described in Methods. The  $^{125}\text{I}$ - $\alpha$  bungarotoxin was replaced with known amounts of unlabelled  $\alpha$  bungarotoxin and the measurement of receptor activity was repeated. The predicted amount of radioactivity bound to the acetylcholine receptor takes into account the biological activity of the  $^{125}\text{I}$ - $\alpha$  bungarotoxin that was used.

Table 2. A comparison of the activities of radiolabelled  $\alpha$  bungarotoxins

Type of $\alpha$ bungarotoxin	Acetylcholine* receptor concentration pmol/ml	Biological activity	Acetylcholine <sup>†</sup> receptor concentration pmol/ml	Correction <sup>§</sup> factor	Specific radio- activity Ci/nmol
High specific activity	3374 $\pm$ 423(6)	50 $\pm$ 1.6%(4)	6748	x 1.1	713
High specific activity	4039 $\pm$ 420(3)	47.6%(2)	8485	x 0.88	720
High specific activity	5597 $\pm$ 193(4)	60 $\pm$ 1.3%(3)	8600	x 0.87	647
Low specific activity	6852 $\pm$ 289(6)	75 $\pm$ 6.2%(6)	9136	x 0.83	3.3
Lactoperoxidase labelled	4896 $\pm$ 422(6)	71%(2)	6895	x 1.09	324
N-[propionyl- <sup>3</sup> H] propionylated	7542 $\pm$ 563(6)	98%	7542	x 1	65

\* Acetylcholine receptor concentration is the experimentally determined value.

<sup>†</sup> Acetylcholine receptor concentration is corrected for 100% biological activity of <sup>125</sup>I- $\alpha$  bungarotoxin.

<sup>§</sup> The correction factor is the value by which the concentration of acetylcholine receptor must be multiplied to give the same value as the N-[propionyl-<sup>3</sup>H] propionylated  $\alpha$  bungarotoxin.

2. Assay for acetylcholine receptor(i) The ammonium sulphate precipitation assay

Initial experiments incubated  $^{125}\text{I}$ - $\alpha$  bungarotoxin and acetylcholine receptor overnight at  $4^{\circ}\text{C}$  before the addition of saturated ammonium sulphate. However, it was found that the prior incubation of the reagents before the addition of saturated ammonium sulphate gave identical results for the measured acetylcholine receptor concentration as when all the reagents were added simultaneously (Table 3 ).

Table 3

Assay Conditions	Acetylcholine receptor pmol/ml
(i) Receptor + toxin overnight $4^{\circ}\text{C}$ (ii) $(\text{NH}_4)_2 \text{SO}_4$ addition	$0.45 \pm 0.03$
(i) Receptor + toxin + $(\text{NH}_4)_2 \text{SO}_4$ addition simultaneously	$0.46 \pm 0.03$

A time course for the precipitation of the receptor-toxin complex by 30% (v/v) ammonium sulphate showed that it was necessary to leave the complex overnight at  $4^{\circ}\text{C}$  for complete precipitation (FIG.11 page 80).

The assay was shown to be linear with protein concentration as long as the  $^{125}\text{I}$ - $\alpha$  bungarotoxin was present in excess.



Non-specific binding was normally measured in the presence of 0.1mM d-tubocurarine but both 0.1mM benzoquinonium and 0.1mM pancuronium bromide were equally effective in the inhibition of specific  $^{125}\text{I}$ - $\alpha$  bungarotoxin binding. The inhibition of binding by 0.1mM d-tubocurarine was of the order of 80%.

It was thus usual practice to add all reagents simultaneously and to leave at 4°C overnight before filtration. The precipitation of the acetylcholine receptor with ammonium sulphate solution (30% (v/v) final concentration) was chosen since this concentration gave the most reproducible results with minimal non-specific binding.

(ii) The DEAE cellulose filtration assay

A time course for the binding between  $^{125}\text{I}$ - $\alpha$  bungarotoxin and purified acetylcholine receptor showed that a plateau was reached after at least 90 min incubation at 23°C (FIG. 12 page 81). Alternatively, an overnight incubation at 4°C gave identical values for the concentration of acetylcholine receptor (Table 4) and this method was often used for convenience.

Table 4

Assay conditions	Acetylcholine receptor pmol/ml
Receptor + toxin 90 min at 23°C	0.14 ± 0.02
Receptor + toxin overnight at 4°C	0.15 ± 0.02

The addition of 0.1mM d-tubocurarine or 0.1mM benzoquinonium inhibited 90-100% of total binding.

(iii) A comparison between the two assay methods

Acetylcholine receptor in the 20000g Triton X100 detergent extract was measured by the two methods. Table 5 shows the results obtained in four different receptor preparations.

Table 5. Acetylcholine receptor concentration determined by two different assay methods

Acetylcholine receptor preparations	Receptor concentration pmol/ml	
	DEAE cellulose filtration assay	Ammonium sulphate precipitation assay
20000g detergent extract A*	0.43 <sup>+</sup>	0.67
20000g detergent extract B*	0.39	0.87
20000g detergent extract C*	0.12	0.45
20000g detergent extract D*	0.09	1.08

\* A, B, C, D are four different preparations of solubilised acetylcholine receptor.

<sup>+</sup> Each determination is the mean of 3 values determined at 3 different protein concentrations and is corrected for specific binding.

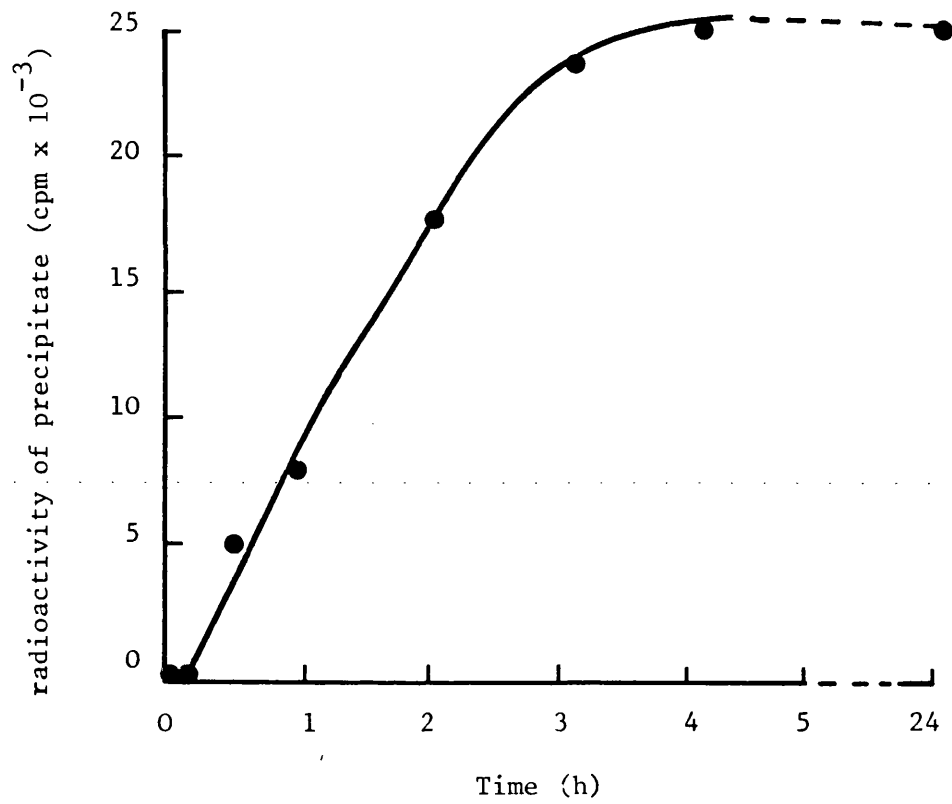


FIG. 11. The time course for the precipitation of the  $^{125}\text{I}$ - $\alpha$  bungarotoxin-receptor complex by 30% (v/v) ammonium sulphate solution at  $4^{\circ}\text{C}$

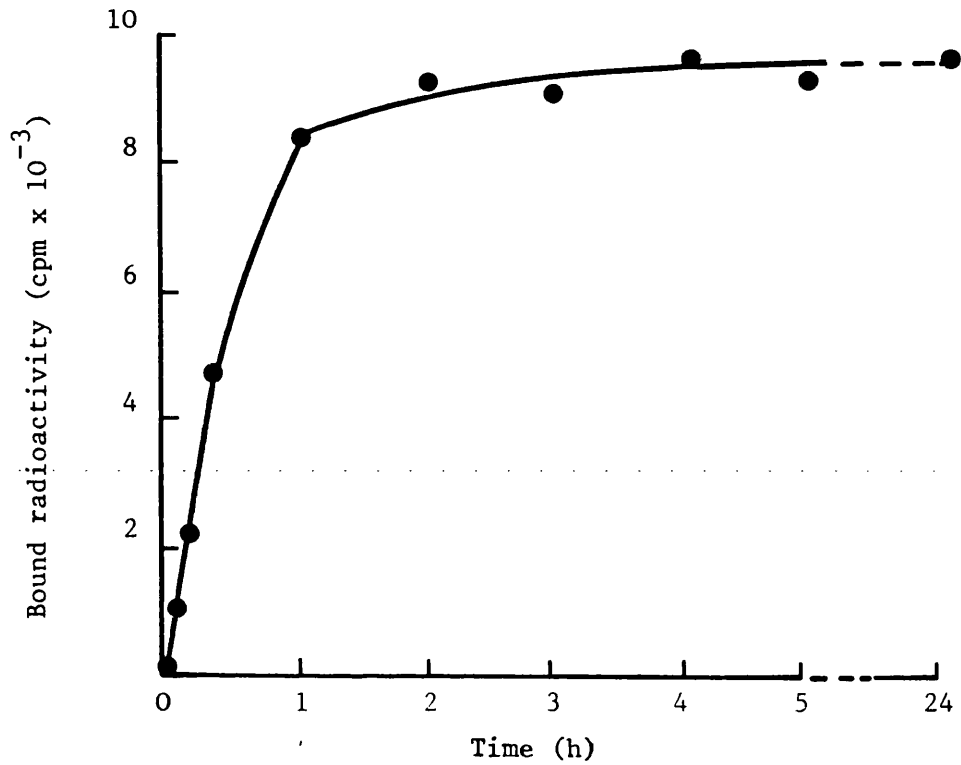


FIG. 12. The time course for the DEAE cellulose filtration assay for the measurement of the activity of the acetylcholine receptor  
Purified acetylcholine receptor (100 $\mu$ l) and <sup>125</sup>I- $\alpha$  bungarotoxin were mixed and incubated for the times as shown above, before filtration.

### 3. Assay of anti-(acetylcholine receptor) antibodies

#### (i) The time course

In an initial experiment, a series of myasthenic serum samples were assayed by the method of Lindstrom (1977). The results obtained showed that the different sera contained varying concentrations of anti-(acetylcholine receptor) antibodies. The serum with the highest antibody titre, where the antibody titre is defined as the moles of  $^{125}\text{I}$ - $\alpha$  bungarotoxin precipitated, was subsequently used to establish the conditions of the radioimmunoassay.

Acetylcholine receptor ( $1.5-2 \times 10^{-10}\text{M}$ ) was incubated for 8h at  $4^{\circ}\text{C}$  with  $^{125}\text{I}$ - $\alpha$  bungarotoxin ( $3-6 \times 10^{-10}\text{M}$ ). The myasthenic serum sample ( $5\mu\text{l}$ ) of known high antibody titre was added and incubated for varying times up to 24h before the addition of goat anti-(human IgG) antiserum. The time course for the reaction showed that the reaction between antigen and antibody was completed after 12h (FIG. 13 page 84).

In a second set of experiments, the solubilised acetylcholine receptor ( $1.5-2 \times 10^{-10}\text{M}$ ),  $^{125}\text{I}$ - $\alpha$  bungarotoxin ( $3-6 \times 10^{-10}\text{M}$ ) and the myasthenic serum sample ( $5\mu\text{l}$ ) of known high antibody titre were added simultaneously and the time course for the reaction was followed as above. The time course obtained was very similar to the one obtained earlier (FIGS. 13 and 14 , pages 84 and 85).

The precipitation of the antigen-antibody complex with the second antibody, goat anti-(human IgG) antiserum, occurred for 16h at  $4^{\circ}\text{C}$  as recommended by Hunter (1978).

The following conditions were used for all subsequent assays:-

- (i) Solubilised acetylcholine receptor and  $^{125}\text{I}$ - $\alpha$  bungarotoxin incubated for 8h at  $4^{\circ}\text{C}$ .

- (ii) Myasthenic serum sample added and incubated overnight at 4°C.  
 (iii) Second antibody added and precipitation allowed to occur overnight at 4°C.

(ii) Concentration of  $\alpha$  bungarotoxin

The effect of increasing  $^{125}\text{I}$ - $\alpha$  bungarotoxin concentration in the anti-(acetylcholine receptor) antibody titre can be seen in FIG. 15 page 86. In the radioimmunoassay, the working concentration range of  $^{125}\text{I}$ - $\alpha$  bungarotoxin is  $3-6 \times 10^{-10}\text{M}$  and it can be seen from FIG. 15 that over this range there is incomplete radiolabelling of receptor. The supply of  $^{125}\text{I}$ - $\alpha$  bungarotoxin was limited and it was not possible to use a higher concentration ( $20 \times 10^{-10}\text{M}$ ) routinely and therefore a correction factor was determined. This factor was defined as:-

Correction factor =

$$\frac{\text{Antibody titre determined with } ^{125}\text{I}\text{-}\alpha \text{ bungarotoxin } (20 \times 10^{-10}\text{M})}{\text{Antibody titre determined with } ^{125}\text{I}\text{-}\alpha \text{ bungarotoxin } (3-6 \times 10^{-10}\text{M})}$$

The value for the ratio was 2.5 for two different myasthenic serum samples and all subsequent antibody measurements took account of this (Lindstrom 1977).

(iii) The reproducibility of the radioimmunoassay

Seven myasthenic sera were taken at random and the anti-(acetylcholine receptor) antibody titres were measured with four different preparations of detergent solubilised acetylcholine receptor. The results are shown in Table 6 , page 87.

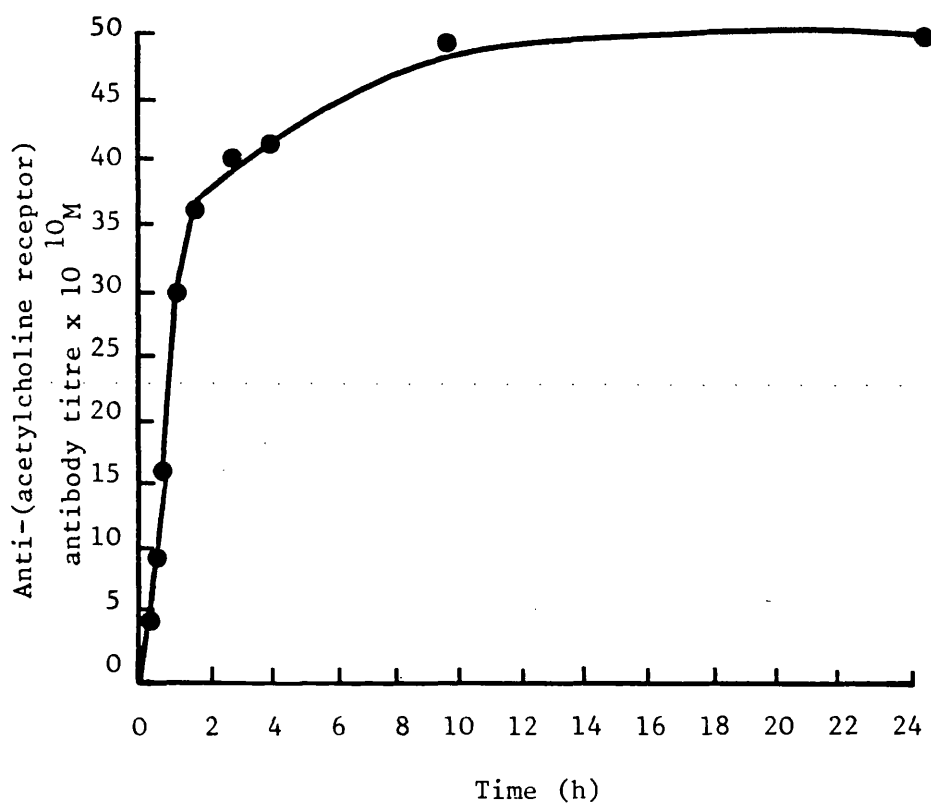


FIG. 13. The time course for the reaction of anti-(acetylcholine receptor) antibody with labelled antigen at 4°C following preincubation of acetylcholine receptor with <sup>125</sup>I-α bungarotoxin for 8h at 4°C

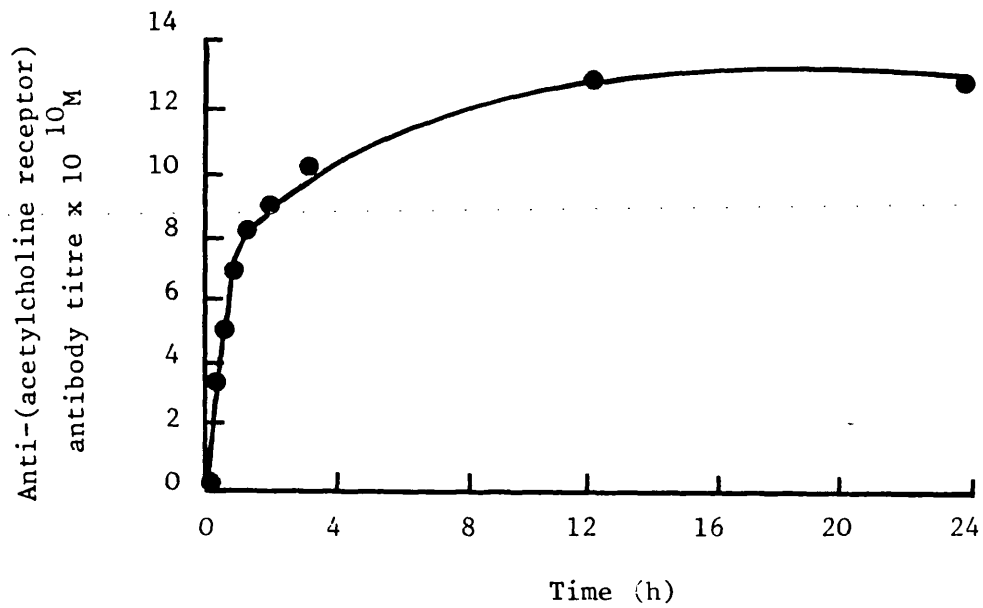


FIG. 14. The time course for the reaction of anti-(acetylcholine receptor) antibody with labelled antigen, following the simultaneous addition of all reactants



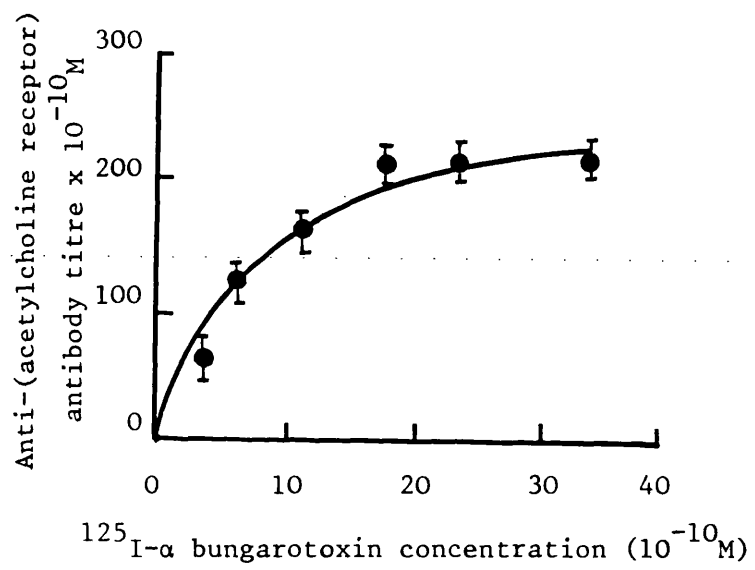


FIG. 15. The effect of increasing the  $^{125}\text{I-}\alpha$  bungarotoxin concentration on the anti-(acetylcholine receptor) antibody titre

Table 6. A comparison of the antibody titre of seven myasthenic sera with four different preparations of solubilised acetylcholine receptor

Serum sample	Antibody titre $\times 10^{10}M$ * A	Antibody titre $\times 10^{10}M$ * B	Antibody titre $\times 10^{10}M$ * C	Antibody titre $\times 10^{10}M$ * D	Mean Ab. titre $\times 10^{10}M$	S.E.M.
1	87	139.5	132	100	114.6	12.58
2	21	27.5	16.5	10.95	18.99	3.5
3	455	305	175.5	191.5	281.75	129
4	26	8.5	17	19	17.63	3.6
5	437.5	515	156	230.5	334.8	84.59
6	82	127.5	132	87.5	107.3	13.07
7	6	0	20.5	10.5	9.25	4.32

\* A, B, C, D are four different preparations of solubilised acetylcholine receptor.

4. Anti-(acetylcholine receptor) antibody titres in the sera of myasthenic patients

(i) Myasthenic patients in general

The serum samples that were assayed for anti-(acetylcholine receptor) antibody activity were measured by a blind method and comprised samples of normal serum, sera from patients with polymyositis and myasthenic serum samples. The myasthenic patients were classified according to the severity of both cranial and generalised symptoms on a scale of 0 to 3 where 3 represents the most severely affected state.

The range of antibody titres for the control patients was found to be  $0-2.5 \times 10^{-10}$  M (17 patients). From an initial population of 46 myasthenic patients, 87% were found to have a titre which was significantly different from control values. FIG. 16 shows the relationship between the disease severity and antibody titre for 39 patients. It was found that there was no direct relationship between the antibody titre and disease severity. However, the median value for the antibody titre was found to increase with disease severity (FIG. 16 page 91).

A statistical analysis of the control population and the myasthenic population was carried out. The mean anti-(acetylcholine receptor) antibody titre for the normal population was  $1.29 \times 10^{-10}$  M ( $\pm 0.086$ ) (17) and the mean anti-(acetylcholine receptor) antibody titre for the myasthenic population was  $141.3 \times 10^{-10}$  M  $\pm 11.4$  (59). The mean values were found to be significantly different (student t test,  $p < 0.01$ ).

(ii) Plasma-exchange patients

In a second series of measurements, the anti-(acetylcholine receptor) antibody was measured in the sera of patients during a course of plasma-exchange. For each exchange, 4ℓ of the patient's plasma were replaced by plasma protein fraction and this process was repeated for up to six times at approximately 2.5 day intervals. At the end of the first week of exchange, each patient was placed on prednisolone (100mg) daily and azathioprine (150mg daily). The azathioprine was maintained for three months but the steroids were gradually reduced over the three month period.

Serum samples were taken immediately before and after each plasma-exchange and were assayed for anti-(acetylcholine receptor) antibodies using the same preparation of antigen for each exchange series. Table 7 shows the antibody titre during plasma-exchange for eight patients, and FIG. 17 (patient 2) and FIG. 18 (patient 3) emphasise the trend of antibody titres during plasma-exchange that was typical of patients 1-5. The overall trend was down and all patients showed marked clinical improvement.

Patient 6 did not respond to 10mg of edrophonium chloride given intravenously and had no clinical improvement when treated with up to 900mg of pyridostigmine daily. Prednisolone given in doses from 60-100mg daily for two months was similarly without any improvement. However, after plasma-exchange, patient 6 showed marked sensitivity to edrophonium and other anti-cholinesterases. Patients 7 and 8 both achieved complete remission of symptoms after five plasma-exchanges despite the apparent absence of anti-(acetylcholine receptor) antibodies in their serum throughout the whole series of exchanges.

Once this procedure had been established in the laboratory, the routine analysis of myasthenic sera was taken over by a technician and some of the subsequent data are reported in Carter *et al* (1980).

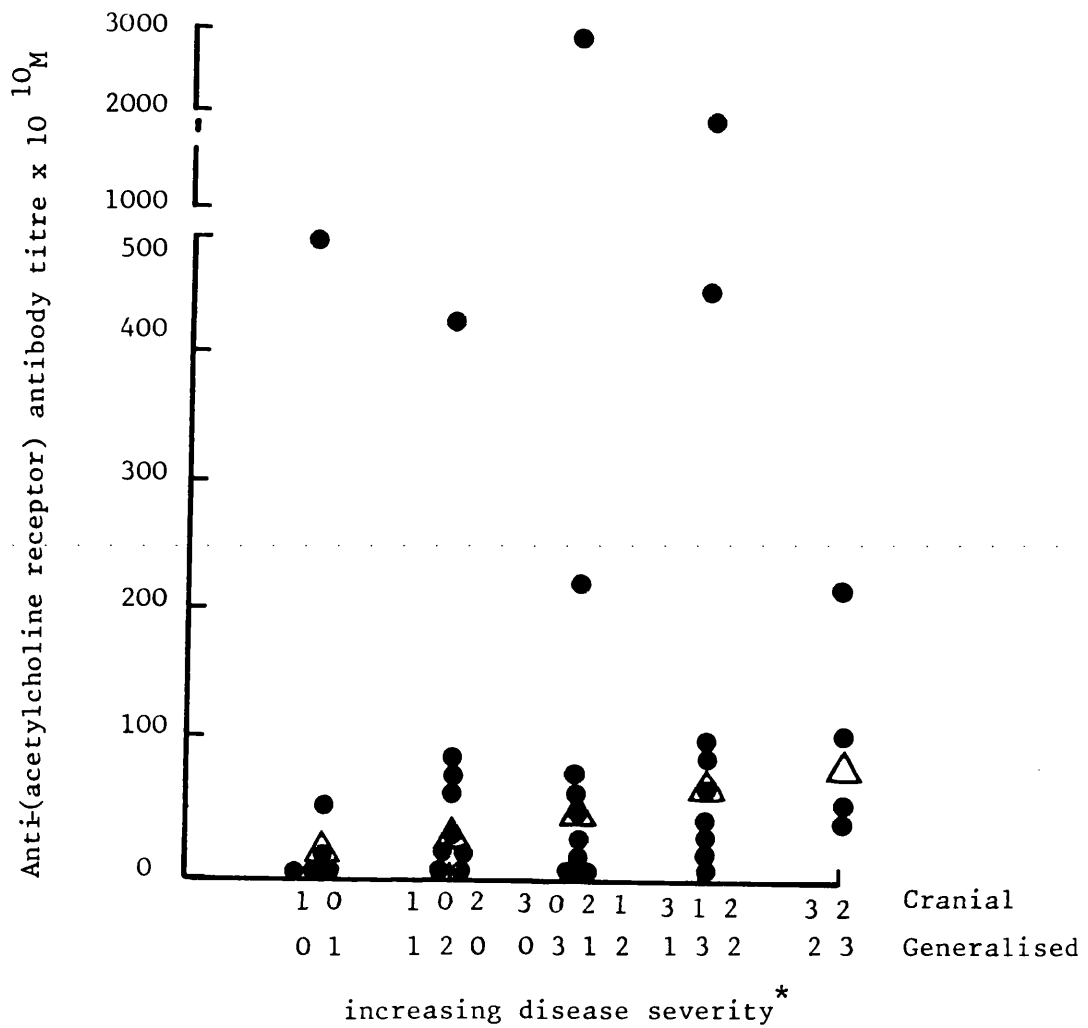


FIG. 16. The relationship between disease severity and anti-(acetylcholine receptor) antibody titre for patients with myasthenia gravis

●● Antibody titre x 10<sup>-10</sup>M

△ Median value for each clinical classification

\* disease severity assessed on a scale 0-3 where 3 is the most severe (see Barkas *et al* 1979)

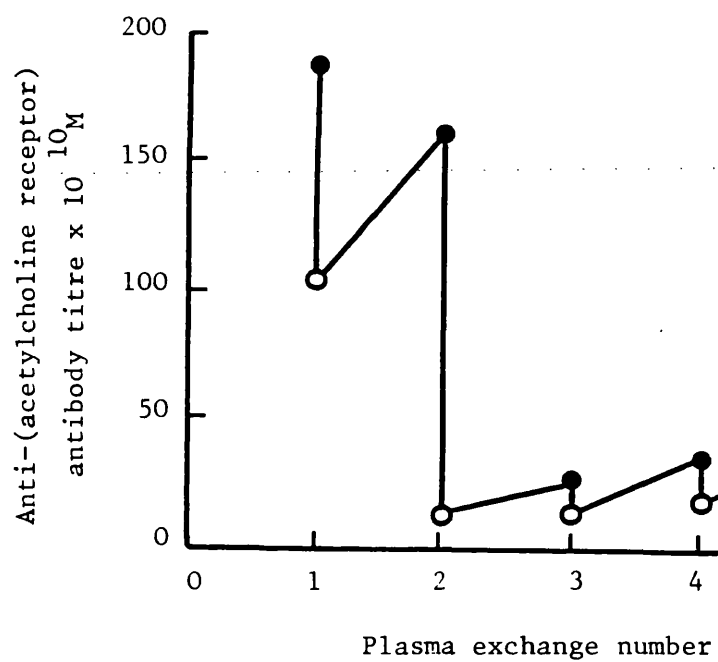


FIG. 17. The variation in anti-(acetylcholine receptor) antibody titre during plasma exchange for patient number 2 in Table 7

- Pre-plasma exchange
- Post-plasma exchange

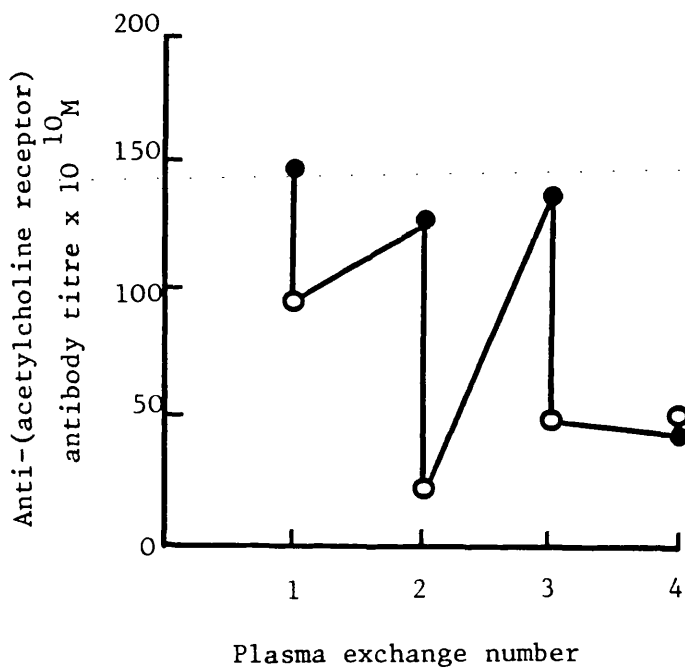


FIG. 18. The variation in anti-(acetylcholine receptor) antibody titre during plasma exchange for patient number 3 in Table 7

- Pre-plasma exchange
- Post-plasma exchange



Table 7. Antibody titres from myasthenic patients undergoing successive plasmaphereses

Patient no.	Age	Sex	Clinical state		Antibody titre $10^{-10}$ M ( $\alpha$ bungarotoxin binding sites)																	
					1st exchange		2nd exchange		3rd exchange		4th exchange		5th exchange		6th exchange							
					Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post						
1	52	F	3	0	128.5	35.5	81.5	15	30.5	3.75	4.4	2.65										
2	56	F	3	3	189	113.5	164	14.5	28	19.5	38.5	23	43	13								
3	66	M	3	2	149	98	129.5	27.5	141.5	51	44.5	46										
4	35	F	3	3	48	7		4	39	16												
5	68	F	3	3	234	43	35.5	16	220.5	26.5												
6	62	M	2	1	71	54	95	59	60	26	79.5	30.5	12	149	8.5	33.5						
7	29	F	3	3	7	2	0.5	0.5	1	1.5	0.5	0	0.5	1.5	2.5							
8	44	F	2	2	1.5	11.5	2.5	2	2.5	1	0	1	3.5	0.5								

5. Alternative assays for anti-(acetylcholine receptor) antibodies

(i) Assay for a sub-population of antibodies directed at the  $\alpha$  bungarotoxin binding site of the acetylcholine receptor

Twenty myasthenic serum samples were taken at random and assayed as follows: myasthenic serum (10 $\mu$ l) was added to a solution containing solubilised acetylcholine receptor (100 $\mu$ l) and left at 4°C overnight.  $^{125}$ I- $\alpha$  bungarotoxin (50 $\mu$ l, 1nM final concentration) was added and the ammonium sulphate precipitation assay was completed as described previously (page 63). Samples of the receptor preparation were assayed simultaneously in the absence of myasthenic serum but in the presence of normal human serum. The inhibition of  $^{125}$ I- $\alpha$  bungarotoxin binding was expressed as:-

Inhibition=

$$\left[ \frac{(\text{Binding in presence of normal serum}) - (\text{Binding in presence of myasthenic serum})}{\text{Binding in the presence of normal human serum}} \right] \times 100\%$$

The results given in Table 8 show that all myasthenic sera inhibit the binding of toxin over the range 1.4-74%. Three sera with comparatively low antibody titres, numbers 1, 9, 15, inhibit the binding of toxin by 52%, 34%, 55% whilst serum with a very high titre, for example number 16, has a low inhibitory capacity of 26%.

(ii) The anti-(acetylcholine receptor) antibody *in vivo*

Some work was done in collaboration with A.L. Harvey and colleagues at the University of Strathclyde, into the effects of myasthenic serum on the acetylcholine sensitivity of chick muscle in culture (Harvey *et al* 1978).

Sera from seven patients with myasthenia gravis and from six control subjects were assayed for the presence of anti-(acetylcholine receptor) antibodies and for the ability to reduce acetylcholine-induced depolarisation of embryonic chick skeletal muscle cells in culture. All the myasthenic sera caused a dramatic reduction in sensitivity to acetylcholine with a mean value of  $89 \pm 4\%$  compared to the control value of  $15 \pm 7\%$ . Three of the myasthenic samples had very low antibody titres but were equally effective in reducing the sensitivity of the muscle cells (Table 9 page 98).

Table 8. The inhibition of  $^{125}\text{I}$ - $\alpha$  bungarotoxin binding to acetylcholine receptor in the ammonium sulphate precipitation assay

Serum Sample	* Clinical Classification	Antibody titre $\times 10^{10}\text{M}$	% inhibition of $^{125}\text{I}$ - $\alpha$ bungarotoxin binding	Ratio of antibody titre to % inhibition
1		1.5	52	.029
2		28.9	66	.438
3		31.2	58	.54
4		183.9	74	2.48
5		253.4	40	6.33
6		176.6	41	4.3
7		3.5	24	.146
8		7.5	19	.395
9		5.5	34	.162
10		6.3	1.4	4.5
11		64	26	2.46
12		47.9	11	4.35
13		48.5	45	1.07
14		75.6	16	4.73
15		17.1	55	.31
16		951.3	26	36.59
17		44.3	25	1.77
18		32.7	35	.934
19		37.3	26	1.43
20		7.7	17	.45

\* Clinical data not yet available

Table 9. Reduction of acetylcholine sensitivity caused by addition of myasthenic and of control sera

Case number	Severity of myasthenia *	Percentage Reduction †	Antibody titre $\times 10^{10} \text{M}$
1	I	$98 \pm 1$	0.6
2	I	$94 \pm 3$	9.8
3	I	$80 \pm 7$	0
4	IV	$98 \pm 1$	69.3
5	IIb	$91 \pm 7$	12.8
6	IIa	$90 \pm 3$	0.3
7	III	$67 \pm 11$	14.7
1	-	$0 \pm 16$	0.3
2	-	$0 \pm 20$	0
3	-	$0 \pm 11$	0
4	-	$15 \pm 11$	0
5	-	$37 \pm 11$	0.3
6	-	$39 \pm 5$	1.0

\* Grading scale according to Osserman (1958)

† Percentage reduction of acetylcholine response, at 50% level of control dose-response curve, shown by addition of human sera. Values are given as mean  $\pm$  S.E.M. determined in at least 10 fibres in a single culture.

The second series of patients 1-6 are controls.

Section B

The purification and characterisation of the human acetylcholine

receptor

METHODS1. The purification of  $\alpha$  toxin from *Naja naja siamensis* venom

$\alpha$  toxin was purified from the crude venom of *Naja naja siamensis* by a modification of the method of Cooper and Reich (1972).

Phosphocellulose was equilibrated with 0.01M potassium phosphate buffer, pH 6.0. It was packed into a column (30cm x 2.5cm) at 4°C and left to settle overnight. The packed column was washed with 0.01M potassium phosphate buffer, pH 6.0 (150ml). Crude venom (1g) was dissolved in distilled water (10ml) and applied to the ion-exchange column. The column was washed with the equilibrating buffer (300ml) to remove non-bound material. The proteins were then eluted with a linear gradient (10mM-50mM) of potassium phosphate buffer, pH 6.0 at a rate of 48ml/h. Fractions (14ml) were collected and the optical density at 280nm measured. The peak tubes were pooled and to the first two eluted protein peaks, solid ammonium sulphate was slowly added to give a saturated solution. This solution was left to stir overnight at 4°C and centrifuged at 30000g for 10 min. The pellet was dissolved in distilled water and dialysed overnight at 4°C against 3 x 4l of distilled water. The diffusate was freeze-dried and stored at 4°C until use.

The activity of the  $\alpha$  toxin was assayed as follows. Purified acetylcholine receptor from *Torpedo marmorata* (0.2pmol, 100 $\mu$ l) in 0.01M potassium phosphate buffer, pH 7.4, containing 1% (v/v) Triton X100 and 0.1% (w/v) bovine serum albumin was incubated for 30 min at 23°C with a sample (50 $\mu$ l) that was to be assayed for  $\alpha$  toxin activity. <sup>125</sup>I- $\alpha$  bungarotoxin (0.23pmol, 50 $\mu$ l) in the above buffer was added and the mixture was incubated for 1h at 23°C. The amount of <sup>125</sup>I- $\alpha$

bungarotoxin bound was then determined by the DEAE cellulose filtration assay (Methods page 64). The samples that were assayed for  $\alpha$  toxin binding activity were serially diluted until a 50% inhibition of  $^{125}\text{I}$ - $\alpha$  bungarotoxin binding was observed.

## 2. The preparation of the affinity columns

### (i) The $\alpha$ toxin affinity column

$\alpha$  toxin purified from *Naja naja siamensis* crude venom was coupled to Sepharose 4B following the method of March *et al* (1974).

Sepharose 4B (50ml packed beads) was washed with 0.1M NaCl (1l) and distilled water (500ml). The beads were resuspended in cold water (100ml total volume) and 2M sodium carbonate (100ml) added. The solution was left to stir at 4°C.  $\alpha$  toxin (25mg) was dissolved in 0.2M sodium hydrogen carbonate (100ml), pH 9.4, and the optical density at 280 nm measured. Cyanogen bromide (3g) dissolved in acetonitrile (1.5ml) was added to the Sepharose 4B solution and stirred for 2 min at 4°C. The mixture was rapidly filtered, washed with ice cold water (500ml) and the Sepharose 4B beads added to the  $\alpha$  toxin solution and stirred overnight at 4°C. The beads were washed with water (400ml) and resuspended in 2M glycine, pH9 (200ml) and stirred overnight at 4°C. The affinity beads were collected by filtration and washed alternately with 0.1M acetate buffer, pH 4.0 containing 1M NaCl (150ml) and 0.1M borate buffer, pH 8.0 containing 1M NaCl (150ml). This process was repeated three times and the affinity beads were equilibrated with 0.01M potassium phosphate buffer, pH 7.4, containing 0.1% (v/v) Triton X100. The beads were stored at 4°C in the presence of 0.02% (w/v)  $\text{NaN}_3$ . After use, the affinity column was regenerated by washing



with 0.01M potassium phosphate buffer, pH 7.4, containing 1M NaCl (300ml) and 0.01M potassium phosphate buffer, pH 7.4 (500ml).

(ii) The lectin affinity columns

Concanavalin A, lens culinaris and soybean lectin (Type VI) (25mg in each case) were covalently coupled to Sepharose 4B exactly as described above (Methods page 100) except that all lectin-affinity columns were equilibrated with 0.075M potassium phosphate buffer, pH 7.8, containing 75mM NaCl, 5mM MgCl<sub>2</sub>, 1.5mM CaCl<sub>2</sub> and 0.2% (v/v) Triton X100 (Dolly 1979).

(iii) The antibody affinity column

IgG was prepared from the serum of a myasthenic patient as described earlier (Methods, 64). The purified IgG (50mg) was covalently coupled to Sepharose 4B exactly as described above (Methods page 100). The antibody-affinity column was equilibrated with 0.01M potassium phosphate buffer, pH 7.4, containing 0.1% (v/v) Triton X100 and was stored at 4°C.

3. Iodination of purified acetylcholine receptor

The purified protein (20-350 fmol <sup>125</sup>I-α bungarotoxin binding sites) was directly labelled with <sup>125</sup>I by the method of Urbaniak *et al* (1973) as described earlier (Methods page 60) except that all solutions contained 0.1% (v/v) Triton X100 and Na <sup>125</sup>I (5μl) was used.

4. Isoelectric focussing of the acetylcholine receptor

Isoelectric focussing was carried out in polyacrylamide disc

gels (6.5cm x 0.5cm) prepared from 4% (w/v) acrylamide and containing 4% (v/v) Triton X100 with 3% (v/v) Ampholines (pH 4-6; pH 3.5-10). Gels were pre-run at 350 V for 1h with 0.4% (v/v)  $H_2SO_4$ , pH 2.0, containing 1% (v/v) Triton X100 in the anode chamber and 0.2% (v/v) ethanolamine, pH 10.6, containing 1% (v/v) Triton X100 in the cathode chamber. The samples (either  $^{125}I$ -acetylcholine receptor (100 $\mu$ l);  $^{125}I$ -Triton X100 (100 $\mu$ l) or purified acetylcholine receptor (50 $\mu$ l) and  $^{125}I$ - $\alpha$  bungarotoxin (0.25 pmol, 50 $\mu$ l) in 0.01M potassium phosphate buffer, pH 7.4, containing 1% (v/v) Triton X100 and 0.1% (w/v) bovine serum albumin preincubated for 90 min at 23°C) containing 0.02% (w/v) bromophenol blue as the tracking dye were applied to the top of each gel with a syringe. They were focussed for 1.5h at 23°C under the conditions described above. Gels were removed and sliced manually at 1mm intervals then suspended in double distilled water (200 $\mu$ l) for determination of radioactivity and pH. Slices to be examined by SDS-PAGE (Methods page 102) were incubated in 0.01M potassium phosphate buffer (100 $\mu$ l), pH 7.4, containing 0.1% (w/v) sodium dodecyl sulphate, 0.1% (v/v) Triton X100 at 4°C overnight.

##### 5. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Polyacrylamide gel electrophoresis was carried out under denaturing conditions according to the method of Weber and Osborn (1969). All samples were boiled for 5 min in 0.01M potassium phosphate buffer, pH 7.4, containing 5% (w/v) sodium dodecyl sulphate and 2.5% (v/v)  $\beta$  mercaptoethanol. A list of the samples applied is given below and all contained 0.02% (w/v) bromophenol blue and 10% (w/v) sucrose:-

1. Standard marker enzymes (10 $\mu$ l of 1mg/ml).

2.  $^{125}\text{I}$ -acetylcholine receptor eluted from isoelectric focussing gels (100 $\mu\text{l}$ ).
3. Purified acetylcholine receptor (100 $\mu\text{l}$ ).
4. Detergent solubilised acetylcholine receptor (5 $\mu\text{l}$ ) diluted in 0.01M potassium phosphate buffer, pH 7.4, (95 $\mu\text{l}$ ).
5. Human muscle membrane fragments labelled with [ $^3\text{H}$ ]-MBTA (100 $\mu\text{l}$ ).
6. Purified acetylcholine receptor labelled with [ $^3\text{H}$ ]-MBTA (100 $\mu\text{l}$ ).

Electrophoresis was carried out in disc gels (8cm x 0.5cm) using 10% (w/v) acrylamide and 6mA/gel at an operating voltage of 30V for 5h at 23°C. Gels were fixed and stained simultaneously with 0.25% (w/v) Coomassie blue R in 54% (v/v) methanol/9% (v/v) acetic acid at 23°C overnight. They were destained with 5% (v/v) methanol/7% (v/v) acetic acid by heating in a boiling water bath and scanned densitometrically at 620nm. Alternatively, gels were immediately frozen at -20°C, sliced at 1mm intervals and the slices counted in an LKB 1280 Ultrogamma counter. Bovine serum albumin (68000), catalase (E.C.1.11.1.6) (60000), liver alcohol dehydrogenase (E.C.1.1.1.1) (41000) and haemoglobin (15500) were used as standard markers.

#### 6. Non-denaturing polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed by the method of Davis (1964). Electrophoresis was carried out in disc gels (8cm x 0.5cm) using 7% (w/v) acrylamide in the running gel at pH 8.9 and 2.5% (w/v) acrylamide in the stacking gel, pH 6.7. All solutions contained 0.1% (v/v) Triton X100. The samples were prepared by incubation of purified acetylcholine receptor (50fmol, 50 $\mu\text{l}$ ) with  $^{125}\text{I}$ - $\alpha$  bungarotoxin (180fmol, 50 $\mu\text{l}$ ) in 0.01M potassium phosphate buffer containing 1% (v/v)

Triton X100 for 90 min at 23°C. Bromophenol blue was added (0.02% (w/v) final concentration) and sucrose (10% (w/v) final concentration). Samples (100µl) were electrophoresed at 2mA/gel for 1.75h at 23°C. Gels were stained and destained as described previously (Methods page 103), sliced at 1mm intervals and counted.

#### 7. Sedimentation in a sucrose density gradient

Continuous sucrose density gradients were prepared by making a five step discontinuous gradient with 4-20% (w/v) sucrose in 0.01M potassium phosphate buffer, pH 7.4, containing 0.1M NaCl and 0.5% (v/v) Triton X100 and leaving to stand overnight at 23°C. The samples were prepared by preincubation of purified acetylcholine receptor (50fmol, 50µl) with <sup>125</sup>I-α bungarotoxin (180fmol, 50µl) in 0.01M potassium phosphate buffer, pH 7.4, containing 1% (v/v) Triton X100 and 0.1% (w/v) bovine serum albumin, for 90 min at 23°C. The samples (100µl) were mixed with the standard marker enzymes, β-galactosidase (E.C.3.2.1.23) ( $s_{20,w} = 16$ )(140µg, 28µl); catalase ( $s_{20,w} = 11.4$ )(800µg, 32µl) and yeast alcohol dehydrogenase ( $s_{20,w} = 7.4$ )(400µg, 40µl) and the mixture (160µl) was layered carefully on the gradients. Velocity sedimentation was performed in the SW:40 rotor of a Beckman L5-65 ultracentrifuge and runs were carried out at 150000g ( $r_{av} = 112.7\text{mm}$ ) for 7h at 23°C. After centrifugation, the sucrose gradients were pumped from the tube bottom with a Gilson peristaltic pump and 5-drop fractions (76µl  $\bar{v}$ ) were collected. Fractions were then counted in an LKB 1280 Ultragamma counter, assayed for marker enzyme activity (Bergmeyer et al 1974) and the refractive index was measured.

## 8. Affinity labelling of muscle membranes

### (i) The preparation of muscle membranes

Human muscle (100g) was chopped and homogenised for 1 min at 4°C in buffer (100ml) which contained 0.01M potassium phosphate, pH 7.4, 0.1M NaCl; 1mM EDTA; 2mM benzamidine hydrochloride; 0.1mM benzethonium chloride; 0.1mM phenylmethylsulphonyl fluoride, 0.02% (w/v)  $\text{NaN}_3$  and 0.002% (w/v) pepstatin. The homogenate was centrifuged at 20000g for 60 min at 4°C. The pellet was rehomogenised in 100ml of the above buffer. The homogenate was centrifuged at 700g. The pellet was discarded and the supernatant recentrifuged at 20000g for 20 min. The resulting pellet was washed by resuspension in 0.01M potassium phosphate buffer, pH 7.4, containing 0.15M NaCl and 1mM EDTA and recentrifugation at 20000g for 20 min. The pellet was then resuspended in the same buffer.

### (ii) The affinity labelling procedure

The labelling procedure followed the method of Barrantes *et al* (1975). The affinity ligand 4-(N-maleimido)-benzyl trimethylammonium iodide (MBTA) was synthesised with a tritium label by Dr. R. Harrison by the method of Karlin (1971) to a specific activity of 126 $\mu\text{Ci}/\mu\text{mol}$  MBTA.

Membranes (1ml) were reduced by addition of 1mM dithiothreitol in 0.01M Tris HCl buffer (4ml), pH 7.3, containing 0.1M NaCl, 1mM EDTA and left on ice for 10 min. The samples were then centrifuged at 100000 g for 20 min in an MSE superspeed 50 centrifuge. The pellets were resuspended in 0.01M potassium phosphate buffer, pH 7.4, containing 0.15M NaCl, 1mM EDTA and recentrifuged at 100000g. Again the pellets

were resuspended and  $\alpha$  bungarotoxin (100nmol) was added to the control sample. The membranes were left on ice for 1h. [ $^3$ H]-MBTA (1nmol) dissolved in 0.1mM HCl (25 $\mu$ l) was added to each membrane sample and incubated at 23 $^{\circ}$ C for 2 min. Excess  $\beta$ mercaptoethanol (10 $\mu$ l) was added and the membranes were centrifuged at 100000g for 20 min. The pellets were resuspended in 0.01M potassium phosphate buffer, pH 7.4, containing 0.15M NaCl, 1mM EDTA and recentrifuged at 100000g for 20 min. The washing procedure was repeated once more. The final pellets were dissolved in 0.01M potassium phosphate buffer, pH 7.4, containing 5% (w/v) sodium dodecyl sulphate, 5% (v/v)  $\beta$ mercaptoethanol and boiled for 30 min. Each sample was then subjected to polyacrylamide gel electrophoresis under denaturing conditions (Methods, page 102). After electrophoresis, the gels were sliced at 1mm intervals and each slice was incubated for 48h at 23 $^{\circ}$ C with a solubilisation/scintillation cocktail which contained toluene (1 $\mu$ l), hyamine hydroxide (10ml), soluene 350 (10ml), 2,5-diphenyloxazole (6g), (Aloyo 1979). The samples were then counted in a Packard scintillation spectrometer.

#### 9. Affinity labelling of purified acetylcholine receptor

Two methods were employed to label purified acetylcholine receptor with the affinity ligand [ $^3$ H]-MBTA.

(i) The first method employed was that of Froehner *et al* (1977(b)). The affinity reagent [ $^3$ H]-MBTA (200 $\mu$ M) in acetonitrile was evaporated to dryness and dissolved in 0.1M HCl (1ml). It was further diluted in 0.019M sodium phosphate buffer, pH8, containing 0.15M NaCl, 1mM EDTA and 0.2% (v/v) Triton X100 to give a final concentration of 5 $\mu$ M. Purified acetylcholine receptor (4pmol  $^{125}$ I- $\alpha$  bungarotoxin binding activity) was dialysed for 2h at 4 $^{\circ}$ C against 0.019M sodium phosphate

buffer (1ℓ), pH 8, containing 0.15M NaCl, 1mM EDTA and 0.2% (v/v) Triton X100. The diffusate was divided into two and each sample was incubated with 0.4mM dithiothreitol (500μl) for 10 min at 23°C. α-Bungarotoxin (4μg) was added to the control sample and both samples were left on ice for 1h. Then 0.53M sodium phosphate buffer, pH 6.7, (100μl) and 5μM [<sup>3</sup>H]-MBTA (400μl) were added and the mixture incubated for 1 min at 23°C. The samples were chilled on ice for 5 min and 0.1M β mercaptoethanol (20μl) and poly-aspartic acid (5μg) added. Each sample was dialysed for 1h at 4°C against 0.01M potassium phosphate buffer (1ℓ), pH 7.4, containing 1% (v/v) Triton X100. Acetone (10ml) was added and a precipitate left to form at -20°C for 30 min. The precipitate was collected by centrifugation in a bench centrifuge and dissolved in 0.01M potassium phosphate buffer, pH 7.4, containing 5% (w/v) sodium dodecyl sulphate and 2.5% (v/v) β mercaptoethanol. Poly-acrylamide gel electrophoresis under denaturing conditions was carried out as described earlier (Methods, pages 102, 106 respectively).

(ii) The second method used was that described by Rubsamen *et al* (1978). Acetylcholine receptor (35pmol <sup>125</sup>I-α bungarotoxin binding sites) was reduced for 10min at 23°C with 0.2mM dithiothreitol in 0.019M sodium phosphate buffer (1ml), pH 8.0 containing 0.13M NaCl, 1mM EDTA and 0.2% (v/v) Triton X100. The reaction was then slowed by the addition of 0.53M potassium phosphate buffer, pH 5.5 (100μl) and by cooling in ice. The receptor was immediately separated from the dithiothreitol by the passage through a Sephadex G25 (superfine) column (20cm x 0.5cm) which was equilibrated with the same buffer that was used for the reduction except that it was at pH 7. Fractions (500μl) were collected and assayed for receptor activity.

The peak fraction (500 $\mu$ l) was incubated with [ $^3$ H]-MBTA (10 $\mu$ l, 45 pmol) for 10 min at 23 $^{\circ}$ C and transferred to ice. A control sample was pre-incubated with  $\alpha$  bungarotoxin (100pmol) for 30 min at 23 $^{\circ}$ C and then labelled with [ $^3$ H]-MBTA as described. Excess 12mM  $\beta$  mercaptoethanol (100 $\mu$ l) was added and the free and bound label separated using another Sephadex G25 (superfine) column (20cm x 0.5cm) which was equilibrated with 0.1mM potassium phosphate buffer, pH 7.5, containing 0.2% (v/v) Triton X100. Fractions (500 $\mu$ l) were collected and samples (20 $\mu$ l) counted in a Packard scintillation spectrometer. The bound material was dialysed overnight at 4 $^{\circ}$ C against 0.1mM potassium phosphate buffer pH 7.5, containing 0.2% (v/v) Triton X100. The diffusates were then subjected to polyacrylamide gel electrophoresis under denaturing conditions (Methods 5, 8 pages 102 and 106 respectively).

#### 10. Binding properties of human acetylcholine receptor

The binding properties of both the detergent solubilised acetylcholine receptor and the purified protein were examined. Details are given in Results (pages 162 to 174) and all reagents are dissolved in assay buffer which contains 0.01M potassium phosphate buffer, pH 7.4, 1% (v/v) Triton X100 and 0.1% (w/v) bovine serum albumin.

#### 11. The raising of anti-(acetylcholine receptor) antibodies

The procedure for immunisation was followed for the preparation of goat anti-(human IgG) antiserum as described earlier (Methods page 65) except that purified acetylcholine receptor from human muscle (3pmol) was the antigen and rabbits were used. The rabbits were bled at regular intervals and the serum assayed for the presence of anti-(acetylcholine receptor) antibodies (Methods page 66).



12. Assay for acetylcholinesterase

Acetylcholinesterase activity was measured by the method of Ellman *et al* (1961).

13. Protein determination

(i) Protein determination was by the method of Lowry *et al* (1951). In the presence of Triton X100, reagent A contained 5% (w/v) sodium dodecyl sulphate. Bovine serum albumin was used as the standard protein and FIG. 19 shows the standard calibration curve.

(ii) The protein content of purified acetylcholine receptor was measured by a Coomassie G250 micro-assay (Sedmak and Grossberg 1977).

The dye was prepared by making a solution of 0.06% (w/v) Coomassie brilliant blue G250 B1131 in 1.9% (w/v) perchloric acid and filtering through Whatman filter paper No. 1. This reagent (500 $\mu$ l) was added to the protein sample (25 $\mu$ l), mixed and the optical density at 620nm measured. Bovine serum albumin was used as the standard protein and it was always dissolved in the sample buffer which contained 0.01M potassium phosphate, pH 7.4, 0.5M NaCl, 1mM EDTA, 0.1mM benzethonium chloride, 0.1mM phenylmethylsulphonyl fluoride, 0.02% (w/v) NaN<sub>3</sub> and 0.1% (v/v) Triton X100. FIG.19 shows the standard calibration curve.

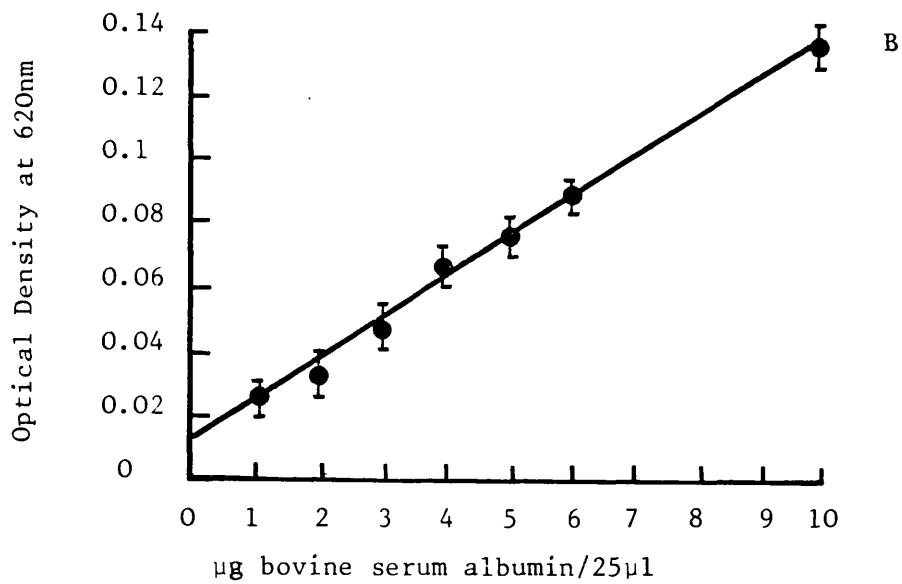
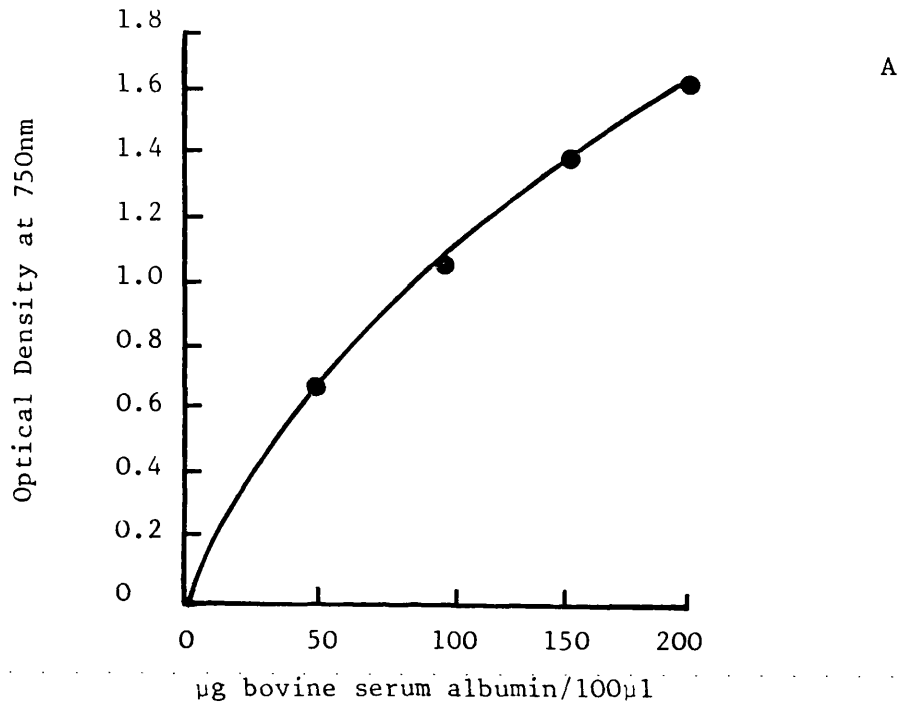


FIG. 19. Standard curves for protein determination

A Lowry *et al* (1951)

B Sedmak and Grossberg (1977)

EXPERIMENTAL AND RESULTS1. The purification of  $\alpha$  toxin from *Naja naja siamensis* venom

$\alpha$  toxin was purified as described in Methods (page 99). The elution profile of the toxin from the ion-exchange column is shown in FIG. 20. Table 10 summarises the results obtained. After ion-exchange chromatography, the  $\alpha$  toxin activity was eluted in two peaks, peak D (100ml) and peak E (78ml). The peaks were pooled and the purification procedure completed with a recovery of 13% of the protein.

FIG. 20. The elution profile of the phosphocellulose ion exchange column in the purification of  $\alpha$  toxin from *Naja naja siamensis* venom

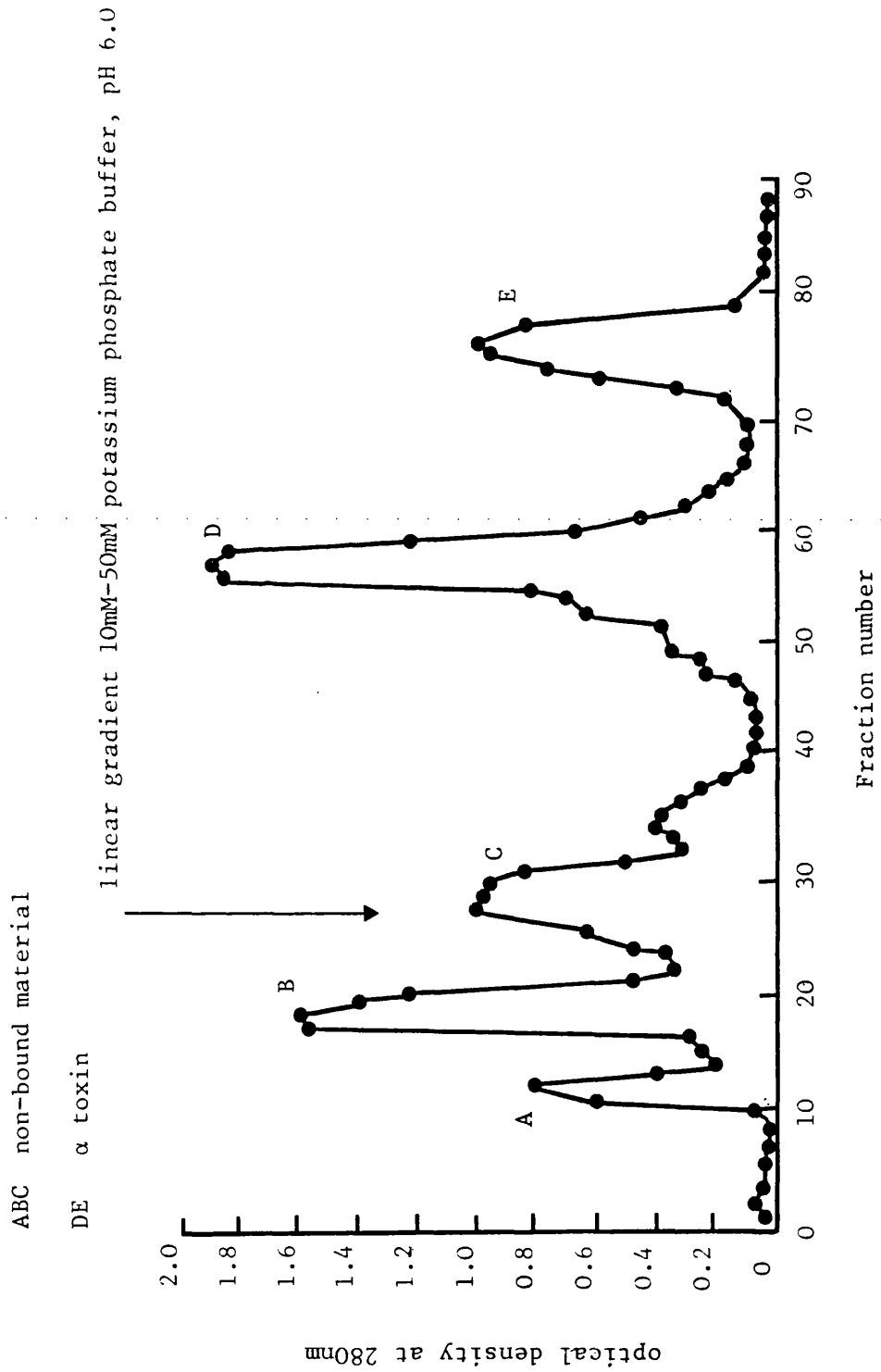
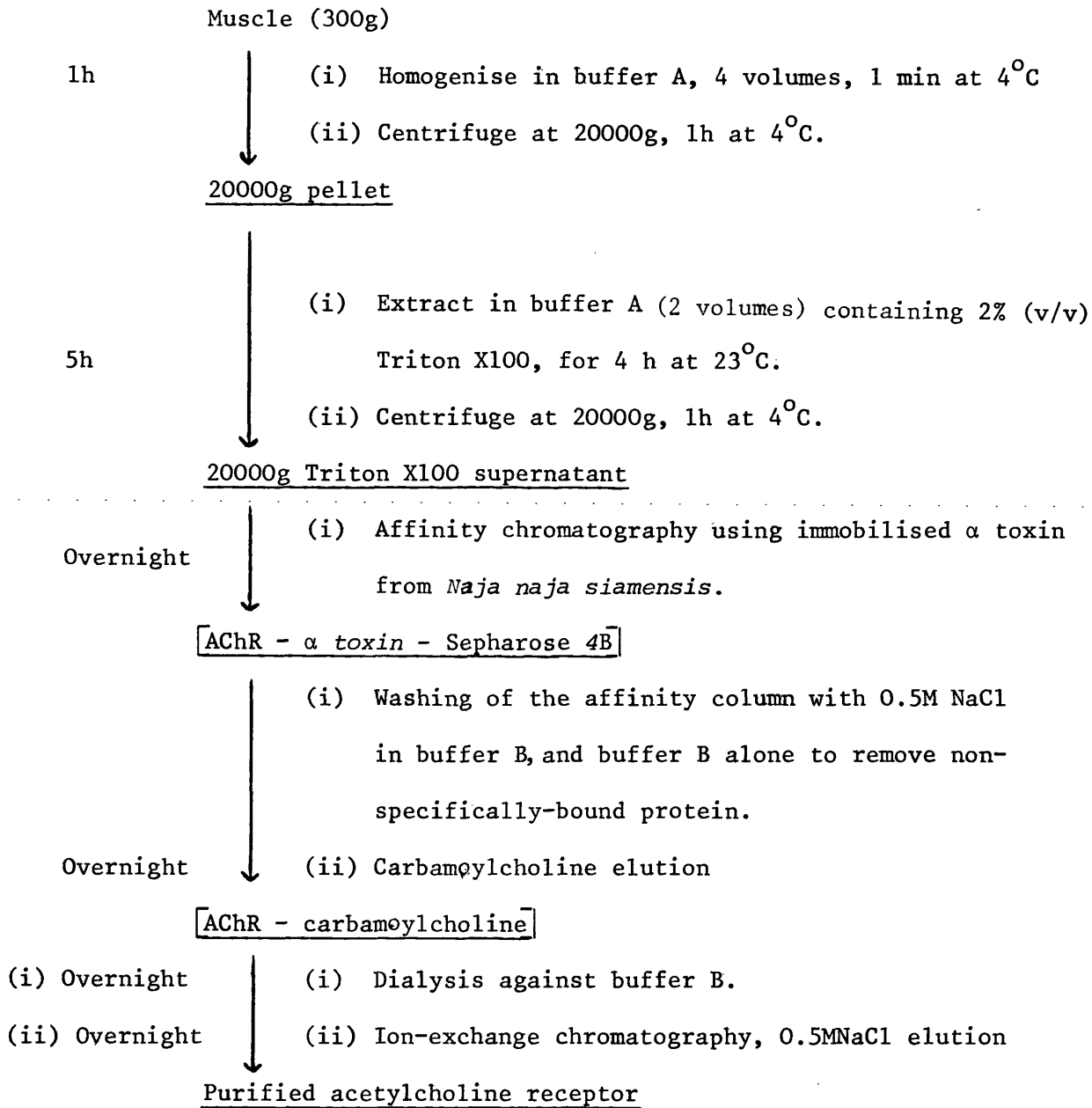


Table 10. Purification of  $\alpha$  toxin from *Naja naja siamensis* venom

Sample	Dilution for 50% inhibition $^{125}\text{I}$ $\alpha$ bungarotoxin binding	Total protein mg	Recovery
Crude venom	$10^{-6}$	130	100%
Peak A	Neat	N.D.	
Peak B	Neat	N.D.	
Peak C	Neat	N.D.	
Peak D	$10^{-4}$	[ 34 ] [ 26.5 ]	46%
Peak E	$10^{-4}$		
Peak D + E lyophilised	1mg/ml diluted 1 in $10^4$	17	13%

FIG. 21. The basic procedure for the purification of the acetylcholine receptor



## 2. The purification of the human acetylcholine receptor

The basic method was derived from the earlier immunological studies using detergent-solubilised acetylcholine receptor extract combined with the methods used in the laboratory for the routine isolation of acetylcholine receptor from *Torpedo marmorata*. FIG.21 is a flow diagram of the basic method details of which are described below.

In initial studies, buffer A contained 0.01M potassium phosphate, pH 7.4, 0.1M NaCl, 1mM EDTA and 0.01M  $\text{NaN}_3$ . The solubilised acetylcholine receptor 20000g supernatant was applied as a batch to the affinity beads and stirred overnight at 4°C. The beads were washed with 0.5M NaCl (300ml) in buffer B where buffer B was 0.01M potassium phosphate buffer, pH 7.4, containing 1mM EDTA, 0.02% (w/v)  $\text{NaN}_3$ , 0.1% (v/v) Triton X100 followed by buffer B alone (300ml) by a batch method. The elution with 0.5M carbamoylcholine in buffer B (50ml) was done at 4°C overnight and was also a batch method. Dialysis was carried out against 2 x 2ℓ buffer B at 4°C overnight. The residual carbamoylcholine was removed by passage through a column (2cm x 2cm) of DEAE cellulose and the column was washed with buffer B (2ℓ). The acetylcholine receptor was eluted with buffer B (20ml) containing 0.5M NaCl. Fractions of 1ml were collected (FIG. 22). Table 11 shows the results of a typical purification of human acetylcholine receptor using the method outlined above.

Table 11

Sample	Acetylcholine receptor pmol <sup>*</sup>	Protein mg	Specific activity $\mu\text{mol}/\text{g}$ protein	Yield <sup>§</sup>	Purification factor
Detergent solubilised acetylcholine receptor	274	4200	0.000065	100%	1
Purified receptor	1.6 <sup>+</sup>	0.071	0.023 <sup>+</sup>	1.4%	353

In this and all subsequent Tables

\* Acetylcholine receptor activity is expressed as molarity of  $^{125}\text{I}-\alpha$  bungarotoxin binding sites.

+ The data for the purified acetylcholine receptor refer to the peak tube of activity after the elution from the ion-exchange column.

§ The yield refers to the total receptor activity recovered from the fractions obtained after ion-exchange chromatography and is expressed as a percentage of the receptor bound to the affinity column.



The results for the purification of the acetylcholine receptor are expressed in relation to the activity of the acetylcholine receptor in the crude detergent extract. It was not possible to measure the receptor concentration of the total homogenate because of its very fibrous nature. Measurements of the diffusate after dialysis are not quoted as they were found to be complicated by the presence of residual carbamoylcholine (6mM approximately). In a series of control experiments, the diffusate was repeatedly dialysed against 0.01M potassium phosphate buffer, pH 7.4, containing 1mM EDTA, 0.02% (w/v)  $\text{NaN}_3$ , 0.1% (v/v) Triton X100 (buffer B) and was assayed for activity at each change of the dialysis buffer. The activity of the eluate was shown to increase with repeated dialysis (Table 12) but for both preparations of acetylcholine receptor, the total activity measured never exceeded the total amount of the final purified protein obtained after DEAE cellulose chromatography.

Table 12

Sample	Acetylcholine receptor A* fmol/ml	Acetylcholine receptor B* fmol/ml
Diffusate after 1 x 4l exchange	00	5
Diffusate after 2 x 4l exchange	25	22
Diffusate after 3 x 4l exchange	43	30
Diffusate after 4 x 4l exchange	56	54
Diffusate after 5 x 5l exchange	55	60

\* A and B are two different receptor preparations

In the purification outlined in Table 11 it was found that between 51-90% of the acetylcholine receptor activity bound to the affinity column by the measurement of the activity of the detergent extract before and after adsorption. Acetylcholine receptor activity was found neither in the salt wash nor in the buffer wash. Similarly, no protein was detected in the second and final buffer wash before the specific elution.

A series of modifications were made to the basic method in attempts to improve the yield and specific activity of the purified acetylcholine receptor.

(i) Affinity purification procedure

The basic purification outlined above was followed except that after the adsorption of the acetylcholine receptor to the affinity beads, the beads were packed in a column (10cm x 2cm) and washed with 0.5M NaCl in buffer B (300ml) followed by buffer B alone (300ml). The receptor was then eluted with 0.5M carbamoylcholine in buffer B at a flow rate of 6.25ml/h for 8h at 23°C and the eluate was processed as before. Elution continued beyond 8h did not yield any further <sup>125</sup>I- $\alpha$  bungarotoxin binding activity. The Table 13 below gives the results obtained for a typical experiment of this type.

Table 13

Sample	Acetylcholine receptor pmol	Protein mg	Specific activity $\mu\text{mol}/\text{g protein}$	Yield	Purification factor
Detergent solubilised acetylcholine receptor	159.4	5772	0.000028	100%	1
Purified receptor	7.9	0.088	0.089	17.8%	3178

The results show that there was an increased yield of 17.8% and that the specific activity had increased four fold (cf Table 11).

(ii) The addition of anti-protease agents

The results outlined in Table 12 suggested that there was no loss of acetylcholine receptor activity during ion-exchange chromatography and that the conditions for the carbamoylcholine elution were now optimised. It was considered that proteolysis may be important (Silman *et al* 1978). Based on the findings of Silman *et al* (1978), Barnard *et al* (1979), and Lindstrom (1979), the following anti-protease agents were added to both the homogenisation and the extraction buffer: 2mM benzamidine hydrochloride, 0.1mM benzethonium chloride, 0.1mM phenylmethylsulphonyl fluoride, 0.002% (w/v) pepstatin and 0.05% (w/v) bacitracin. Also, 0.1mM benzethonium chloride and 0.1mM phenylmethylsulphonyl fluoride were added to buffer B and this was subsequently used for the washing of the beads, in dialysis and the washing of the ion-exchange column. A stock solution of 0.1M phenylmethylsulphonyl

fluoride dissolved in propan-2-ol was kept at 4°C and added to the buffers immediately prior to use. The purification procedure was then completed as before employing the column elution of the affinity beads at ambient temperature.

Table 14

Sample	Acetylcholine receptor pmol	Protein mg	Specific activity μmol/ g protein	Yield	Purification factor
Detergent solubilised acetylcholine receptor	536	3045	0.00018	100%	1
Purified receptor	20	0.24	0.083	15%	461

Table 14 shows the results of an experiment obtained by this method which were very similar to the results obtained in the previous section.

### (iii) Processing time

The addition of anti-protease agents did not alter the results significantly. However the purification time was a period of 5 days and if limited proteolysis could still occur, the prolonged procedure might facilitate such effects. Therefore the process time was reduced to 27h.

The preparation of solubilised acetylcholine receptor was applied to the affinity beads and stirred for 4h at 23°C. The beads were packed in a column (10cm x 2cm), washed as before, and the carbamoylcholine elution was again performed as before, at room temperature

but now took place overnight for 9h. The dialysis stage was reduced to 3h at 4°C and ion-exchange chromatography was as before. This procedure greatly improved the values obtained for the specific activity of the isolated receptor as may be seen from the results of one such experiment in Table 15. In this experiment, the specific activity of the purified receptor protein was 3.5  $\mu\text{mol}$   $^{125}\text{I}$ - $\alpha$  bungarotoxin binding sites per g protein and this represented a thirty fold increase in specific activity with respect to previous methods of purification. However the yield of purified material was the same as was obtained previously.

Table 15

Sample	Acetylcholine receptor pmol	Protein mg	Specific activity $\mu\text{mol}/$ g protein	Yield	Purification factor
Detergent solubilised acetylcholine receptor	196	1724	0.00011	100%	1
Purified receptor	24	0.008	3.5	18%	27272

(iv) The final procedure

The final purification procedure adopted is depicted in FIG. 23, page 125. All the buffers contained the anti-protease agents listed on page 119. The short purification time did not permit optimisation of all stages of the purification. For example, the adsorption of acetylcholine receptor activity by the affinity beads never exceeded 65% (cf. 50-90% overnight at 4°C) but it was successful

in achieving a purified protein with specific activities comparable to those in the literature for receptors purified from other mammalian muscles.

The concentration of acetylcholine receptor in human muscle was found to vary between 0 and 2pmol  $^{125}\text{I}$ - $\alpha$  bungarotoxin binding sites per g muscle (mean value = 0.7pmol/g, n = 30). Loss of receptor activity was not apparent after one month storage of the muscle at  $-80^{\circ}\text{C}$ . The specific activity of the purified acetylcholine receptor was in the range 0.5-3.5 $\mu\text{mol/g}$  protein(6). Acetylcholinesterase had an activity of 17 $\mu\text{mol}$  acetylthiocholine hydrolysed per min per ml in the detergent extract but no activity was observed in the purified protein solution.

Sometimes, the purification was stopped at the solubilised acetylcholine receptor stage and the extract frozen at  $-80^{\circ}\text{C}$  until further purification. No loss of  $^{125}\text{I}$ - $\alpha$  bungarotoxin binding activity or reduction in specific activity of the purified protein occurred when this procedure was adopted.

### 3. An investigation into the low recovery of purified protein

The yield of purified receptor protein never exceeded 16% of the initial detergent-solubilised acetylcholine receptor. Therefore in experiments to investigate the loss of activity, a detergent-solubilised acetylcholine preparation was made in the presence of anti-protease agents and a sample of the extract was frozen at  $-80^{\circ}\text{C}$  immediately. The Triton extract was stored at  $4^{\circ}\text{C}$  and samples were later frozen at 1, 5, 7 day intervals at  $-80^{\circ}\text{C}$ . The samples (100 $\mu\text{l}$ ) were then examined by sodium dodecyl sulphate polyacrylamide gel

123.

electrophoresis under denaturing conditions (page 102). The results are shown in the photograph on page 126. The gels of all four samples are very similar and there is little evidence for increased amounts of low molecular weight species with storage at 4°C.

<sup>125</sup>I- $\alpha$  bungarotoxin binding activity was found to decrease by only 7% after seven days storage at 4°C.

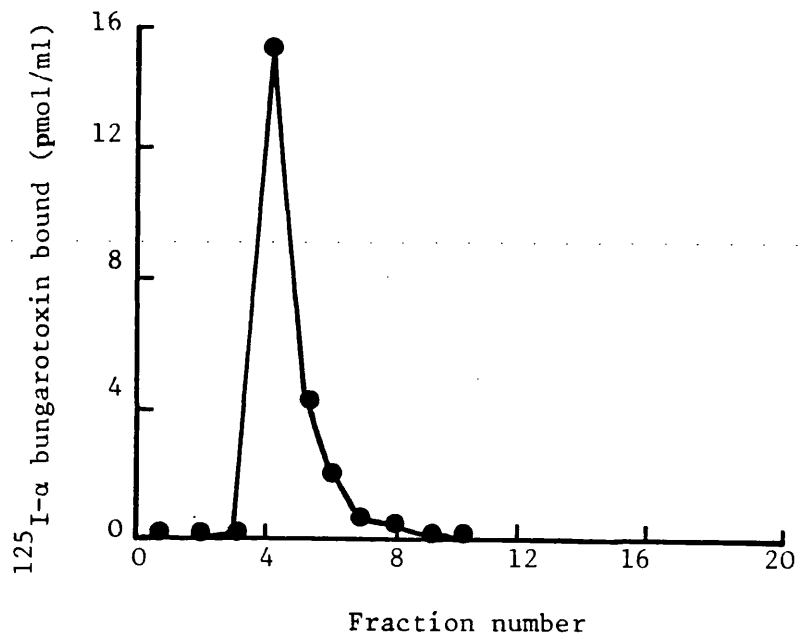


FIG. 22. Ion exchange chromatography on DEAE cellulose of the acetylcholine receptor



FIG. 23. The finally adopted procedure for the purification of the human acetylcholine receptor

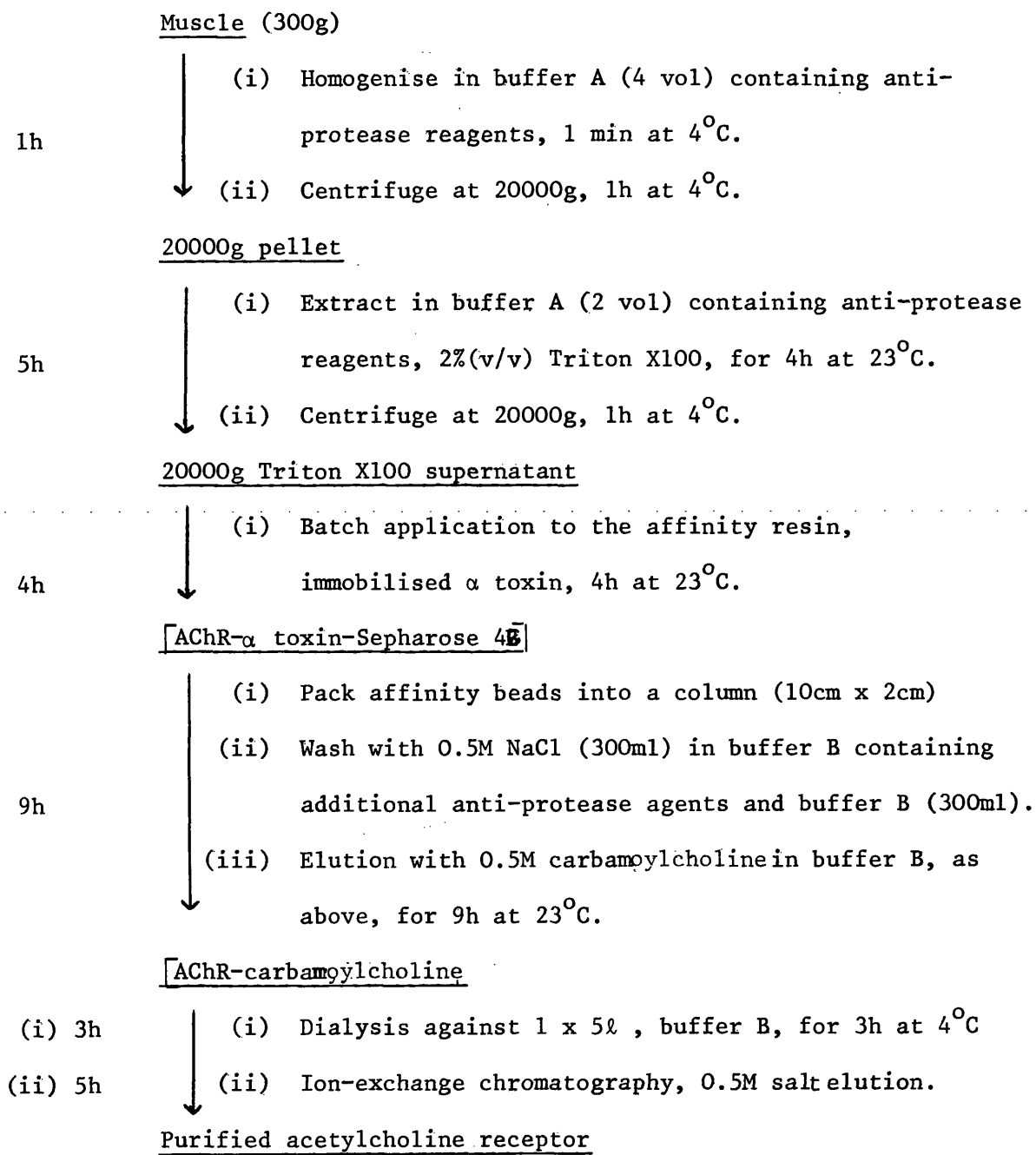




FIG. 24. SDS-PAGE of a detergent extract containing solubilised acetylcholine receptor, after storage at 4°C

The gels are of detergent solubilised acetylcholine receptor after storage of 0, 1, 5, 7 days at 4°C (from left to right).

4. An alternative purification procedure?

The myasthenic serum is a very specific probe for the acetylcholine receptor. There was an abundant supply of myasthenic serum in our laboratory and therefore it was thought that it may be possible to exploit the two factors and to produce an antibody affinity column to purify the acetylcholine receptor. The results reported below are those of a preliminary investigation into the use of an anti-(acetylcholine receptor) antibody affinity column.

(i) Purification of IgG from myasthenic serum and the preparation of the affinity column

IgG was purified from myasthenic serum (50ml) as described earlier (page 64). The anti-(acetylcholine receptor) antibody concentration was measured before and after the ammonium sulphate precipitation and it was found that 99% of the specific antibody was precipitated.

	<u>Anti-(acetylcholine receptor)antibody titre x 10<sup>10</sup><sub>M</sub></u>
Initial serum sample	390
Supernatant after (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> precipitation	2.4

The total yield of purified IgG was 225mg as determined by the optical density at 280nm.

IgG (25mg) was covalently coupled to Sepharose 4B (page 101) and the measurement of the optical density at 280nm before and after the coupling procedure showed that 77% of the IgG was immobilised. The affinity column was then prepared for use (page 101).

(ii) An initial purification attempt

A detergent extract containing solubilised acetylcholine receptor was prepared (page 66) and its activity was measured by the ammonium sulphate precipitation assay (page 63). The extract (350ml) was stirred with the antibody-affinity resin (50ml packed beads) overnight at 4°C and the supernatant was collected by centrifugation and assayed for acetylcholine receptor activity. A control sample of detergent extract was stirred overnight at 4°C in the absence of affinity beads and assayed as above.

Table 16

Sample	Acetylcholine receptor activity pmol/ml
Original detergent extract	1.26
Detergent extract after stirring overnight at 4°C	1.26
Supernatant from the affinity beads	0.96

The results in Table 16 showed that there was a 24% decrease in activity of the supernatant after affinity adsorption and a total of 105pmol bound to the beads. The Sepharose 4B was packed in a column and washed extensively with 0.01M potassium phosphate buffer, pH 7.4, containing 1mM EDTA, 0.1mM phenylmethylsulphonyl fluoride, 0.1mM benzenonium chloride, 0.02% (w/v)  $\text{NaN}_3$  and 0.1% (v/v) Triton X100. The column was then eluted with a solution of 0.5M NaCl in the same buffer and the optical density at 280nm was recorded. Two peaks were eluted from the affinity column neither of which contained acetylcholine receptor activity.

##### 5. Radiolabelling of the purified acetylcholine receptor with $^{125}\text{I}$

Initial experiments following the iodination procedure described in Methods (page 60) were done to radiolabel bovine serum albumin at a concentration comparable to that of purified acetylcholine receptor (2 $\mu\text{g}/\text{ml}$ ). Polyacrylamide gel electrophoresis under denaturing conditions of both  $^{125}\text{I}$ -bovine serum albumin and bovine serum albumin gave identical results when the distribution of radioactivity and the stained gel were compared (FIG.25 page 130).

Purified human acetylcholine receptor was labelled by the same procedure to a specific activity of  $2145 \pm 664 \text{ Ci}/\mu\text{mol}$  (10) with  $21 \pm 3.62\%$  (10) incorporation of total  $^{125}\text{I}$  added. A sample containing 0.05M potassium phosphate buffer, pH 7.4, 0.1% (v/v) Triton X100 was iodinated as a control and the incorporation of total  $^{125}\text{I}$  added into the peak corresponding to the labelled Triton X100 was 3% (mean of 3 values).

Using the same procedure, purified acetylcholine receptor from *Torpedo marmorata* (38fmol) was iodinated to a specific activity of 2909  $\text{Ci}/\mu\text{mol}$  and purified acetylcholine receptor from rabbit muscle (20fmol) was iodinated to a specific activity of 1500  $\text{Ci}/\mu\text{mol}$ .

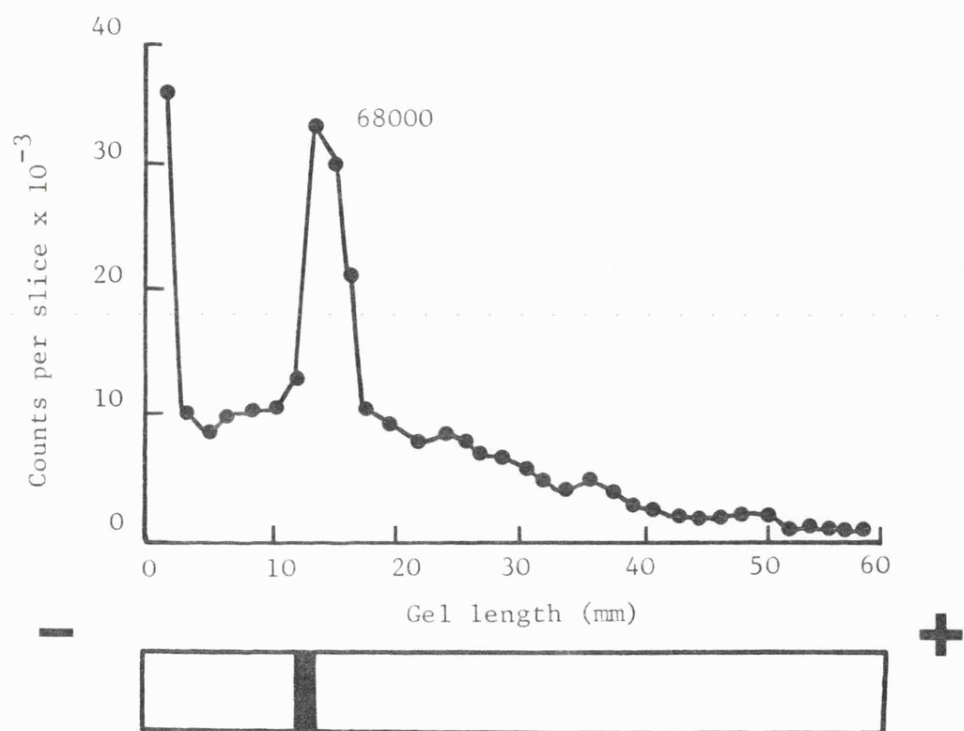


FIG. 25. SDS-PAGE of <sup>125</sup>I-bovine serum albumin

## 6. Isoelectric focussing of the acetylcholine receptor

Initially the purified human  $^{125}\text{I}$ -acetylcholine receptor was focussed according to the procedure described in the Methods section (page 101) overnight at 350V and at 23°C. However, it appeared that under these conditions, the pH gradient of the polyacrylamide gel was not maintained. In a control experiment, samples were focussed for 1.5h, 2.5h, 3.5h and on termination of the procedure, the samples were analysed for both pH gradient and the distribution of radioactivity. FIG.26 shows that the pH gradient decreases from a range of pH 7.9-2.5 for 1.5h to pH 6.2-2.9 for 3.5h. Simultaneously the peak of radioactivity was seen to regress to the origin with increased focussing time. Focussing for 1.5h gave the most satisfactory result and was used in all subsequent experiments.

The first measurements of the isoelectric point of the acetylcholine receptor showed that the protein focussed as a sharp band at pH 5.6. However, it was noticed that if the receptor was radio-labelled and focussed immediately after purification, then the  $^{125}\text{I}$ -acetylcholine receptor focussed as a sharp band at pH 6.6. These results were investigated in a series of experiments in which acetylcholine receptor was purified and half the sample was immediately radio-iodinated. Samples of the labelled receptor preparations were examined by isoelectric focussing immediately. Both preparations were then stored at 4°C and electrofocussed (immediately after iodination in the case of the unlabelled receptor) after one and three weeks.

The results are shown in the sequence of FIGS.27 to 29. Radio-labelling and isoelectric focussing of iodinated acetylcholine receptor immediately following purification gave a very sharp focussing pattern

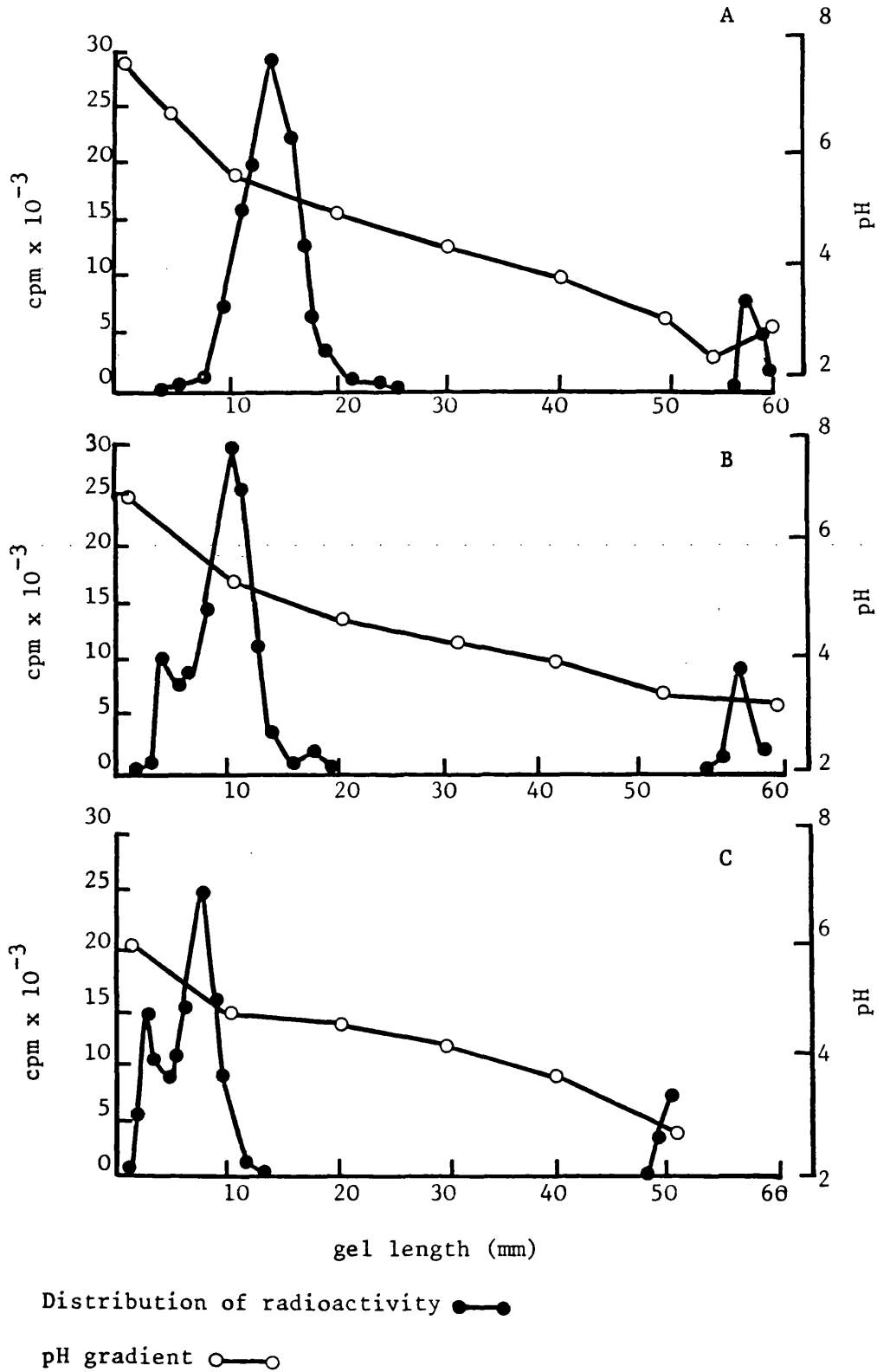
with a peak of radioactivity at pH 6.6 (FIG.27 ). After storage of the  $^{125}\text{I}$ -acetylcholine receptor for one week at  $4^{\circ}\text{C}$ , a second band at pH 5.3 was detected and after three weeks storage at  $4^{\circ}\text{C}$ , the same two peaks of radioactivity were present in equal proportions.

Acetylcholine receptor that was stored at  $4^{\circ}\text{C}$  for one week before radio-iodination, gave a similar distribution of radioactivity (FIG.28 ) to that shown by stored  $^{125}\text{I}$ -acetylcholine receptor (FIG.28 ). However, storage of unlabelled purified receptor at  $4^{\circ}\text{C}$  for three weeks led to only a single major electrofocussed peak at pH 5.6 together with a lower radioactivity at higher pI values (FIG.29). It was thus concluded that in this system, the measurement of the isoelectric point must be made as quickly as possible following the preparation of the purified receptor protein. This will be discussed more fully later (page 202).

Purified receptor labelled with  $^{125}\text{I}$ - $\alpha$  bungarotoxin focussed as a sharp band at pH 5.1. This pattern is shown together with the peak of freshly purified and radio-iodinated receptor in FIG.31. Control experiments showed that the  $^{125}\text{I}$ - $\alpha$  bungarotoxin pI 9.0 electrophoresed into the cathode chamber and  $^{125}\text{I}$ -Triton X100 focussed as a band at the acid extremity of the gel (FIG.30). Purified  $^{125}\text{I}$ -acetylcholine receptor from *Torpedo marmorata* focussed as a sharp band at pH 4.9 and the corresponding  $^{125}\text{I}$ - $\alpha$  bungarotoxin labelled receptor as a sharp band at pH 5.65 (FIG.32). Similarly, purified  $^{125}\text{I}$ -acetylcholine receptor from rabbit skeletal muscle gave a broad band at pH 5.7 and the corresponding  $^{125}\text{I}$ - $\alpha$  bungarotoxin labelled receptor gave a broad band at pH 5.6 (FIG.33).



FIG. 26. The time course for isoelectric focussing of  $^{125}\text{I}$ -acetylcholine receptor



A, B, C are focussing times of 1.5h, 2.5h, 3.5h respectively

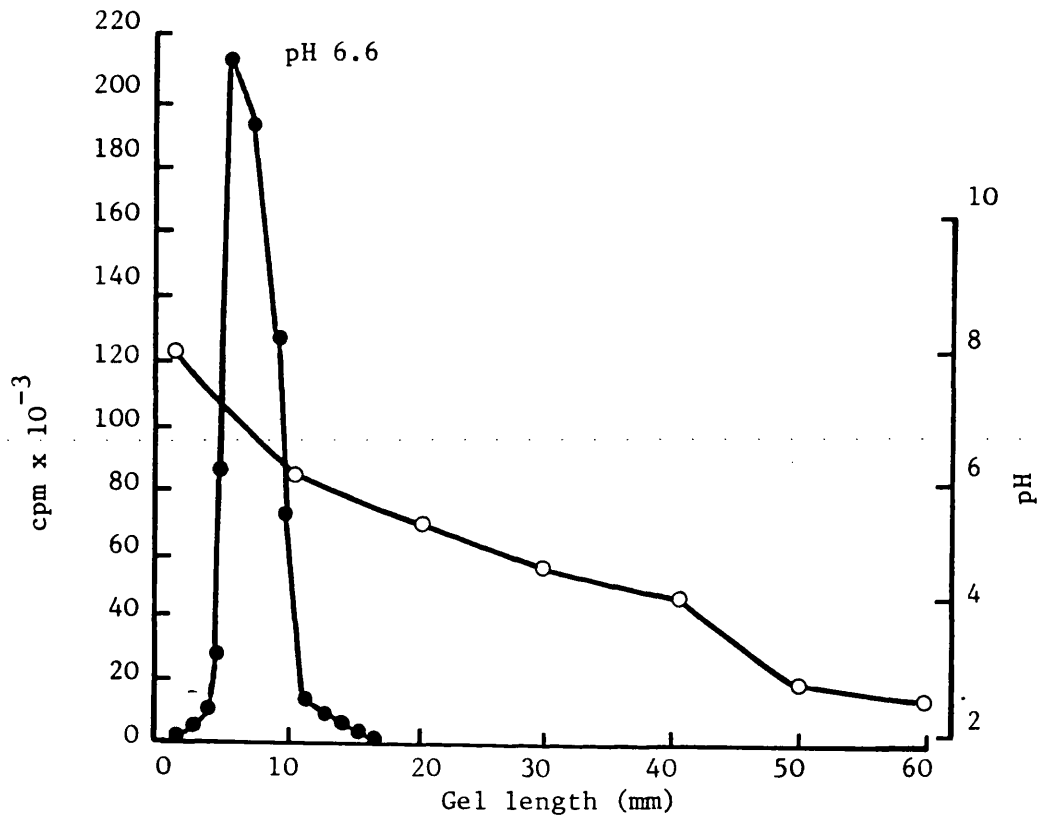
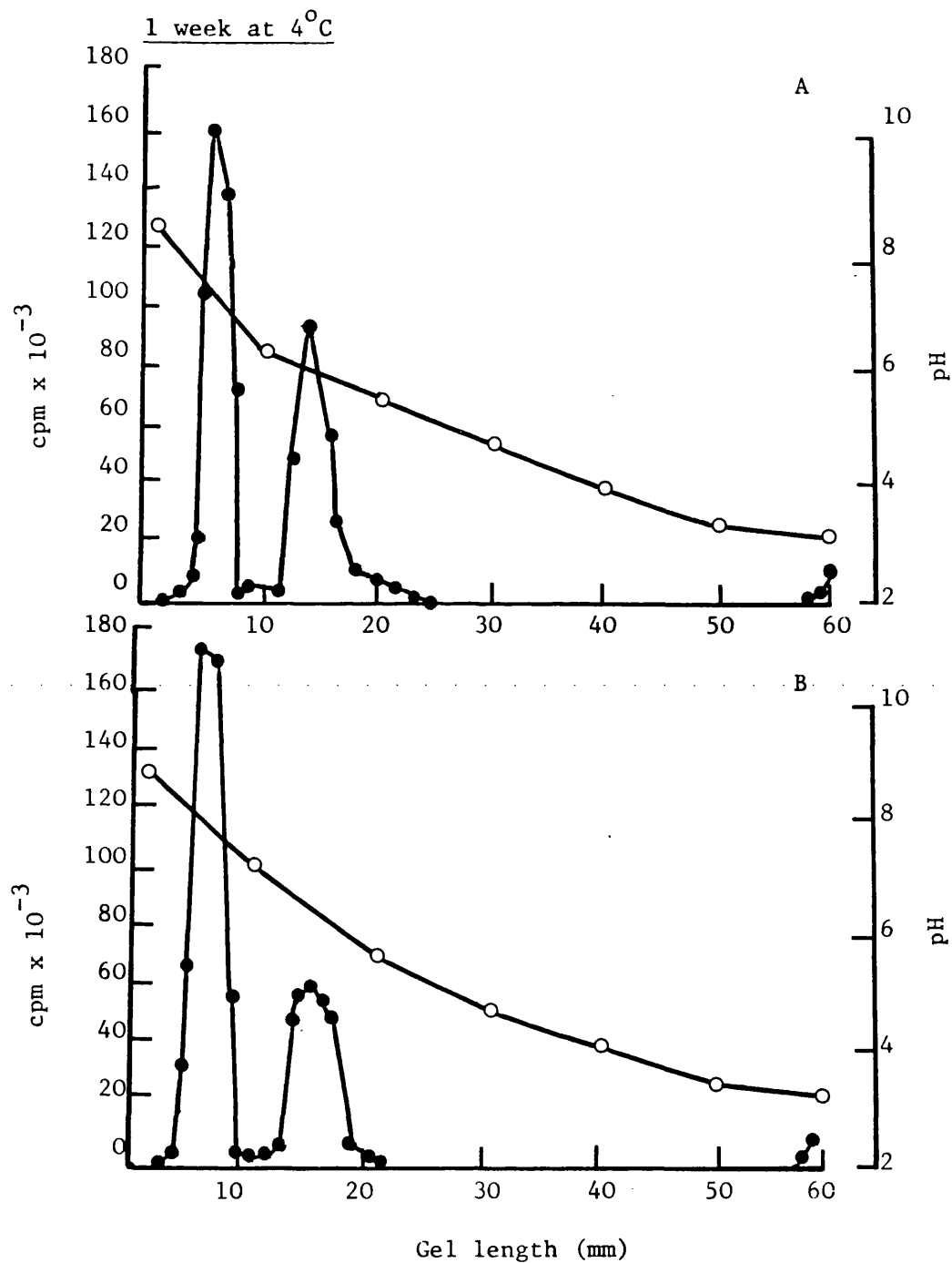


FIG. 27. The distribution of radioactivity in an isoelectric focussing gel of <sup>125</sup>I-acetylcholine receptor that had been radiolabelled immediately following purification

- Distribution of radioactivity
- pH gradient

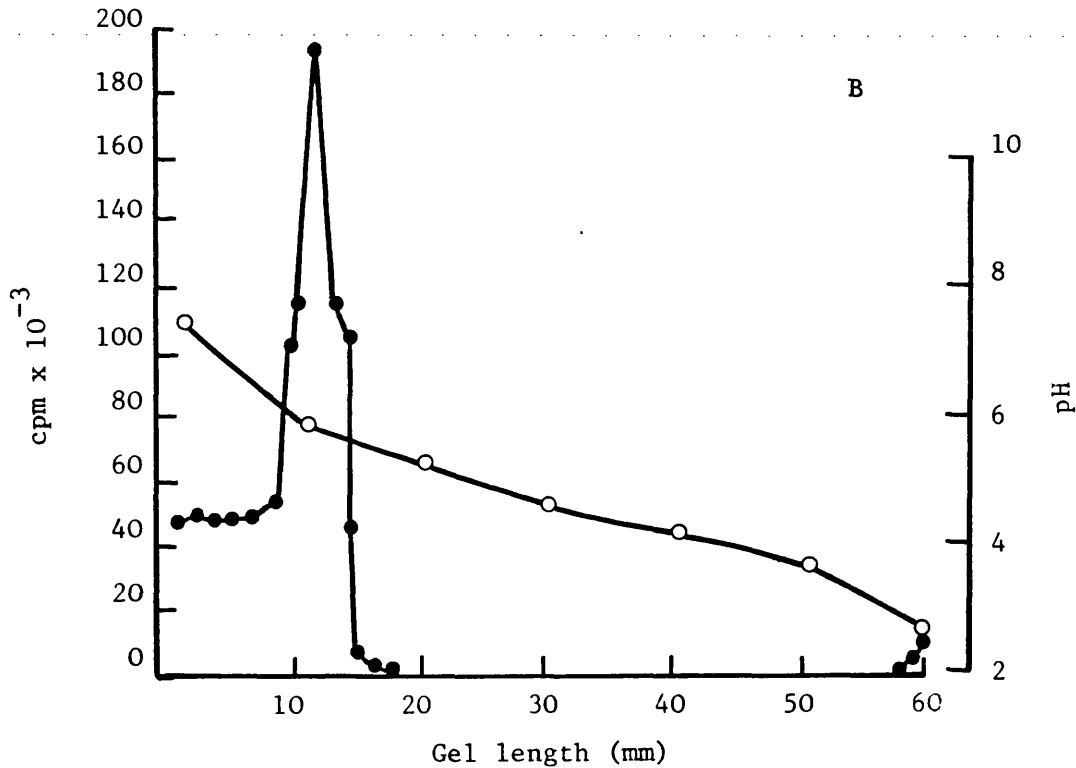
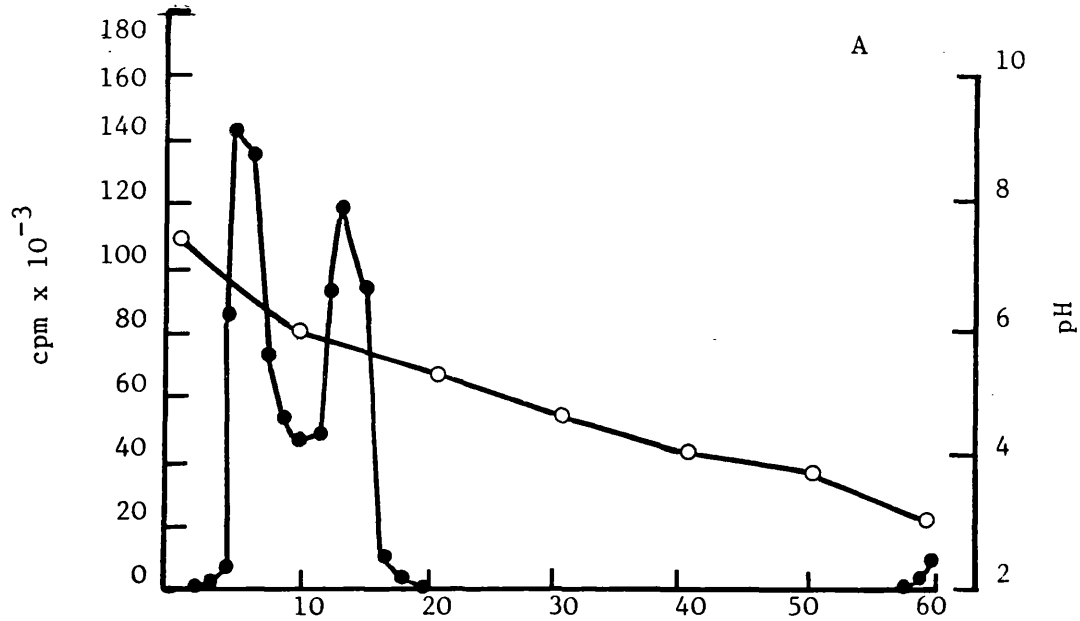
FIG. 28. The distribution of radioactivity in isoelectric focus gels of  $^{125}\text{I}$ -acetylcholine receptor after storage for



A Isoelectric focus gel of  $^{125}\text{I}$ -acetylcholine receptor after 1 week storage at  $4^{\circ}\text{C}$

B Isoelectric focus gel of  $^{125}\text{I}$ -acetylcholine receptor after storage of unlabelled acetylcholine receptor at  $4^{\circ}\text{C}$  for 1 week

The distribution of radioactivity in isoelectric focus gels of  $^{125}\text{I}$ -acetylcholine receptor after storage for 3 weeks at  $4^{\circ}\text{C}$



● Radioactivity

○ pH gradient

A Isoelectric focus gel of  $^{125}\text{I}$ -acetylcholine receptor after 3 weeks at  $4^{\circ}\text{C}$

B Isoelectric focus gel of  $^{125}\text{I}$ -acetylcholine receptor after storage of unlabelled acetylcholine receptor at  $4^{\circ}\text{C}$  for 3 weeks

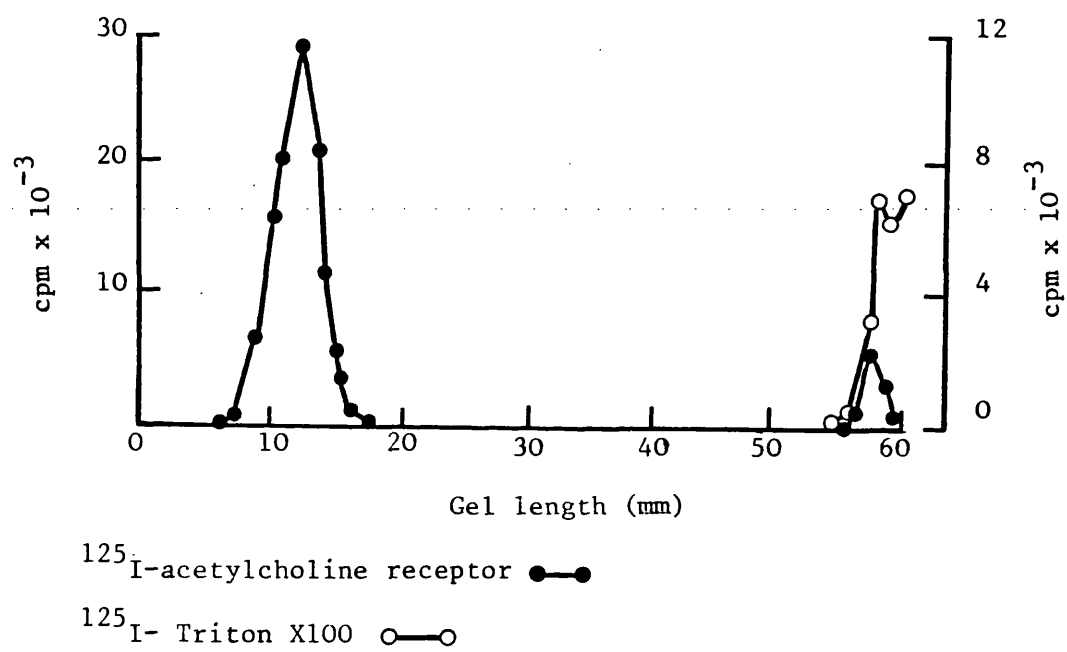


FIG. 30. The distribution of radioactivity in isoelectric focus gels of <sup>125</sup>I-acetylcholine receptor and <sup>125</sup>I-Triton X100

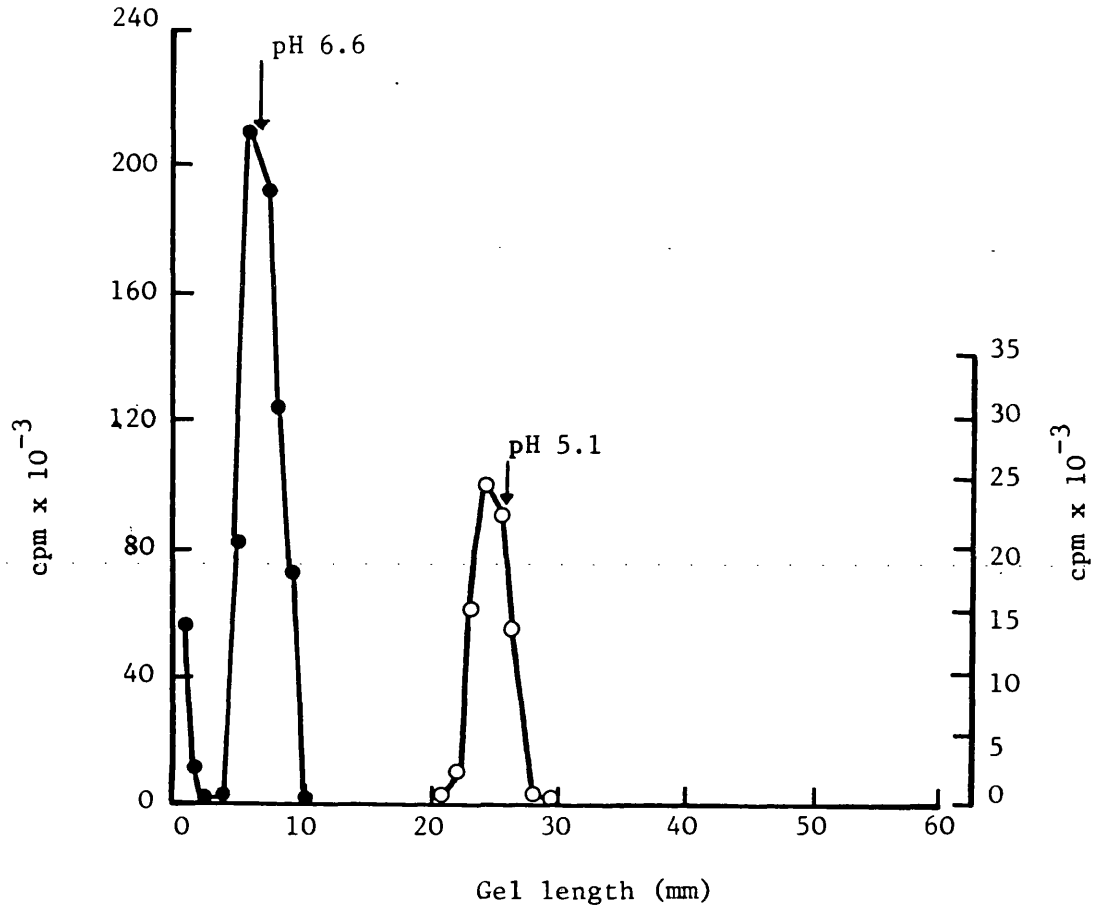


FIG. 31. Isoelectric focus gels of  $^{125}\text{I}$ -acetylcholine receptor and  $^{125}\text{I}$ - $\alpha$  bungarotoxin-acetylcholine receptor, purified from human muscle

$^{125}\text{I}$ -acetylcholine receptor (cpm on left hand scale) ●—●  
 $^{125}\text{I}$ - $\alpha$  bungarotoxin-acetylcholine receptor ○—○  
 (cpm on right hand scale)

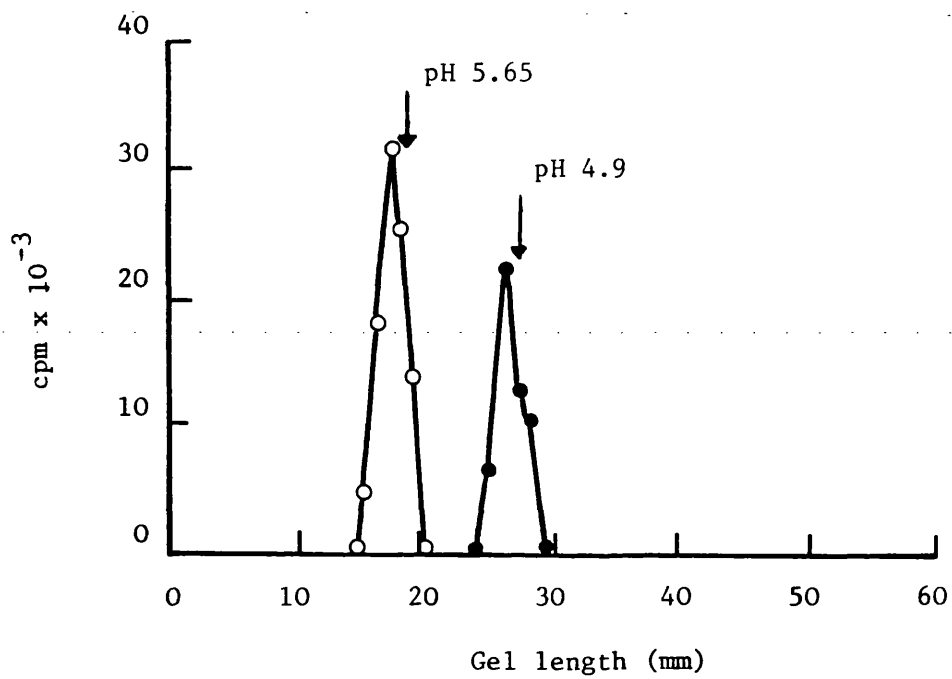


FIG. 32. Isoelectric focus gels of  $^{125}\text{I}$ -acetylcholine receptor and  $^{125}\text{I}$ - $\alpha$  bungarotoxin-acetylcholine receptor, purified from *Torpedo marmorata*

$^{125}\text{I}$ -acetylcholine receptor ●—●

[ $^{125}\text{I}$ - $\alpha$  bungarotoxin-acetylcholine receptor] ○—○

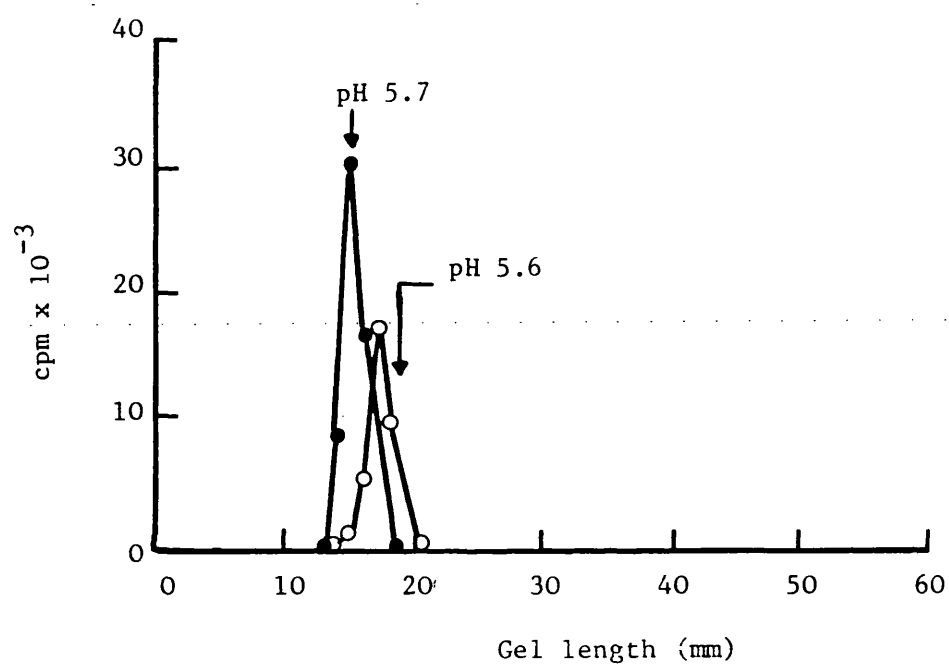


FIG. 33. Isoelectric focus gel of  $^{125}\text{I}$ -acetylcholine receptor and  $^{125}\text{I}$ - $\alpha$  bungarotoxin-acetylcholine receptor purified from rabbit muscle

$^{125}\text{I}$ -acetylcholine receptor ●—●

$^{125}\text{I}$ - $\alpha$  bungarotoxin-acetylcholine receptor ○—○



7. Sedimentation of purified acetylcholine receptor-<sup>125</sup>I-α bungarotoxin in a sucrose density gradient

Sedimentation in a sucrose density gradient was carried out as described in Methods (page 104). A plot of the sedimentation coefficient against the fraction number with peak enzyme activity for each standard marker enzyme, gave a straight line (linear correlation coefficient = 0.96, FIG. 35 ).

<sup>125</sup>I-α Bungarotoxin-(purified human acetylcholine receptor) complex sedimented as a single radioactive component with  $s_{20,w} = 9.5s$  corresponding to a molecular weight of 175-200000, using the approximation,

$$\frac{(s_{20,w})_1}{(s_{20,w})_2} = \left[ \frac{\text{Molecular weight}_1}{\text{Molecular weight}_2} \right]^{2/3}$$

Schachman (1959)

The corresponding complex from purified *Torpedo marmorata* acetylcholine receptor sedimented as a single peak  $s_{20,w} = 9.0 s$  (molecular weight 175-200000). Free <sup>125</sup>I-α bungarotoxin remained at the top of the gradient.

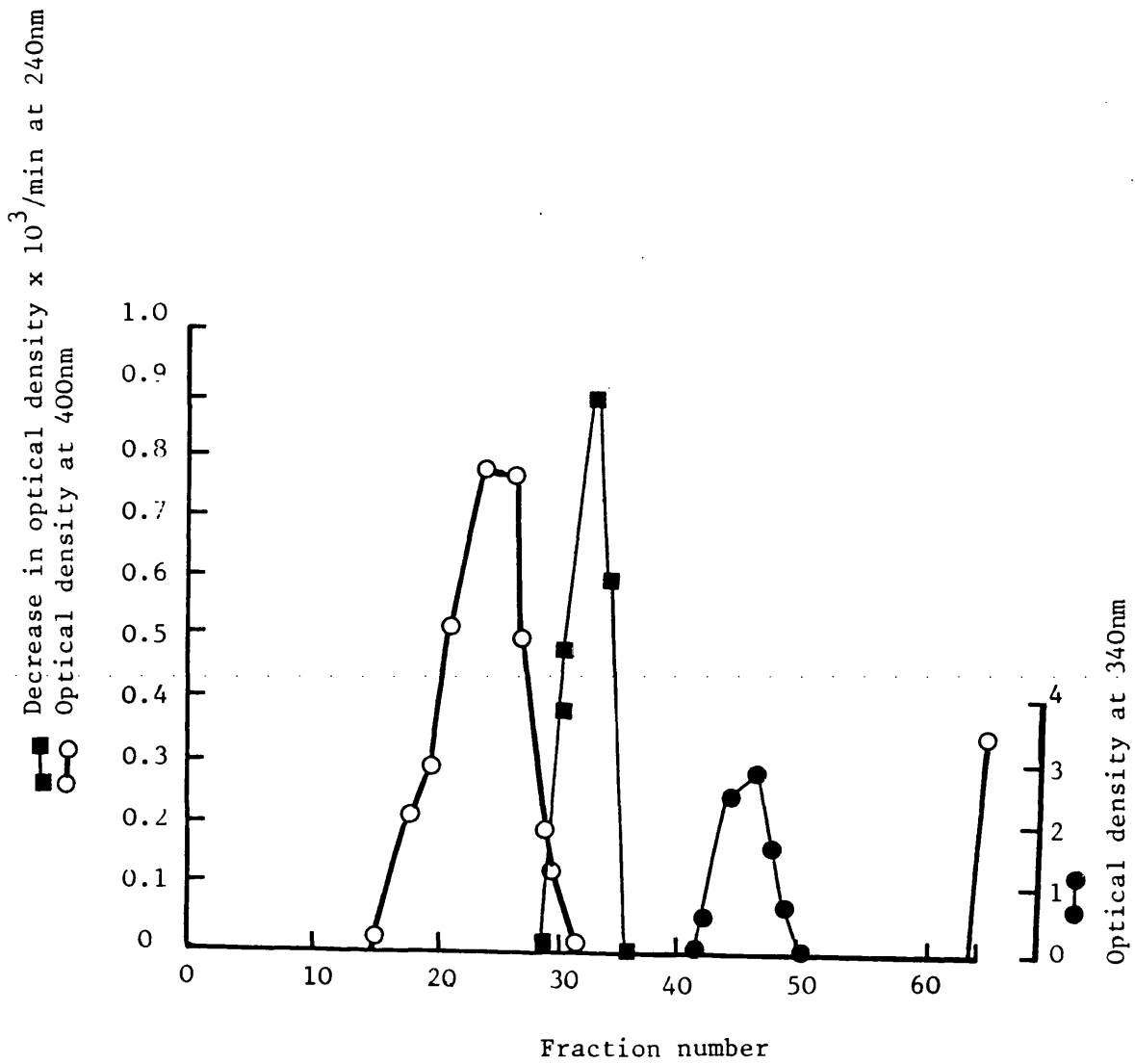


FIG. 34. The activities of the enzyme markers after sedimentation in a sucrose density gradient

β galactosidase ( $S_{20,w} = 16$ ) ○—○

catalase ( $S_{20,w} = 11.4$ ) ■—■

yeast alcohol dehydrogenase ( $S_{20,w} = 7.4$ ) ●—●

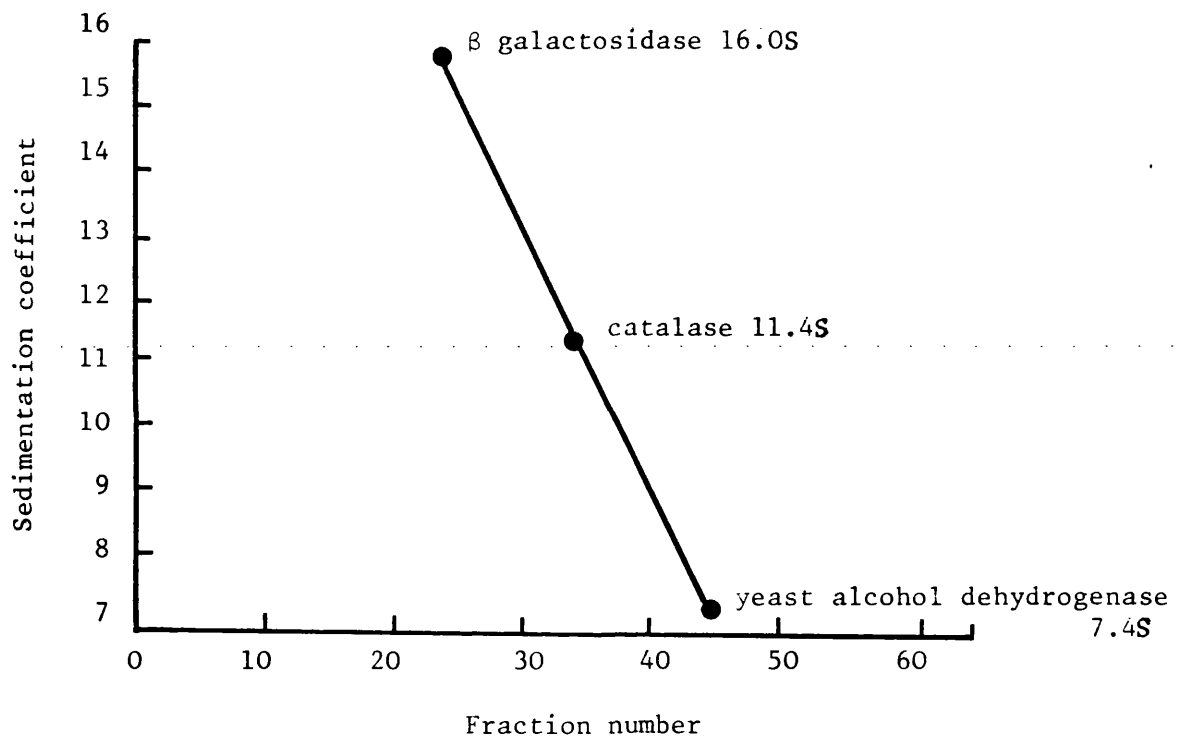


FIG. 35. The standard line for the marker enzymes after sedimentation in a sucrose density gradient

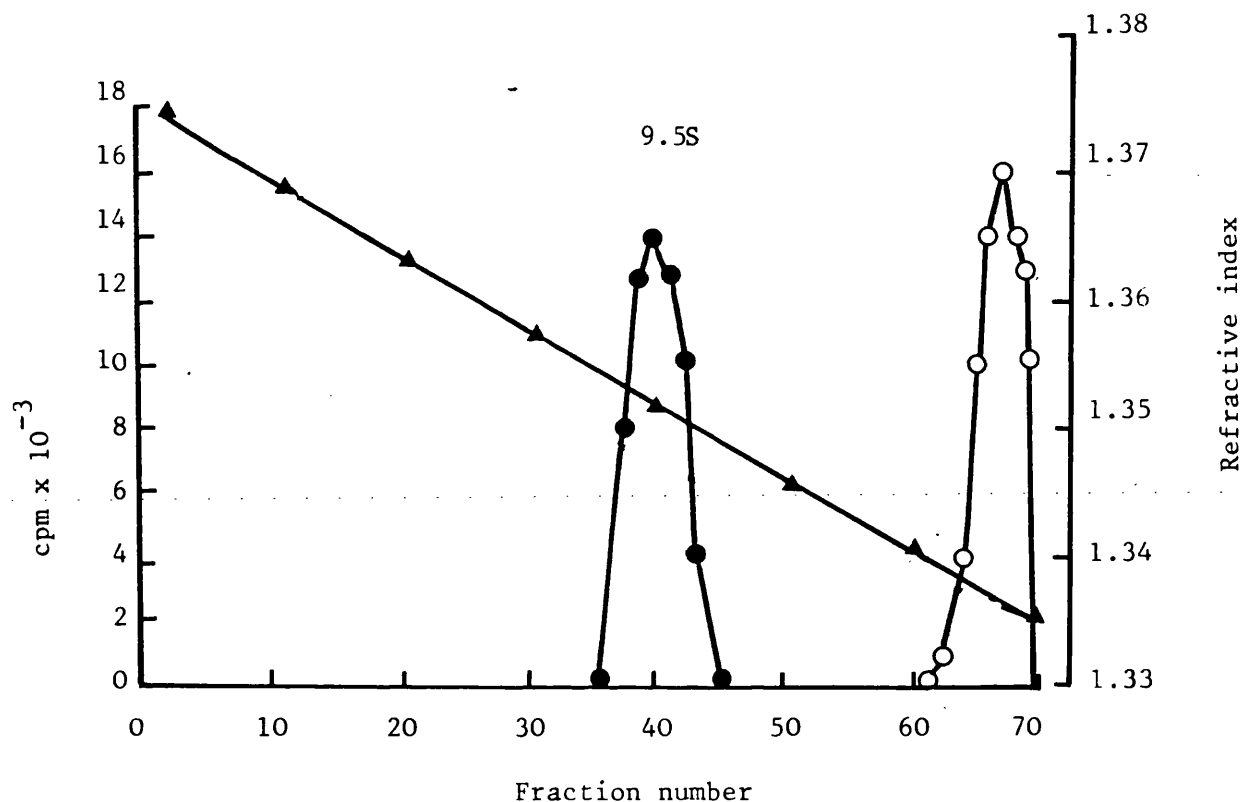


FIG. 36. The distribution of radioactivity after sedimentation in a sucrose density gradient of [<sup>125</sup>I-α bungarotoxin and of [<sup>125</sup>I-α bungarotoxin acetylcholine receptor] purified from human muscle

- [<sup>125</sup>I-α bungarotoxin-acetylcholine receptor]
- <sup>125</sup>I-α bungarotoxin
- ▲—▲ refractive index

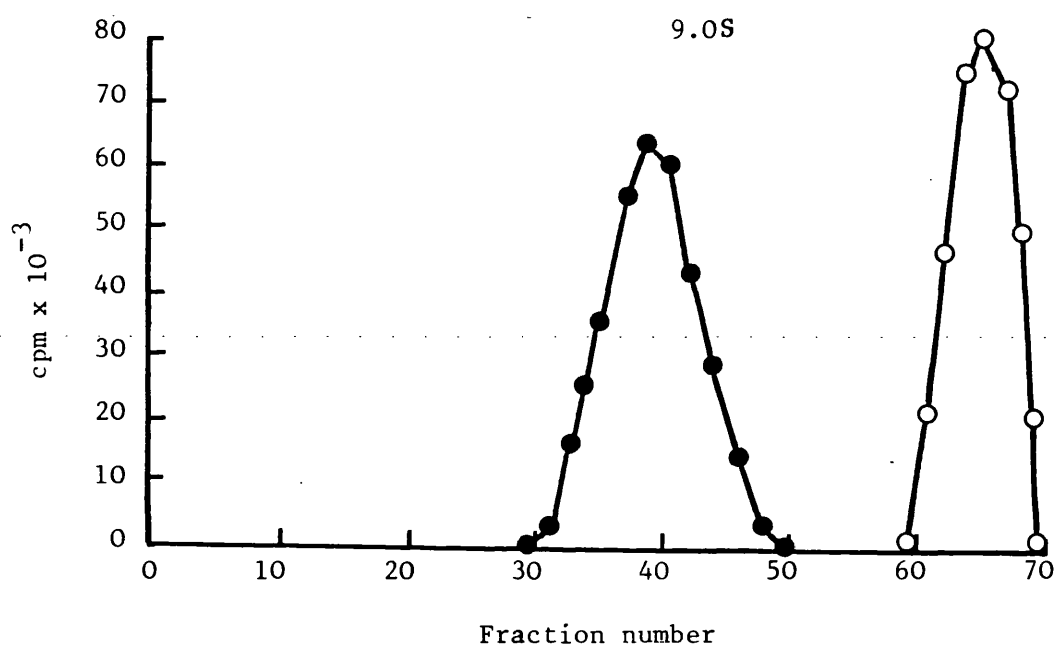


FIG. 37. The distribution of radioactivity after sedimentation in a sucrose density gradient of [<sup>125</sup>I-α bungarotoxin-acetylcholine receptor] purified from *Torpedo marmorata*

●● [<sup>125</sup>I-α bungarotoxin-acetylcholine receptor]

○○ <sup>125</sup>I-α bungarotoxin

8. Polyacrylamide gel electrophoresis of purified acetylcholine receptor-

<sup>125</sup>I α bungarotoxin complexes

Polyacrylamide gel electrophoresis of purified human acetylcholine receptor-<sup>125</sup>I-α bungarotoxin complex was carried out as in Methods (page 103). Staining of the gels with Coomassie G250 was unsuccessful because of the presence of 1% (w/v) bovine serum albumin in the <sup>125</sup>I-α bungarotoxin buffer. However, the distribution of radioactivity gave a broad peak at 0.18 with respect to the tracking dye. Similar analysis of purified acetylcholine receptor -<sup>125</sup>I α bungarotoxin complex from *Torpedo marmorata* gave a single band at 0.22. Gels containing <sup>125</sup>I-α bungarotoxin only showed no radioactivity in the gel. However radioactivity was detected in the cathode buffer (FIG.38 ).

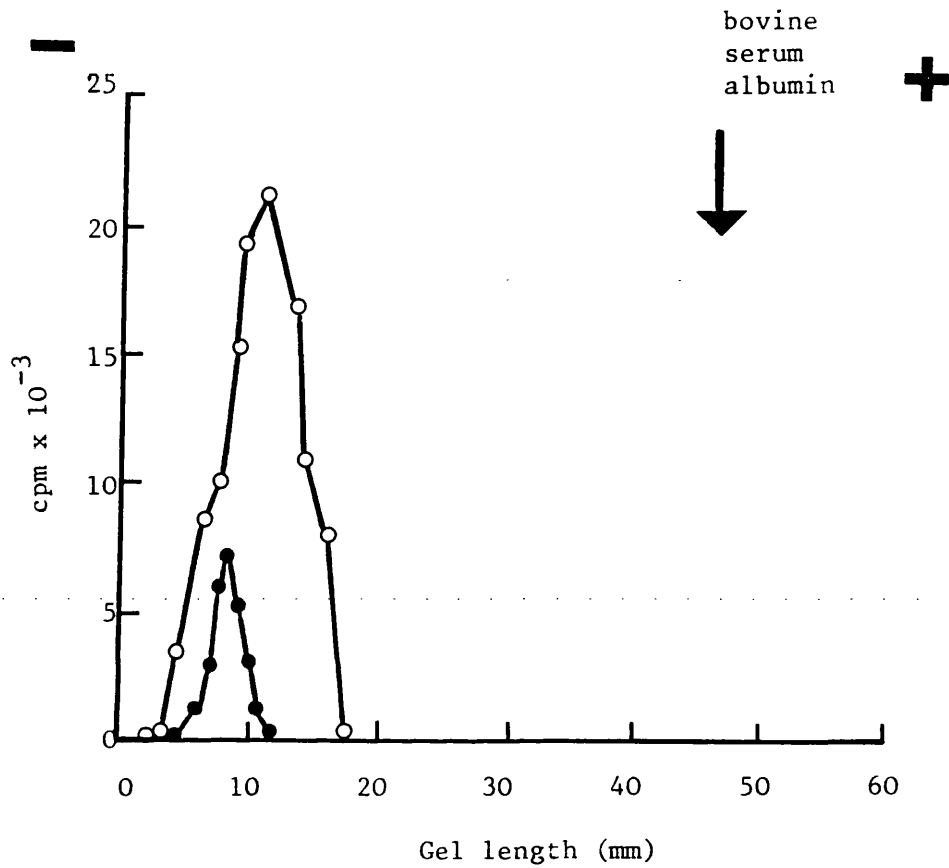


FIG. 38. Polyacrylamide gel electrophoresis under non-denaturing conditions of the receptor-toxin complex

[<sup>125</sup>I- $\alpha$  bungarotoxin-acetylcholine receptor] purified from  
*Torpedo marmorata* ○—○

[<sup>125</sup>I- $\alpha$  bungarotoxin-acetylcholine receptor] purified from  
human muscle ●—●

9. Sodium dodecyl sulphate polyacrylamide gel electrophoresis of purified acetylcholine receptor

The molecular weights of the receptor subunits were calculated from the standard calibration line (FIG. 39). The relative mobility was calculated from the formula:

Relative mobility =

$$\frac{\text{Distance of protein migration}}{\text{Length of gel after destaining}} \times \frac{\text{Length of gel before staining}}{\text{Distance of bromophenol blue migration}}$$

An analysis of the two extreme lines through the points of the standard calibration graph showed that the error in the determination of the molecular weight was 5% of the mean. Therefore, all values quoted are plus and minus 5%.

In view of the very small amounts of purified acetylcholine receptor initially available, early examination of acetylcholine receptor by polyacrylamide gel electrophoresis under denaturing conditions used  $^{125}\text{I}$ -acetylcholine receptor but no reproducible pattern for the distribution of radioactivity could be obtained. However, better results were achieved when the  $^{125}\text{I}$ -acetylcholine receptor was electrofocussed prior to SDS-PAGE. The gel slice corresponding to the peak of radioactivity (FIG. 27) was extracted with 0.01M potassium phosphate buffer, pH 7.4, containing 0.1% (w/v) sodium dodecyl sulphate and 0.1% (v/v) Triton X100 (Methods page 102) leading to 45% solubilisation of the total radioactivity and the extract was subjected to SDS-PAGE as described in Methods (page 102). The molecular weights of the two bands (FIG. 40) observed were  $66000 \pm 3300$ ;  $42000 \pm 2100$ . The relative



intensity, measured by the total radioactivity in each peak of the two bands was not always constant. But, the same distribution of radioactivity was observed when either the low specific activity receptor or the high specific activity receptor were examined in this system. A control experiment was run in which Triton X100 alone was radioiodinated and electrofocussed. The slice corresponding to the position of  $^{125}\text{I}$ -acetylcholine receptor was extracted as described above and subjected to SDS-PAGE where no peaks of radioactivity could be detected.

The subsequent improvement in purification procedures for the acetylcholine receptor led to increased yields which enabled the subunit composition of the protein to be examined by SDS-PAGE using direct staining (Methods page 102) without prior iodination. The densitometric analysis of two different preparations of purified acetylcholine receptor is shown in FIG.41 and also in the photograph on page 154. Usually two major bands with molecular weights  $68000 \pm 3400$ ;  $42000 \pm 2100$  were observed. Sometimes an additional band with molecular weight  $56000 \pm 2800$  was apparent (FIG.41) and occasionally a third band was observed at  $85000 \pm 4250$ .

To detect if any of these bands was a contaminating polypeptide, a control was prepared which consisted of material that was non-specifically bound to the affinity resin. In the purification procedure, the solubilised acetylcholine receptor extract was divided and one half incubated with a ten-fold excess of cold  $\alpha$  bungarotoxin prior to further purification.  $\alpha$  bungarotoxin binding activity was recovered in the purified control (8% of the normal sample). Gel scans of the purified acetylcholine receptor showed the two major bands as normal but a gel scan of the control sample showed scarcely visible

150.

bands of molecular weights  $68000 \pm 3400$  and  $42000 \pm 2100$ .

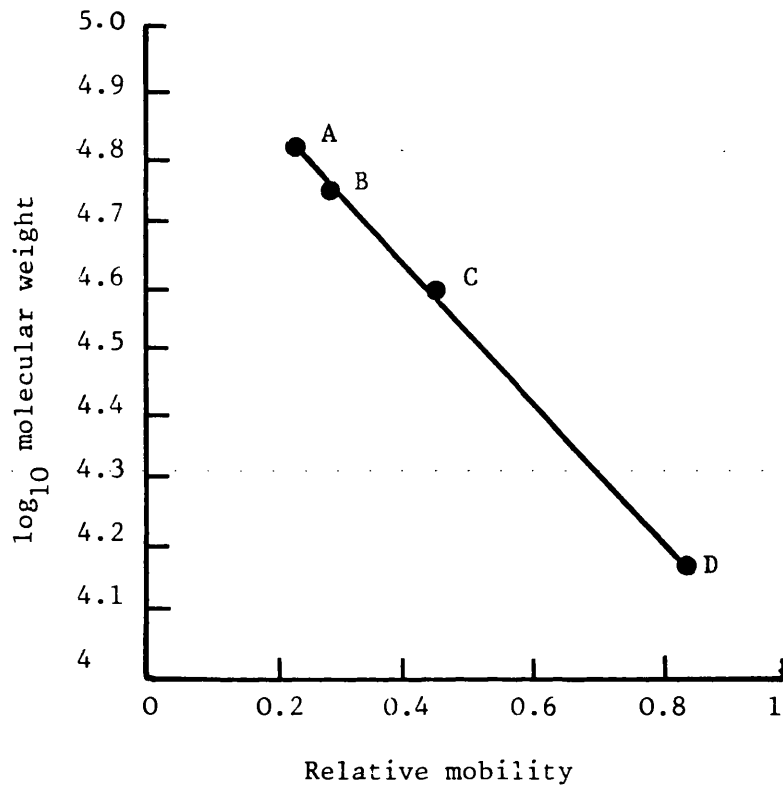


FIG. 39. The standard line for SDS-PAGE under denaturing conditions

- A Bovine serum albumin (68000)
- B Catalase (60000)
- C Liver alcohol dehydrogenase (41000)
- D Haemoglobin (15500)

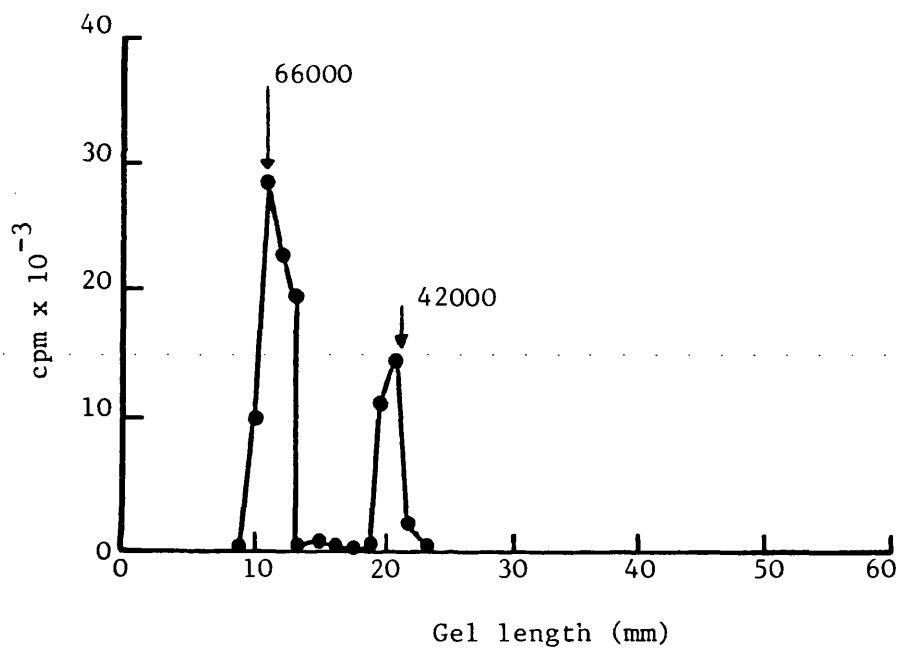


FIG. 40. Distribution of radioactivity in SDS-PAGE of <sup>125</sup>I-acetylcholine receptor eluted from the peak of the isoelectric focus gel shown in FIG. 26

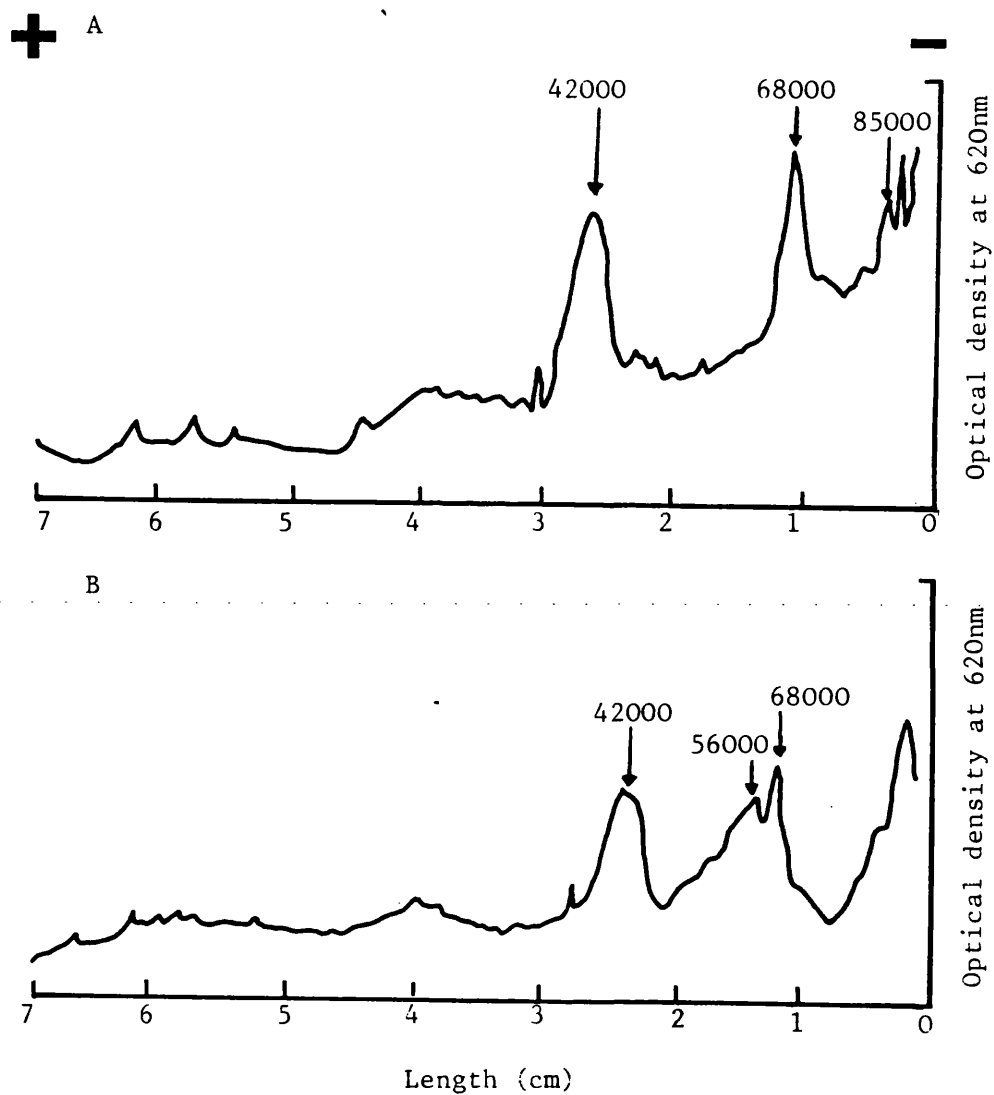


FIG. 41. Densitometric analysis of SDS-PAGE gels of purified acetylcholine receptor from human muscle

A and B are two different preparations of purified acetylcholine receptor from human muscle.

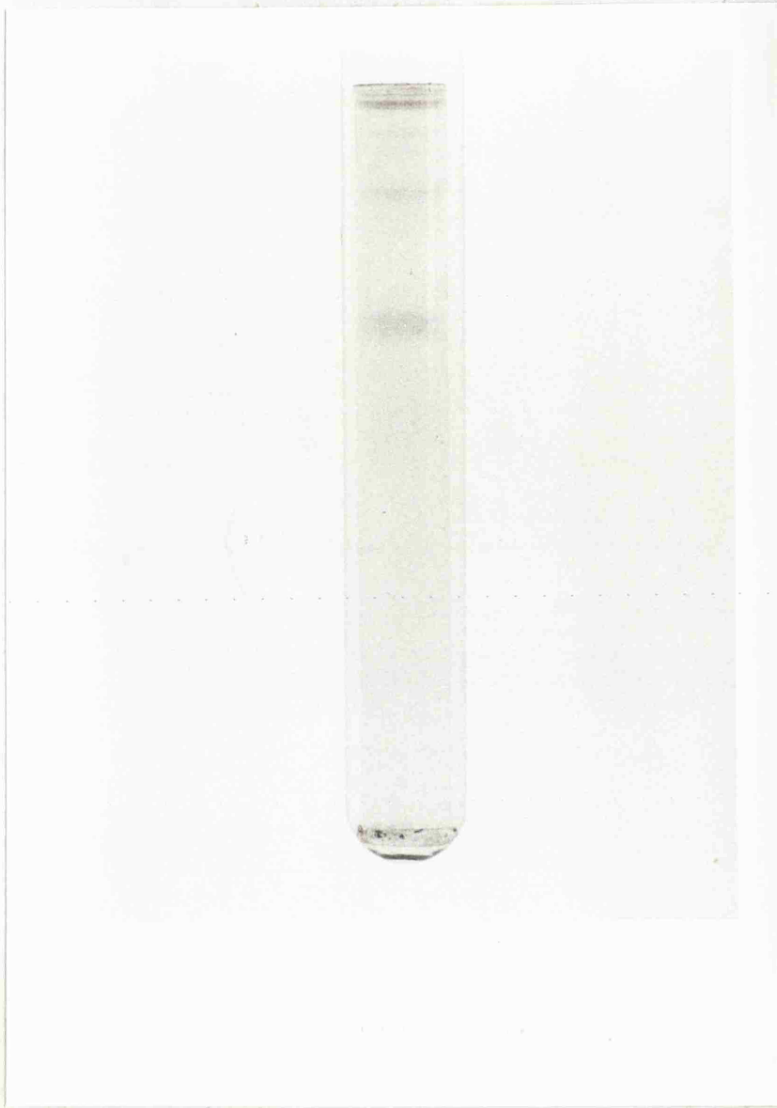


FIG. 42. An SDS-PAGE gel of purified acetylcholine receptor  
from human muscle

#### 10. Affinity labelling of human acetylcholine receptor

Purified membrane fragments from human muscle (Methods page 105) were labelled with [ $^3\text{H}$ ]-MBTA as described in the Methods page 105. An analysis of the distribution of radioactivity in SDS-PAGE gels of the [ $^3\text{H}$ ]-MBTA-labelled membranes (Methods page 106) showed a single peak with molecular weight  $40000 \pm 2000$  (FIG.43 ). In control samples where the membrane fragments were pre-treated with  $\alpha$  bungarotoxin before the affinity alkylation procedure, no peaks of radioactivity were observed in the SDS-PAGE analysis of the membranes.

Affinity labelling of purified human acetylcholine receptor as described in the Methods, page 106 led to stained patterns identical to those observed in SDS-PAGE of purified unlabelled receptor: that is two major bands with molecular weights  $66000 \pm 3300$ ;  $42000 \pm 2100$  (FIG. 41). No consistent distribution of radioactivity was observed over several individual experiments. Affinity labelling of purified *Torpedo marmorata* acetylcholine receptor (in amounts five times those employed above) by the same procedure again led to an SDS-PAGE stained pattern of bands identical with that shown by unlabelled receptor, i.e. in this case four major bands of molecular weights  $40000 \pm 2000$ ;  $50500 \pm 2535$ ;  $59700 \pm 2985$ ;  $67400 \pm 3370$  with no clear pattern of distribution of radioactivity.

In view of the apparently non-specific contamination of all precipitated protein bands with free label, this was removed by purification on Sephadex G25 prior to SDS-PAGE according to the procedure described in Methods, page 107. FIG.44 (i) shows the elution profile of the reduced receptor from the Sephadex G25 column and FIG.44 (ii) shows the elution of the [ $^3\text{H}$ ]-MBTA-receptor from an identical column

(all details in Methods, page 107). When a sample from the peak tube of the [<sup>3</sup>H]-MBTA acetylcholine receptor was examined by sodium dodecyl sulphate polyacrylamide gel electrophoresis, it was found that two major bands, molecular weights  $66000 \pm 3300$ ;  $42000 \pm 2100$ , were visible when stained with Coomassie G250 as for unlabelled receptor (FIG. 45).

However, the distribution of radioactivity showed that only one of the bands, that at  $42000 \pm 2100$ , was labelled with [<sup>3</sup>H]-MBTA. In control samples in which the receptor was preincubated with  $\alpha$  bungarotoxin before the affinity ligand no radioactivity was associated with the 42000 band.



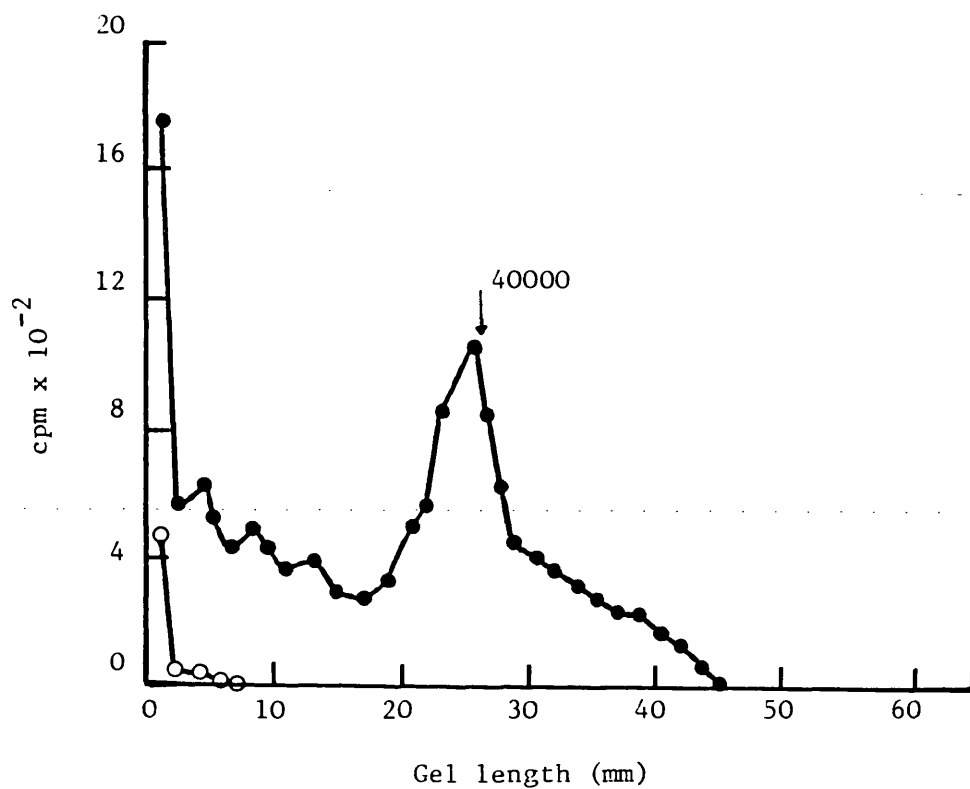
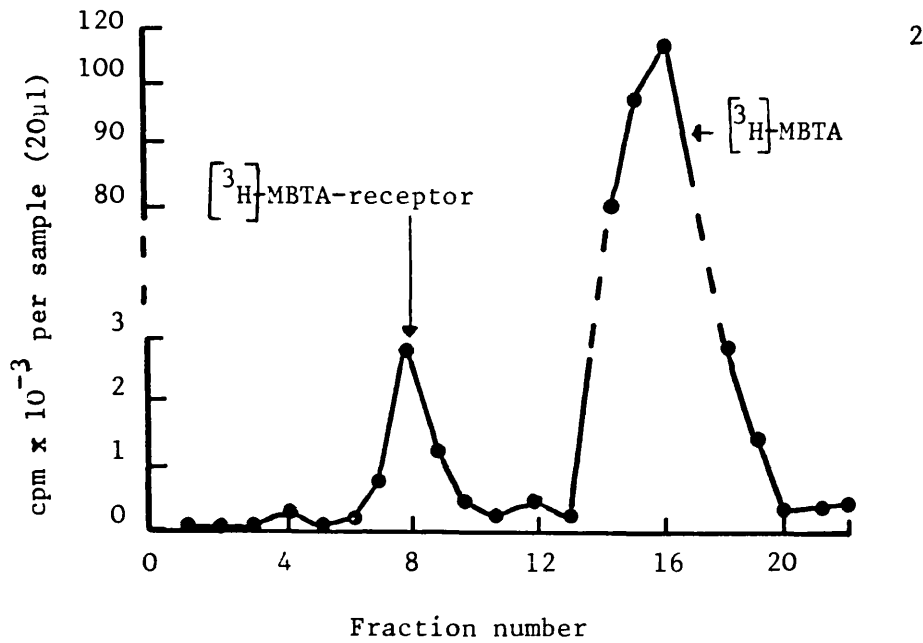
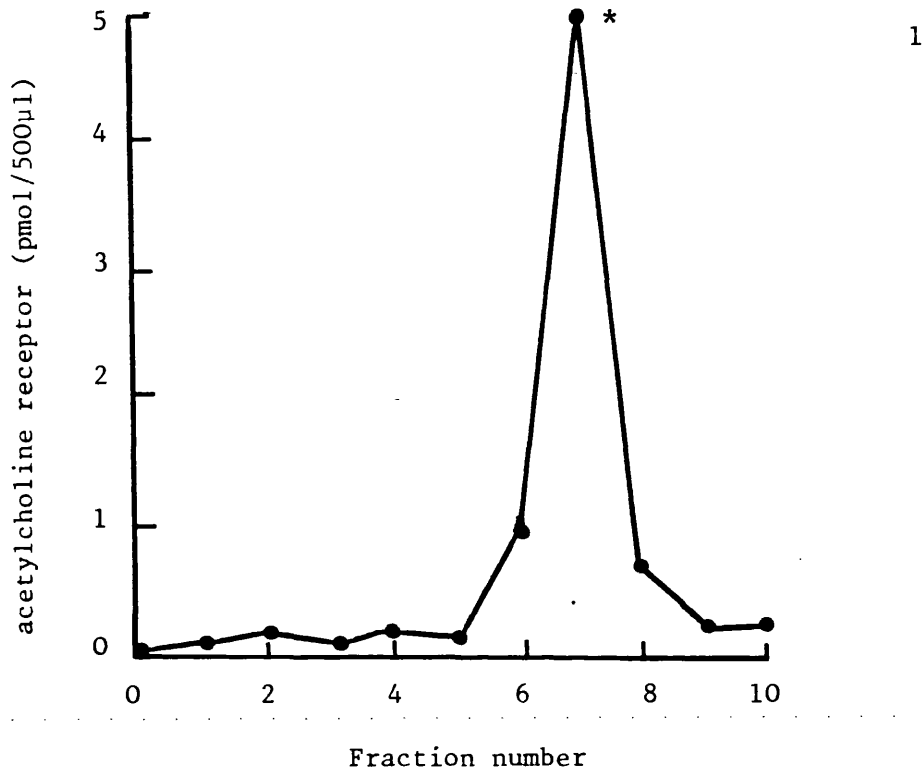


FIG. 43. The distribution of radioactivity in SDS-PAGE gels of muscle membranes labelled with the affinity ligand [<sup>3</sup>H]-MBTA

●—● <sup>3</sup>H-MBTA-labelled membrane fragments

○—○ membrane fragments preincubated with unlabelled  $\alpha$  bungarotoxin

FIG. 44. Gel filtration of acetylcholine receptor in the affinity labelling procedure

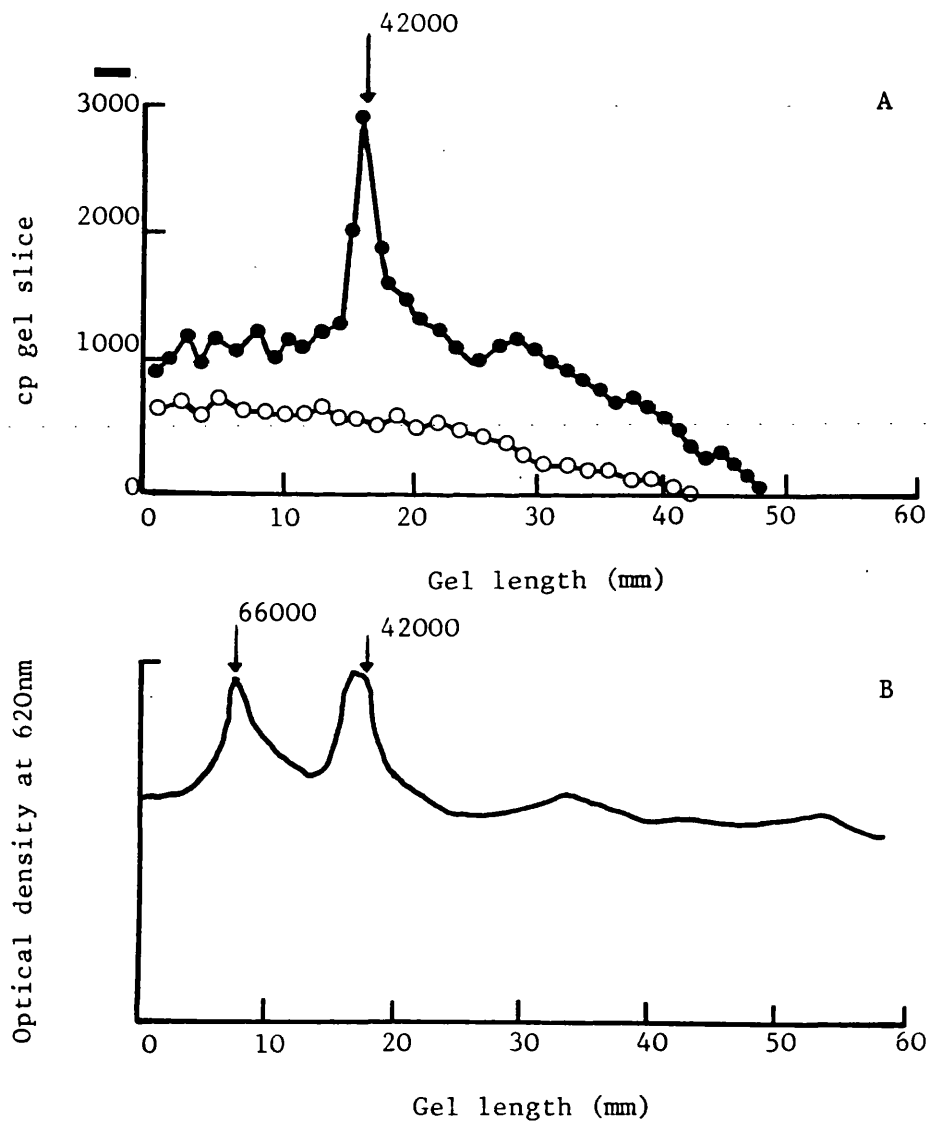


\* This value for the acetylcholine receptor concentration is an underestimation as in the assay for receptor activity, the reduced receptor was present in excess

1 Gel filtration of reduced receptor

2 Gel filtration of [<sup>3</sup>H]-MBTA receptor

FIG. 45. SDS-PAGE of  $[^3\text{H}]\text{MBTA}$  acetylcholine receptor



A Distribution of radioactivity in SDS-PAGE gels of  $[^3\text{H}]\text{MBTA}$  acetylcholine receptor

B Densitometric analysis of  $[^3\text{H}]\text{MBTA}$  labelled acetylcholine receptor

●—●  $[^3\text{H}]\text{MBTA}$  labelled purified receptor

○—○ Purified receptor preincubated with unlabelled  $\alpha$  bungarotoxin

### 11. The glycoprotein nature of the acetylcholine receptor

The glycoprotein nature of the receptor was examined using immobilised lectins. Each of the three lectins, concanavalin A (specific for mannose and glucose); lens culinaris (specific for mannose and glucose); soybean lectin (type VI) (specific for galactose) was immobilised onto Sepharose 4B as described earlier (Methods page 101 ). The measurement of the optical density of 280nm of the lectin solution before and after the coupling process showed that  $40 \pm 5\%$  of each lectin was immobilised.

A detergent extract containing solubilised human acetylcholine receptor was prepared as described earlier (page 67). The activity of the detergent extract was measured by the ammonium sulphate precipitation assay (Methods page 63). The extract was divided into four and each quarter (100ml) was stirred overnight at  $4^{\circ}\text{C}$  with an immobilised lectin (50ml packed bead volume). The control experiment was run in which underivatized Sepharose 4B was stirred with receptor solution. The supernatant was in each case collected by centrifugation at 3000g for 10min and was assayed for acetylcholine receptor activity as before.

The results shown in Table 17 show that there is a 13% loss of acetylcholine receptor activity from the detergent extract in the control sample. This was shown to be due to non-specific adsorption to the Sepharose 4B as the detergent extract had exactly the same activity when measured on consecutive days. The percentage activity absorbed by the lectins relative to the control value are, concanavalin A 37%, lens culinaris 46% and soybean lectin (type VI) 3%.

Table 17

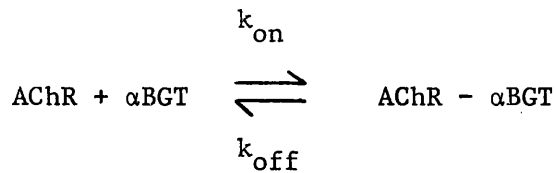
Sample	Acetylcholine Receptor pmol/ml	Percentage AChR bound to lectin
Original detergent extract	0.32 <sup>*</sup>	100%
Supernatant after exposure to Concanavalin A- Sephadex 4B	0.16 <sup>*</sup>	50%
Supernatant after exposure to lens culinaris - Sephadex 4B	0.13 <sup>*</sup>	59%
Supernatant after exposure to soybean lectin (Type VI) - Sephadex 4B	0.27 <sup>*</sup>	16%
Supernatant after exposure to underivatized Sephadex 4B	0.28 <sup>*</sup>	13%

\* Each acetylcholine receptor concentration is the mean value of six determinations at three different protein concentrations.

## 12. Binding properties of human acetylcholine receptor

The binding properties of both the detergent solubilised acetylcholine receptor and the purified protein were examined.

- (i) The determination of the rate constant ( $k_{on}$ ) for the binding of  $^{125}\text{I}$ - $\alpha$  bungarotoxin to the acetylcholine receptor



The rate of association between purified acetylcholine receptor and  $^{125}\text{I}$ - $\alpha$  bungarotoxin was determined from the progress curves of the reaction. Purified acetylcholine receptor (30fmol, 100 $\mu$ l) in assay buffer (Methods page 108) was incubated with  $^{125}\text{I}$ - $\alpha$  bungarotoxin (160fmol, 50 $\mu$ l) in assay buffer for varying times of up to 120 min at 23 $^{\circ}$ C. The amount of toxin bound was measured by the DEAE cellulose filtration assay (Methods page 64). The results were plotted and the  $k_{on}$  constant determined by two analyses.

Firstly, the  $k_{on}$  constant was determined directly from the initial rate of reaction (FIG. 46) where pseudo-first order reaction kinetics were assumed. The  $k_{on}$  value was determined from the equation:-

$$v = k_{on} [R_0] [T_0]$$

where  $v$  = the initial velocity of the reaction

$R_0$  = the initial receptor concentration

$T_0$  = the initial  $^{125}\text{I}$ - $\alpha$  bungarotoxin concentration

A linear regression analysis of the initial rate showed that the correlation coefficient for the experimental points was 0.998 and the  $k_{on}$  determined from the line of best fit was  $4.83 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ .

Secondly, the reaction between solubilised rat denervated receptor and  $^{125}\text{I}$ - $\alpha$  bungarotoxin has been shown to follow second order reaction kinetics (Colquhoun and Rang 1976) and therefore the results were plotted according to the integrated second-order rate equation which is:-

$$\log_{10} \frac{[R_0][T]}{[T_0][R]} = \frac{(T_0 - R_0) k_{on} t}{2.303}$$

where  $R_0$  = the initial receptor concentration  
 $R$  = the receptor concentration at time  $t$   
 $T_0$  = the initial  $^{125}\text{I}$ - $\alpha$  bungarotoxin concentration  
 $T$  = the  $^{125}\text{I}$ - $\alpha$  bungarotoxin concentration at time  $t$   
 $t$  = time in seconds

FIG. 46 shows the results obtained. A linear regression analysis of the results showed that the correlation coefficient was 0.96 and the  $k_{on}$  determined from the line of best fit was  $2.9 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ .

The experiment was repeated twice more and values obtained for  $k_{on}$  by the integrated second-order rate equation were  $1.6 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$  (linear correlation coefficient 0.98);  $5.9 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$  (linear correlation coefficient 0.95) (mean value =  $3.46 (3)$ ).

(ii) The determination of the rate constant ( $k_{\text{off}}$ ) for the dissociation of the receptor- $^{125}\text{I}$ - $\alpha$  bungarotoxin

The rate of dissociation ( $k_{\text{off}}$  or  $k_{-1}$ ) for the dissociation of receptor-toxin complex was determined.

Purified acetylcholine receptor (70fmol, 100 $\mu$ l) in assay buffer was incubated for 90 min at 23°C with  $^{125}\text{I}$ - $\alpha$  bungarotoxin (180fmol, 50 $\mu$ l) in assay buffer. Excess of unlabelled  $\alpha$  bungarotoxin (1.25nmol, 10 $\mu$ l) was added and the time course of displacement of the labelled ligand by the cold ligand was followed. The assay that was used to determine the amount of  $^{125}\text{I}$ - $\alpha$  bungarotoxin bound to the receptor was the DEAE cellulose filtration assay (Methods page 64). FIG.47 shows the profile for the rate of displacement of radioactive ligand by cold ligand. The dissociation rate was calculated using the relationship

$$v = k_{\text{off}} [\text{RT}]$$

where  $v$  = the dissociation rate

$[\text{RT}]$  = the initial concentration of receptor- $^{125}\text{I}$   $\alpha$  bungarotoxin.

A value of  $3.6 \times 10^{-6} \text{ s}^{-1}$  was obtained.

(iii) The determination of the dissociation constant

The dissociation constant ( $K_D$ ) was determined between both purified acetylcholine receptor and  $^{125}\text{I}$ - $\alpha$  bungarotoxin and detergent solubilised acetylcholine receptor and  $^{125}\text{I}$ - $\alpha$  bungarotoxin.

The acetylcholine receptor (60fmol, 100 $\mu$ l) was incubated for 90 min at 23°C, with a range of concentrations of  $^{125}\text{I}$ - $\alpha$  bungarotoxin which varied between 0.36-8nM (toxin volume - 50 $\mu$ l). Both ligand and receptor were in assay buffer (Methods page 108). The amount of toxin bound was determined either by the ammonium sulphate precipitation



assay for detergent-solubilised receptor (Methods page 63 ) or by the DEAE cellulose filtration assay (Methods page 64) for purified receptor.

Firstly, the concentration of labelled receptor measured was plotted as a function of the concentration of  $^{125}\text{I}$ - $\alpha$  bungarotoxin in the assay. For both purified and detergent-solubilised acetylcholine receptor, the binding of  $^{125}\text{I}$ - $\alpha$  bungarotoxin was found to be saturable (FIG. 48 ). Secondly, the results obtained were subjected to a Scatchard analysis for the determination of the dissociation constant, using the equation

$$\frac{[\text{RT}]}{[\text{T}]} = -1/K_D [\text{RT}] + 1/K_D [\text{R}_0]$$

where RT = concentration of  $^{125}\text{I}$ - $\alpha$  bungarotoxin bound

T = concentration of  $^{125}\text{I}$ - $\alpha$  bungarotoxin free

$\text{R}_0$  = the initial receptor concentration

$K_D$  = the dissociation constant

The ratio of  $\frac{[\text{Bound}]}{[\text{Free}]}$  ligand was plotted against  $[\text{bound}]$  ligand and the dissociation constant calculated from the gradient (FIG. 48 ). The value determined for the  $K_D$  between detergent-solubilised acetylcholine receptor and  $^{125}\text{I}$ - $\alpha$  bungarotoxin was  $6 \times 10^{-10}$  M (mean of 2 determinations). The Scatchard plot for each determination was analysed by linear regression and the correlation coefficients for the experimental points were 0.96 and 0.97. Similarly, the value determined for the  $K_D$  between purified acetylcholine receptor and  $^{125}\text{I}$ - $\alpha$  bungarotoxin was  $5 \times 10^{-10}$  M (mean of 2 determinations) and the correlation coefficients were 0.97 and 0.98.

A value can also be obtained for  $K_D$  using the rate constants of the reaction, since  $K_D = \frac{k_{\text{off}}}{k_{\text{on}}}$ . A value of  $6.9 \times 10^{-10} \text{M}$  was obtained by inserting the experimentally determined rate constants.

Thirdly, the results obtained were subjected to a Hill plot which was derived to quantitate the deviations of receptor-ligand interactions from the classic mass-action, rectangular hyperbola behaviour. The Hill plot in the logarithmic form is:-

$$\log_{10} \frac{B}{(B_{\text{MAX}} - B)} = n \log_{10} T - \log_{10} K'_D$$

where  $B = RT$  = concentration of  $^{125}\text{I}$ - bungarotoxin bound

$B_{\text{max}}$  = the maximum number of binding sites and is determined from the intercept of the y axis in the Scatchard plot

$T$  = concentration of  $^{125}\text{I}$ -bungarotoxin free

$K'_D$  = a composite constant composed of the intrinsic dissociation constant  $K_D$  and interaction factors that determine the degree to which  $K_D$  is altered at each discrete binding step.

From an experiment for the  $K_D$  between purified receptor and  $^{125}\text{I}$ -bungarotoxin,  $B_{\text{MAX}}$  was found to be  $1.15 \text{lnM}/\mu\text{g}$  protein. A plot of  $\log_{10} \frac{B}{(B_{\text{MAX}} - B)}$  against  $\log_{10} T$  was found to be a straight line (FIG.49 ) with correlation coefficient 0.97 and gradient = 0.84.

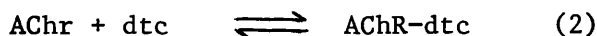
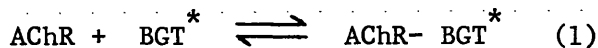
(iv) The determination of the dissociation constant  $K_D$  between purified acetylcholine receptor and d-tubocurarine

Two methods for the determination of the  $K_D$  between purified acetylcholine receptor and d-tubocurarine were used.

(a) The displacement of  $^{125}\text{I}$ - $\alpha$  bungarotoxin binding at equilibrium

D-tubocurarine ( $2 \times 10^{-5}\text{M}$ ,  $15\mu\text{l}$ ) was added to purified receptor ( $60\text{fmol}$ ,  $100\mu\text{l}$ ).  $^{125}\text{I}$ - $\alpha$  bungarotoxin was added in a concentration range which varied between  $0.5\text{-}8\text{nM}$  final concentration (toxin volume =  $50\mu\text{l}$ ) and the samples were incubated at  $4^\circ\text{C}$  overnight. All reagents were dissolved in assay buffer (Methods page 108). The amount of  $^{125}\text{I}$ - $\alpha$  bungarotoxin bound to the purified receptor was determined in each case by the DEAE cellulose filtration (Methods page 64).

In the treatment of the results obtained, d-tubocurarine was treated as a competitive inhibitor. There are two competing reactions,



It is possible to derive a relationship between the bound and free ligands as in a Scatchard plot, by considering the concentrations of the reactants at equilibrium, which is:-

$$[\text{RT}] = [\text{R}_0] - \frac{[\text{RT}]}{[\text{T}]} K_D \left[ 1 + \frac{\text{I}}{K_i} \right]$$

where  $\text{RT} = ^{125}\text{I}$ - $\alpha$  bungarotoxin bound

$\text{T} = ^{125}\text{I}$ - $\alpha$  bungarotoxin free

$\text{R}_0 =$  total acetylcholine receptor concentration

$K_D =$  the dissociation constant between AChR and  $^{125}\text{I}$ - $\alpha$  bungarotoxin

$K_i =$  the dissociation constant between AChR and d-tubocurarine

$\text{I} =$  d-tubocurarine concentration

A plot of the results  $\frac{[\text{Bound}]}{[\text{Free}]}$  against  $[\text{Bound}]$  ligand was made and the  $K_i$  was determined from the gradient. The value used for  $K_D$  was the experimentally determined value,  $5 \times 10^{-10}$  M. In three separate experiments, the values determined for  $K_i$  were  $0.224 \mu\text{M}$ ;  $0.27 \mu\text{M}$ ;  $0.25 \mu\text{M}$  (mean value = 0.25) and the correlation coefficients for each of the plots were 0.92; 0.98 and 0.96 respectively.

(b) The retardation of the initial velocity of reaction

When the reaction of  $^{125}\text{I}-\alpha$  bungarotoxin with purified acetylcholine receptor is performed in the presence of nicotinic ligands, then the rate of reaction is strongly retarded. In the presence of low concentrations of  $^{125}\text{I}-\alpha$  bungarotoxin and nicotinic ligands, it is possible to measure the initial velocity of the reaction and hence determine the protection constant  $K_D$  from the following:-

$$\frac{v_1}{v} = \frac{K_p}{K_p + [L]}$$

where  $v$  = the initial rate measured in the absence of ligand

$v_1$  = the initial rate measured in the presence of ligand

$K_p$  = the protection constant

$[L]$  = the concentration of nicotinic ligand

d-tubocurarine ( $5 \mu\text{M}$  final concentration) was added to purified acetylcholine receptor ( $60 \text{ fmol}$ ,  $100 \mu\text{l}$ ).  $^{125}\text{I}-\alpha$  bungarotoxin ( $240 \text{ fmol}$ ,  $50 \mu\text{l}$ ) was added and the amount of toxin bound was measured at 1 min intervals for the first 5 min of the reaction. The assay used was the DEAE cellulose filtration assay (Methods page 64). FIG. 51 shows the results of one experiment and the value obtained for  $K_p$  was  $0.18 \mu\text{M}$ .

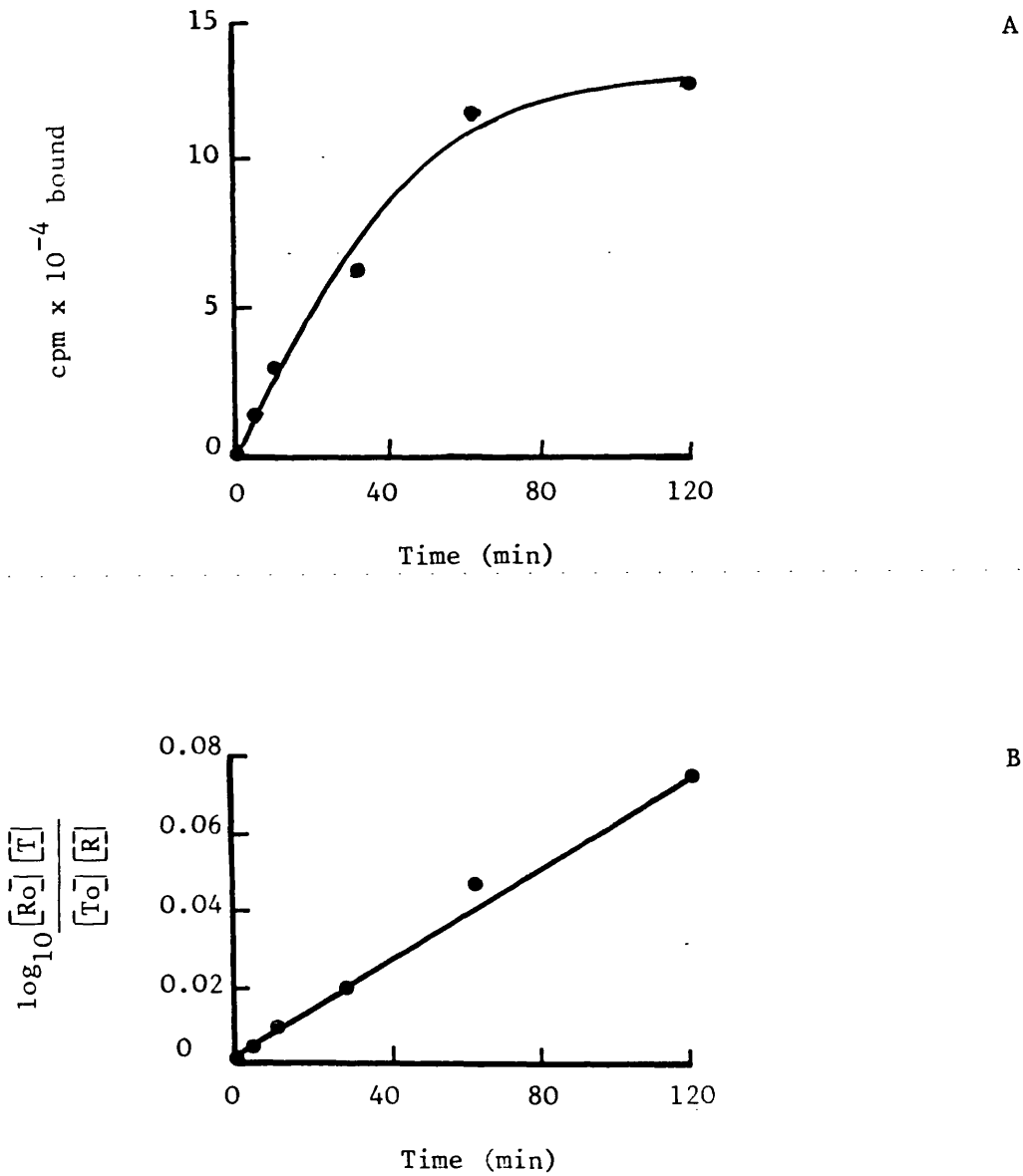


FIG. 46. The determination of the rate of association constant ( $k_{on}$ ) between purified receptor and  $^{125}\text{I}$ - $\alpha$  bungarotoxin

A Primary plot

B Secondary plot

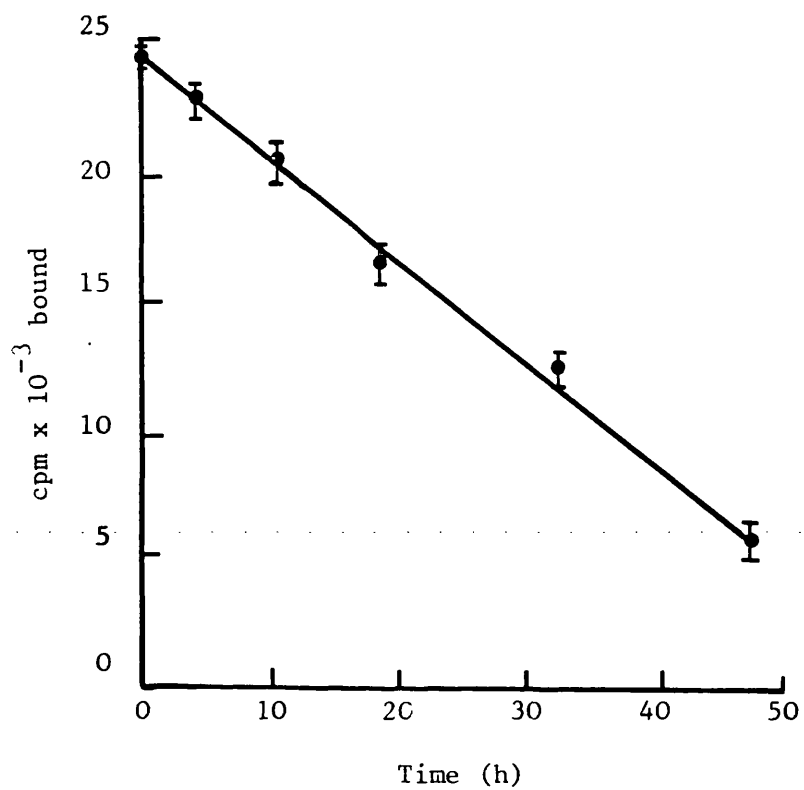
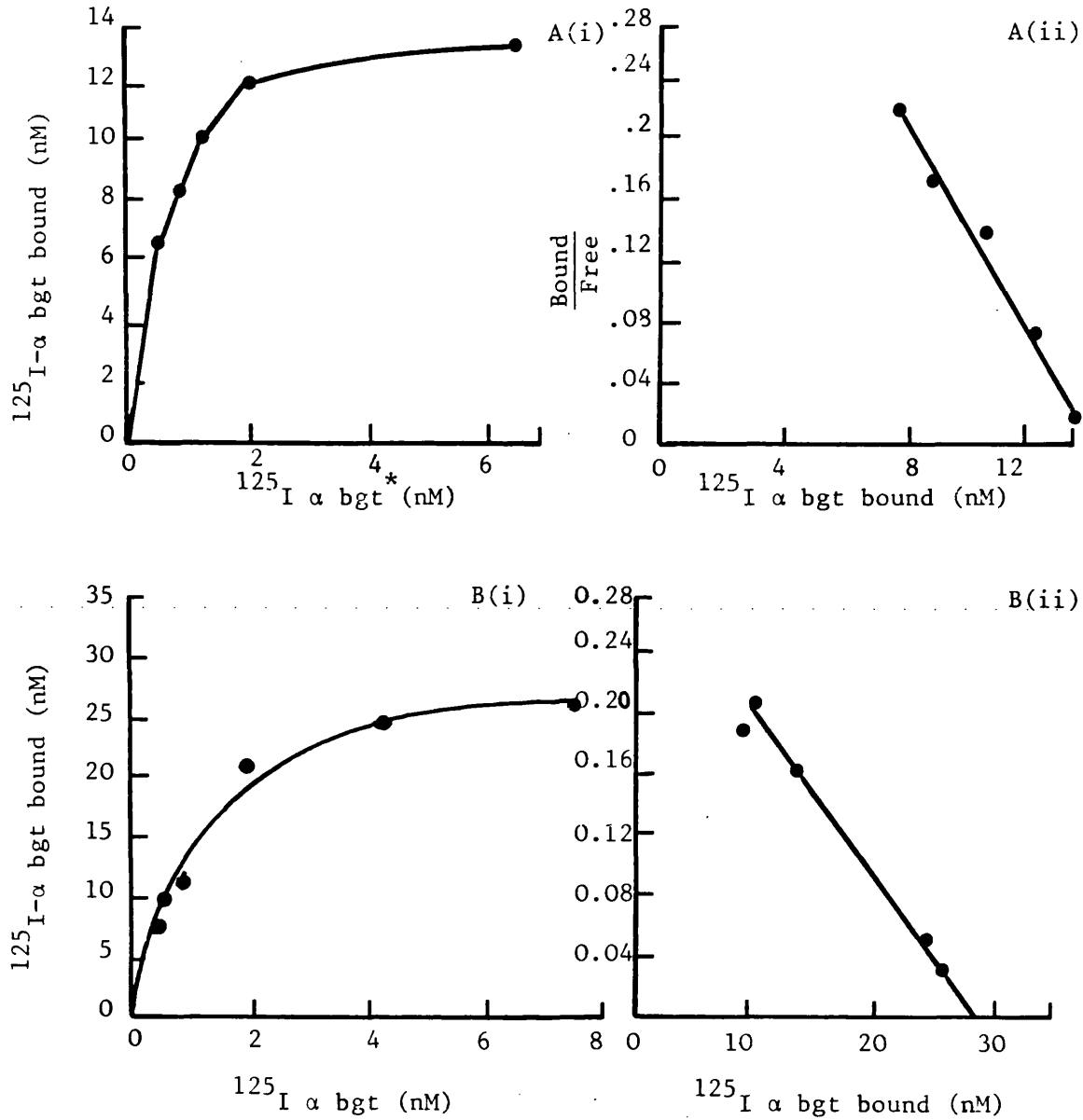


FIG. 47. The determination of the rate of dissociation constant ( $k_{\text{off}}$ ) between purified acetylcholine receptor and  $^{125}\text{I}$ - $\alpha$  bungarotoxin

FIG. 48. The determination of the dissociation constant ( $K_D$ )  
between acetylcholine receptor and  $^{125}\text{I}$ - $\alpha$  bungarotoxin



A(i) Saturation curve for purified acetylcholine receptor

A(ii) Scatchard analysis of the saturation curve for purified acetylcholine receptor

B(i) Saturation curve for detergent solubilised receptor

B(ii) Scatchard analysis of the saturation curve for detergent solubilised receptor

\*  $\alpha$  bgt:  $\alpha$  bungarotoxin

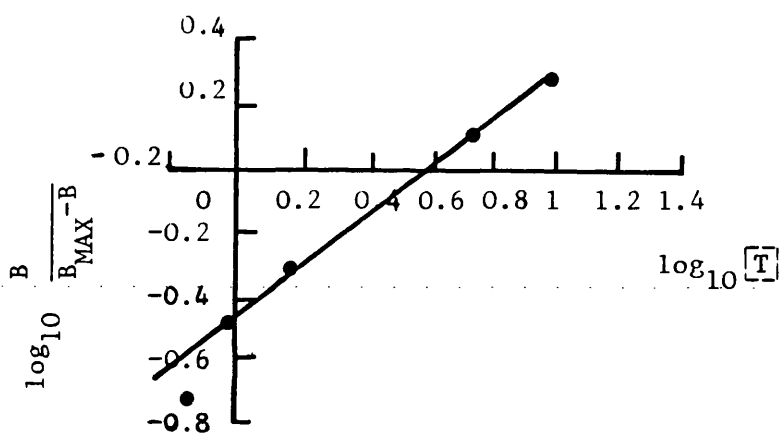


FIG. 49. A Hill plot for the binding of  $^{125}\text{I}$ - $\alpha$  bungarotoxin to purified acetylcholine receptor from human muscle

B = The concentration of  $^{125}\text{I}$ - $\alpha$  bungarotoxin bound

$B_{MAX}$  = The maximum number of  $^{125}\text{I}$ - $\alpha$  bungarotoxin binding sites per mg protein

T = The concentration of  $^{125}\text{I}$ -bungarotoxin free



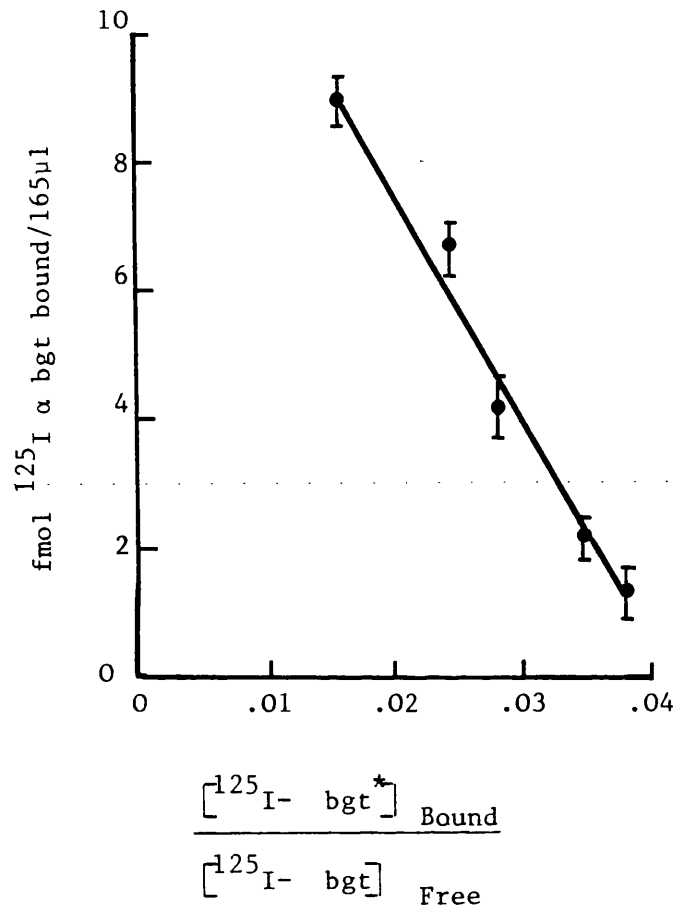


FIG. 50. The determination of the inhibitor constant ( $K_i$ )  
between acetylcholine receptor and d-tubocurarine

\*  $\alpha$  bgt  $\alpha$  bungarotoxin

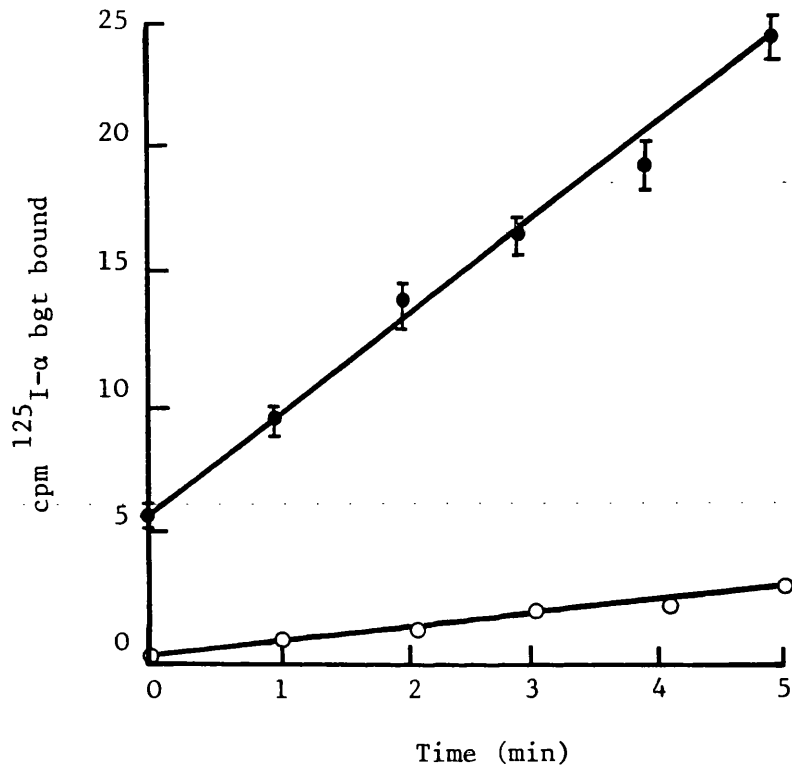


FIG. 51. The determination of the protection constant ( $K_p$ ) between purified acetylcholine receptor and d-tubocurarine by the retardation of the initial rate

- The rate of reaction between purified acetylcholine receptor and  $^{125}\text{I-}\alpha$  bungarotoxin
- The rate of reaction between purified acetylcholine receptor and  $^{125}\text{I-}\alpha$  bungarotoxin in the presence of d-tubocurarine ( $5\mu\text{M}$ )

### 13. The immunological properties of the acetylcholine receptor

The immunological properties of the purified human acetylcholine receptor were examined in two different ways. Firstly, the antigenic activity of the receptor was investigated by the measurement of the protein's ability to precipitate anti-(acetylcholine receptor) antibody from a series of myasthenic sera. Secondly, its ability to provoke an immune response was investigated by the immunisation of experimental animals with the purified protein.

#### (i) The antigenic activity of the acetylcholine receptor

Four myasthenic serum samples were assayed for anti-(acetylcholine receptor) antibody activity as described earlier (Methods page 66). Simultaneously, the same four samples were assayed for anti-(acetylcholine receptor) antibody activity using the purified acetylcholine receptor as the antigen. Purified acetylcholine receptor (60fmol, 100 $\mu$ l) in 0.01M potassium phosphate buffer, pH 7.4, containing 0.1% (w/v) bovine serum albumin, 1% (v/v) Triton X100 was incubated for 8h at 4°C with <sup>125</sup>I- $\alpha$  bungarotoxin (210fmol, 50 $\mu$ l) in the same buffer. Myasthenic serum (5 $\mu$ l) was added and the radioimmunoassay was completed as described previously (Methods, page 66). Table 18 is a comparison between the antibody titres obtained using both detergent solubilised acetylcholine receptor and purified acetylcholine receptor as antigen.

Table 18

Sample	Anti-(acetylcholine receptor) antibody titre 10 <sup>10</sup> M	
	Antigen = detergent solubilised AChR	Antigen = purified AChR
1	137.3 ± 6.9	170.6 ± 45.1
2	154.9 ± 2.2	173.5 ± 12.8
3	3.2 ± 0.8	4.3 ± 0.9
4	21.3 ± 0.6	19.8 ± 5.3
Normal serum	0	0

The antigenicity of the [<sup>3</sup>H]-MBTA-acetylcholine receptor was also examined. [<sup>3</sup>H]-MBTA-acetylcholine receptor (50µl) and 0.01M potassium phosphate buffer, pH 7.4, containing 1% (v/v) Triton X100, 0.1% (w/v) bovine serum albumin (50µl) were incubated overnight at 4°C with myasthenic serum (5µl). Goat anti-(human IgG) antiserum (100µl) was added and a precipitate left to form overnight at 4°C. The precipitate was collected by centrifugation at 3000g for 10 min and washed with 0.01M potassium phosphate buffer (500µl) pH 7.4, containing 0.15M NaCl. The precipitate was dissolved in distilled water (500µl) and added to a scintillation vial. Soluene 350 (200µl) was added to the vial and was left at room temperature for 2h. Scintillant (5ml) which contained PPO (2g); Triton X100 (350ml) and toluene (500ml) was added to each vial and the samples were counted in a Packard scintillation spectrometer. Table 19 shows the results obtained and each result is the mean of two values.

Table 19

Sample	Counts per min precipitated
Myasthenic sample 1	511
Myasthenic sample 2	423
Normal human serum	0

(ii) The immune response to the purified acetylcholine receptor

Three rabbits were injected intramuscularly with purified acetylcholine receptor as described earlier (Methods page 108). The anti-(human acetylcholine receptor) antibody in the rabbit serum was measured by the normal method of radioimmunoassay (Methods page 66) except that antigen-antibody complex was precipitated by sheep anti-(rabbit IgG) serum. FIG. 52 is a profile of the anti-(acetylcholine receptor) antibody titre with time for two rabbits. It can be seen that when there is a reaction to the immunogen, the anti-(human acetylcholine receptor) antibody concentration increases rapidly up to six days following immunisation and then rapidly declines until a further injection is given and the same phenomenon is observed. The production of the specific antibodies was most successful in two rabbits with maximum attained antibody titres of  $200 \times 10^{-10} \text{ M}$  and  $370 \times 10^{-10} \text{ M}$ . The antibody titre in a third rabbit did not exceed  $50 \times 10^{-10} \text{ M}$ . The antibody was also precipitated using purified acetylcholine receptor and  $[^3\text{H}]\text{-MBTA}$ -labelled receptor as antigens. Table 20 shows the results obtained for the antibody titre with one sample of rabbit anti-(human acetylcholine receptor) antiserum.

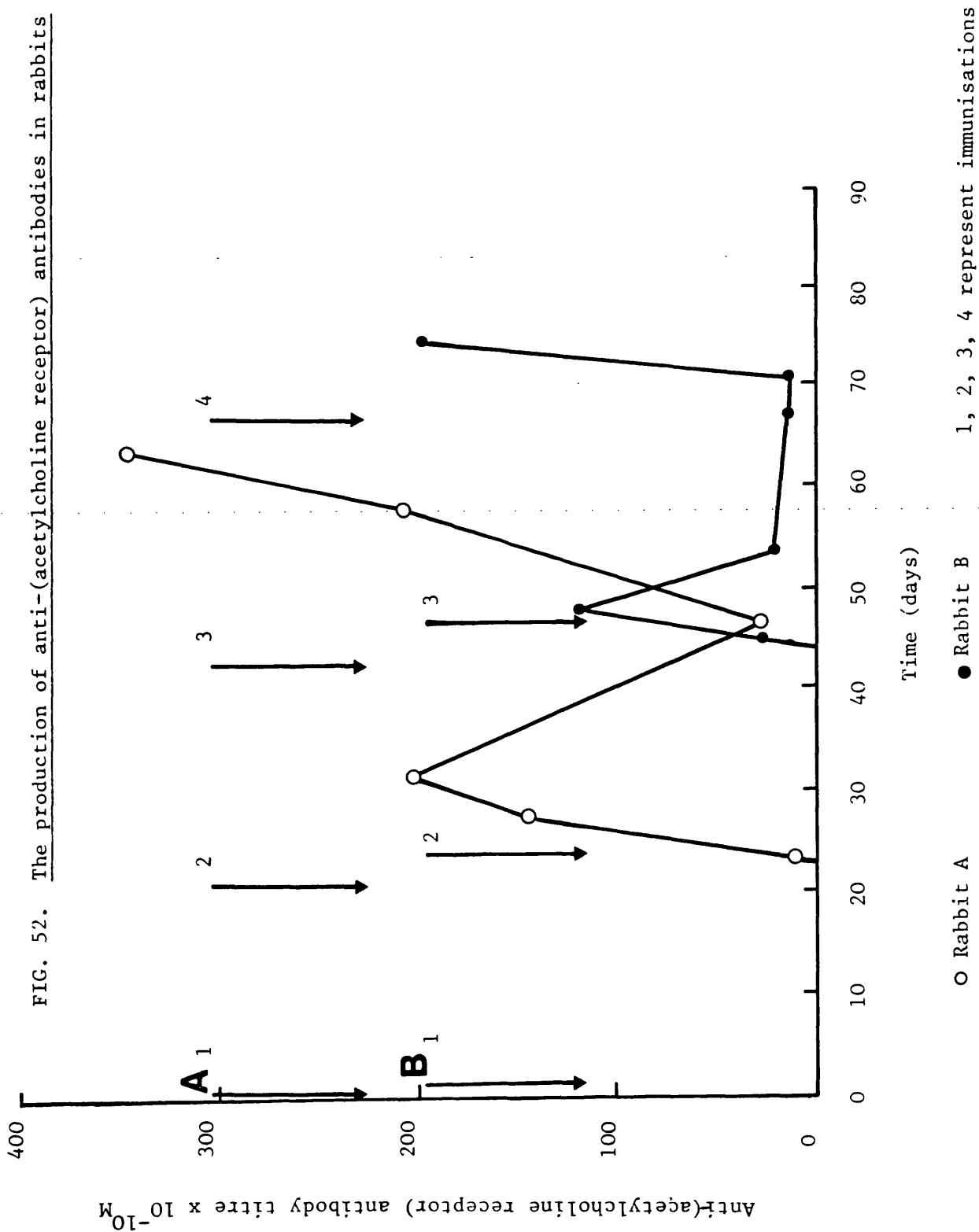
Table 20

Sample	Antigen = detergent solubilised AChR Titre x 10 <sup>10</sup> M	Antigen = purified AChR Titre x 10 <sup>10</sup> M	Antigen = [ <sup>3</sup> H]-MBTA-AChR cpm precipitated
Rabbit antiserum	241 ± 7.4	267 ± 10.1	126
Normal human serum	0	0	0

After each immunisation, the rabbits were observed for signs of paralysis and excess fatiguability but no physical symptoms were seen.

The cross-reactivity of the rabbit immune serum with host acetylcholine receptor was measured using detergent solubilised acetylcholine receptor from rabbit muscle which was prepared as before (Methods page 67). All the different rabbit sera were assayed for rabbit anti-(rabbit acetylcholine receptor) antibody activity but no cross-reactivity was found.

FIG. 52. The production of anti-(acetylcholine receptor) antibodies in rabbits



## DISCUSSION

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DISCUSSIONThe measurement of receptor activity

The physiological function of the nicotinic acetylcholine receptor is to transduce the message embodied in the neurotransmitter, acetylcholine, into a change in the permeability of the post-synaptic membrane. Measurements of this function can be made by electrophysiological methods as previously described (Introduction page 3). The removal of any receptor from its membrane environment necessarily precludes any measurement of its overall function and alternative procedures must be sought both for the identification of the isolated receptor and for the determination of its activity. Two aspects of receptor function have been exploited in the development of an assay procedure for the acetylcholine receptor. Firstly, the isolated receptor carries a highly specific binding site for the natural transmitter and secondly, the binding of ligands to this site may induce changes in the conformation of the receptor molecule. To date, the most widely used method of assay depends on the former property although more recently progress has been made in the detection of the receptor using physical methods (Barrantes 1980).

The methodology of receptor assay parallels to some degree the classical methods of enzymology. Thus analogies are drawn between receptor and enzyme and transmitter and substrate. However, the analogy is not valid beyond the point at which a receptor-ligand complex forms since it is well established that no chemical modification of the transmitter occurs as a result of its interaction with the receptor. The assay methods therefore depend on the ability to measure the amount of receptor-ligand complex that is formed. The

most convenient method for the detection of the complex is to employ a radiolabelled ligand; receptor-ligand complex may be readily separated from free ligand on the basis of size, charge, antigenicity or hydrophobicity. In the case of the acetylcholine receptor, the preferred ligand would be acetylcholine since it is the neuroactivator *in vivo*. In practice, however, acetylcholine does not satisfy the requirements of the assay procedure. The interaction of acetylcholine with its receptor is readily reversible ( $K_D \sim 10^{-5} M$ ) under experimental conditions and furthermore crude preparations of acetylcholine receptor invariably contain high levels of acetylcholinesterase which leads to rapid hydrolysis of acetylcholine (Eldefrawi *et al* 1972). The requirements for a highly specific, irreversible, non-degradable ligand are largely met by  $\alpha$  bungarotoxin. The specificity of  $\alpha$  bungarotoxin has been discussed previously (Introduction, page 8 ); it binds tightly to the receptor ( $K_D \sim 10^{-10} M$ ) and it is readily radiolabelled without serious loss of biological activity.

#### $^{125}I$ - $\alpha$ bungarotoxin as a probe for the acetylcholine receptor

In general two methods, tritiation and radioiodination are used to radiolabel the ligands used in binding studies. Tritium labelling is a particularly convenient method because in most cases the labelled ligand is biologically indistinguishable from the native compound and the long radiochemical half-life of tritium (12.26 years) allows extended storage with no loss of sensitivity resulting from radioactive decay. However, the specific radioactivities obtained with a ligand labelled with tritium are much less than can be achieved by radioiodination. For example, one atom of  $^{131}I$  provides the same number of disintegrations per unit time as 600 atoms of tritium.

$^{131}\text{I}$  has a short half-life (8 days) compared to  $^{125}\text{I}$  (60 days) and  $^{125}\text{I}$  is accordingly the preferred isotope for radiolabelling.

Radioiodinated ligands however suffer from the disadvantage of a greater susceptibility to radiation-induced destruction of molecular structure which may lead to different behaviour of the iodinated substance in analytical systems compared to the parent compound. The isoelectric point of gastrin for example is altered following iodination (Hunter 1978). For these reasons, considerable attention has been devoted to establishing the effective activity of the  $^{125}\text{I}$ - $\alpha$  bungarotoxin that was routinely used in this work.

Firstly, it was shown that the native  $\alpha$  bungarotoxin that was used for the radioiodination was at least 98% pure (T. Barkas, personal communication) as all the  $\alpha$  bungarotoxin was recovered in peak D of the purification profile (Results, page 112). Secondly, the biological activity of  $^{125}\text{I}$ - $\alpha$  bungarotoxin, that is the percentage of radioactivity precipitated by excess of receptor was examined (Results page 70). Concentrations of solutions of acetylcholine receptor subsequently determined by using the toxin were corrected to 100% biological activity (Table 2 page 76 ).

An attempt was made to determine the percentage of radioactivity that corresponded to  $^{125}\text{I}$  non-covalently bound to toxin by precipitating the labelled toxin with 6% (w/v) trichloroacetic acid (Results page 70 ). However, with all the radiolabelled  $\alpha$  bungarotoxins (even N-(propionyl- $^3\text{H}$ ) propionylated  $\alpha$  bungarotoxin which is 98% biologically active) the radioactivity precipitated never exceeded 70% and therefore this parameter has limited use in the assessment of the activity of the toxin in the assays used here.

The method of iodination that was routinely used was the chloramine T method initially devised by Greenwood et al (1963) to produce a radioiodinated product of high specific radioactivity. The  $^{125}\text{I}$ - $\alpha$  bungarotoxin was labelled to a specific radioactivity of  $675 \pm 27$  Ci/mmol and the biological activity of the toxin was found to range between 35-60%. The results shown in Table 2 (page 76) are representative of all the preparations of high specific radioactivity  $^{125}\text{I}$ - $\alpha$  bungarotoxin that were used throughout the three year study. It can be seen that following correction for 100% biological activity, the values for the concentration of acetylcholine receptor purified from *Torpedo marmorata* agree well with the value obtained for the 98% biologically active N-(propionyl- $^3\text{H}$ ) propionylated  $\alpha$  bungarotoxin. Values of activity were in practice further corrected to bring them into line with those determined using the propionylated toxin (Table 2 page 76).

To further explore the concept of biological activity,  $^{125}\text{I}$ - $\alpha$  bungarotoxin was prepared by two different methods each of which labelled the  $\alpha$  bungarotoxin to a lower specific radioactivity than was routinely used. For the low specific radioactivity  $^{125}\text{I}$ - $\alpha$  bungarotoxin, the conditions of the chloramine T method of iodination were manipulated such that the native  $\alpha$  bungarotoxin was not exposed to high concentrations of oxidising agents and similarly, by using the lactoperoxidase enzymic method, this was also so. The results shown in Table 2 (page 76) agree with those for the high specific radioactivity  $^{125}\text{I}$ - $\alpha$  bungarotoxin in that after correction for 100% biological activity, the value obtained for the concentration of the purified solution of acetylcholine receptor approached that obtained for N-(propionyl- $^3\text{H}$ ) propionylated  $\alpha$  bungarotoxin.

The  $^{125}\text{I}$ - $\alpha$  bungarotoxin prepared by the Chloramine T method is a mixture of mono- and di-iodo derivatives. It has been reported that these two derivatives show different binding affinities for acetylcholine receptor (Vogel *et al* 1972) and this could affect the determination of receptor activity. However in our laboratory no differences were seen between mono and di- $^{125}\text{I}$ - $\alpha$  bungarotoxin in this respect (M. Bird personal communication) and this is in agreement with the observations of Vincent and Newsom-Davis (1979).

Despite the limitations inherent in the use of  $^{125}\text{I}$ - $\alpha$  bungarotoxin the procedures outlined above, involving the use of a standard receptor preparation from *Torpedo marmorata* and the N-(propionyl- $^3\text{H}$ ) propionylated  $\alpha$  bungarotoxin, gave a satisfactory working regimen.

#### The acetylcholine receptor assay

Two assay methods were employed for the detection of acetylcholine receptor. This was because the measurement of acetylcholine receptor activity in the detergent extract by the DEAE cellulose filtration assay method was complicated by the saturation of the discs by non-receptor proteins leading to lower values (Table 5 page 79). However both assays satisfied the conditions outlined earlier (Introduction page 18) for an assay for acetylcholine receptor activity in that they were linear with increasing protein concentration (FIG. 9 page 74 and 77 respectively) provided that  $^{125}\text{I}$ - $\alpha$  bungarotoxin was in excess, and in that the addition of nicotinic cholinergic ligands inhibited the total binding in both the ammonium sulphate precipitation assay and the DEAE cellulose filtration assay by a minimum of 80%

(Results, page 78 ).

The radioimmunoassay for anti-(acetylcholine receptor) antibodies

In conjunction with the Institute for Neurological Sciences in Glasgow the radioimmunoassay for the measurement of anti-(acetylcholine receptor) antibodies in the serum of patients with myasthenia gravis was set up to assess the value of such measurements as an aid for diagnosis of myasthenia gravis and as a means of monitoring therapy. Furthermore, the accumulated data may assist in the understanding of the pathogenesis of the disease.

In the radioimmunoassay for the detection of anti-(acetylcholine receptor) antibodies, the antigen used was the human nicotinic acetylcholine receptor which was present in a crude detergent extract. Initially, the concentration of solubilised receptor was routinely of the order  $2 \times 10^{-10}$  M and an assay volume of 1ml was necessary to achieve acceptable sensitivity. The receptor was labelled with  $^{125}\text{I}$ - $\alpha$  bungarotoxin whose advantages as a probe have been outlined earlier (Discussion, page 181). Obviously the use of  $^{125}\text{I}$ - $\alpha$  bungarotoxin is not ideal and it would be preferable to have an antigen that was directly labelled and not to have to rely on an external factor. Furthermore, labelling of the receptor with  $^{125}\text{I}$ - $\alpha$  bungarotoxin occupies the acetylcholine binding site and precludes the detection of anti-(toxin site) antibodies. However, at the time of establishing the assay direct labelling of receptor was not possible (but see section on "the purification of the acetylcholine receptor", page 99 ). A further problem with an external label is that complete labelling of the antigen must be ensured. This condition is normally satisfied if excess ligand is present at a concentration 8-10 times higher than its dissociation

constant with the receptor ( $K_D$   $6 \times 10^{-10}$  M, Results, page 165 ). Under the assay conditions initially employed, this was not possible because of the limited availability of  $^{125}\text{I}$ - $\alpha$  bungarotoxin and the need for an assay volume of 1 ml. Therefore, a correction factor was used (Results, page 72 ) which adjusted the assay to a theoretical situation where all receptor molecules are labelled. This was determined by increasing the  $^{125}\text{I}$ - $\alpha$  bungarotoxin concentration in a radioimmunoassay until no further increase in the antibody titre was seen. This value for the antibody titre is then compared to the value obtained at the normal working concentration of toxin. All subsequent titres were then multiplied by this correction factor. To some extent, this problem has now been circumvented since with the availability of freshly amputated muscle rather than material *post mortem* and the introduction of the anti-protease agents into the preparation of detergent solubilised acetylcholine receptor, it has been possible to obtain receptor-containing extracts of higher specific activity. Thus, the assay volume is reduced with no loss in sensitivity of the assay, and the  $^{125}\text{I}$ - $\alpha$  bungarotoxin concentration is correspondingly increased.

An additional requirement of the radioimmunoassay is that it should provide the neurologist with a rapid result. However, an extensive investigation of the time course of the assay has shown that the minimum time for completion is three days (FIG. 13 and 14 pages 84 and 85 respectively). It is possible that the reaction time could be reduced by carrying out the assay at ambient temperature, but it was thought at the time that the maintenance of  $4^\circ\text{C}$  throughout the complete procedure would minimise proteolysis effects.

The reproducibility of the assay between different preparations of solubilised receptor was examined and the results are shown in Table 6 (page 87 ) for four different antigen-containing Triton X100 extracts. It was shown that while there is some variation between receptor preparations, titres maintain their positions (i.e. high, low, average etc.) relative to the range of values observed.

Once established, the radioimmunoassay was used routinely to analyse myasthenic sera for anti-(acetylcholine receptor) antibodies and to attempt to correlate the antibody titre with disease severity. In agreement with other workers, it was found that 87% of myasthenic serum samples contained elevated levels of circulating anti-(acetylcholine receptor) antibodies (Lindstrom *et al* 1976; Monnier and Fulpius 1977; Lefvert *et al* 1978; Carter *et al* 1980). Further no direct correlation was apparent between overall circulating antibody titre and disease severity. However Lefvert *et al* (1978) and Carter *et al* (1980) both make the point that there may be a good correlation between antibody titre and disease state in individual patients. In general, ocular myasthenic patients were shown to have low antibody titres. This has also been reported by Lindstrom *et al* (1976) and Lefvert *et al* (1978) and is in accordance with the fact that ocular myasthenia is the mildest form of the disease. The median values for the antibody titres (FIG. 16 page 91 ) did increase with increasing disease severity (Barkas *et al* 1979). A second investigation of anti-(acetylcholine receptor) antibody titre measured this parameter in the serum of myasthenic patients undergoing a course of plasma exchange. Plasma exchange was first used as a therapy for myasthenia gravis by Pinching *et al* (1976) following the realisation that a circulating blocking agent may be important in myasthenia gravis (Lindstrom *et al* 1976). It had



previously been employed successfully in the treatment of Goodpasture's syndrome where it was shown to lower the level of autoantibodies to glomerular basement membrane (Lockwood *et al* 1976). Pinching *et al* (1976) observed the effect of plasma exchange in three patients. Two patients showed a dramatic improvement following plasma exchange but the improvement declined when the exchanges were discontinued. The third patient was suffering from congenital myasthenia gravis and no improvement was apparent following 4 x 2ℓ exchanges of plasma.

The results reported by Pinching *et al* (1976) were encouraging enough to promote further investigation by several groups. In the results reported here (pages 89 to 94 ), the anti-(acetylcholine receptor) antibody was measured during the plasma exchange of eight patients. Each patient underwent an intensive course of exchanges where, at each one, 4ℓ of the patient's plasma were replaced and the process was repeated on up to six occasions at 2-4 day intervals. Following the third exchange, the patients received immunosuppressive therapy in the form of prednisolone and azathioprine. Prednisolone in man has been shown to suppress antibody synthesis (McMillan *et al*, 1976) without being cytotoxic (Fauci *et al* 1976) and azathioprine is an antimetabolite that after hepatic conversion into its active form, 6-mercaptopurine, selectively kills dividing cells by interfering with DNA synthesis (Elion 1967).

In Table 7 (page 94 ), for the first six patients, a common trend was observed, that is, following plasma exchange the anti-(acetylcholine receptor) antibody titre decreased dramatically. Subsequently, the antibody titre rose but generally did not attain the values determined prior to the previous exchange. The drop in anti-

body titre was accompanied by an alleviation of symptoms. Further, after the intensive course of plasma exchanges, the patients showed apparently complete remission for up to six months. This was also so for patients 7 and 8 (Table 7) despite the fact that throughout the exchange procedure, their antibody titre did not significantly differ from zero. This régime of plasma exchange plus immunosuppressant therapy has been extended to a further 21 patients (Behan *et al* 1979) and the antibody titres of the patients have been monitored by our laboratory over an extended period (Carter *et al* 1980). Similarly successful results were found. Each of the patients had a total of 16-32ℓ of plasma exchanged over a 2-3 week period. Of the 21 patients reported in Behan *et al* (1979), six patients had a recurrence 3-9 months after the first course of plasma exchange but then obtained complete remission after a second course of exchanges. The anti-(acetylcholine receptor) antibody titre was studied for up to 18 months following the completion of plasma exchange and it was found that in general the antibody titre always remained below the initial pre-exchange value.

Similar results have been reported by other groups, Dau *et al* (1979) found that plasma exchange was of great benefit to five myasthenic patients and that the decline in antibody titre was followed by a lag phase before an improved clinical response was observed. Further, they found that relapses were associated with an increase in antibody titre. Kornfeld *et al* (1979) and Newsom-Davis *et al* (1978) reported similar results also, with an addendum to the latter that there was a minimum delay in clinical improvement of two days and that this amelioration was not always the same in each muscle.

There have been several attempts to explain the beneficial effect of plasma exchange. Firstly, Pinching *et al* (1976) suggested that the removal of circulating antibody could alter the equilibrium between free antibody and antibody bound to receptor. This would allow a larger proportion of the acetylcholine receptors to function free of antibody. Secondly, removal of antibody together with immunosuppressive therapy would allow newly synthesised acetylcholine receptor to be incorporated into the muscle membrane free of bound antibody and thus to be functionally normal. The lag phase observed between the drop in circulating antibody titre and clinical improvement may then be explained in terms of a latent period during which acetylcholine receptors are being synthesised and incorporated into the membrane.

The value of plasma exchange has recently been severely challenged. It has the advantage that it works quickly and is therefore useful in treating severe disease which is causing respiratory embarrassment. The disadvantages are the actual cost of performing the exchange and that in some patients, remission obtained may be short lived. Secondly, there are potential problems arising from the actual exchange technique, for example, a reaction to the foreign products in the plasma protein fraction, an air embolism, infection or haemorrhage and inaccessible veins. Patients have also reported hypotension, sweating attacks, cramp-like pains and lumbago sensations following plasma exchange (Behan *et al* 1979), although some of these symptoms may be psychosomatic. The opponents of the use of plasma exchange argue that its value in the management of myasthenia gravis lies only in its passive ability to lower serum anti-(acetylcholine receptor) antibodies (Newsom-Davis *et al* 1979). Because of the risks associated with the

procedure, its high cost in materials and medical time, they suggest that its use should be limited to the treatment of myasthenia gravis only in crisis. Further, they claim that the same beneficial effects seen in plasma exchange may be observed with immunosuppression therapy alone (Newsom-Davis *et al* 1978).

In the plasma exchange series reported in this thesis, immunosuppressive medication has always been prescribed. Newsom-Davis *et al* (1978, 1979) have attempted to distinguish between the value of plasma exchange and immunosuppression either singly or combined. They found that in two series of patients where one group was treated with immunosuppression alone (both azathioprine and prednisolone) and the other, immunosuppression plus plasma exchange, there was no overall difference in results. Patients treated with azathioprine and prednisolone only showed an overall gradual decline in antibody titre, the rate of which varied with the individual. Patients who were additionally plasma exchanged had transient drops in their specific antibody titre which again coincided with clinical improvement. When the anti-(acetylcholine receptor) antibody titre was compared to the original value before treatment, up to eight months following the commencement of exchanges, the results of the two groups are identical. These findings are in disagreement with the results reported by Dau *et al* (1977) who found that azathioprine and prednisone therapy decreased the antibody titre by only 14% and this decrease was not associated with any clinical remission. However, this was only one observation. Newsom-Davis *et al* (1979(a)) further investigated the effect of an additional immunosuppressant, cyclophosphamide. In a series of exchanges, they combined both azathioprine and cyclophosphamide and found that this was the most effective treatment in extending the time that elapses before

the anti-(acetylcholine receptor) antibody reaches 50% of its initial value.

Thus, in summary, it was found that plasma exchange combined with immunosuppressive therapy was of considerably more value in the treatment of myasthenia gravis than the more traditional methods of antiacetylcholinesterase medication and thymectomy. From the results reported here, it is suggested that plasma exchange in combination with immunosuppression may be able to exert a synergistic action in modifying the immune responses that seem to be responsible for myasthenia gravis. In the generation of an immune response, clonal expansion through the proliferation of lymphocytes with specificity for the initiating antigen is a very early event. Specific clonal expansion can also occur when the feedback inhibition of antibody synthesis is reduced by lowering of the concentration of a given antibody in the circulation. After reduction in titre of an antibody in primarily immunised animals through exchange transfusions, the titre of the antibody removed rebounds to approximately twice its original value within two weeks (Bystryk et al 1971). This rise in titre can be inhibited by cyclophosphamide. Further, in the light of recent observations (review Lindstrom 1979) it can be proposed that the removal of circulating antibody does not result in the liberation of functionally operative receptors but that the process of antigenic modulation is arrested (Introduction page 46). Thus the restoration of normal activity is the result of the integration of newly synthesised acetylcholine receptors into the muscle membrane. If the antibody synthesis is suppressed then neuromuscular transmission occurs normally.

Whilst the presence of circulating elevated levels of anti-(acetylcholine receptor) antibodies in the serum of myasthenic patients and the therapeutic effect of the removal of these antibodies via plasma exchange clearly demonstrates an important role for the antibody in the pathogenesis of myasthenia gravis, it does not provide a total understanding of the aetiology. This is exemplified by the complete remission of patients 7 and 8 in Table 7 (page 94) following plasma exchange despite the fact that their antibody titres did not differ significantly from control values throughout the exchange procedure. One explanation for this observation is that individual patients have widely different threshold values for antibody-initiated impairment of function or that anti-(acetylcholine receptor) antibodies are not the primary agents in the aetiology of myasthenia gravis. An alternative explanation is that, as outlined earlier in the Introduction (page 51), the pathogenic factor is not the total anti-(acetylcholine receptor) antibody population that is important but only a particular sub-population.

The acetylcholine receptor is a macromolecule and as such will possess many antigenic determinants depending upon the steric presentation of the antigen to the circulating lymphocytes of the immune system. Thus it is not unreasonable to expect that the total antibody population directed at the acetylcholine receptor will be heterogeneous. Indeed several groups have established the heterogeneous nature of the anti-(acetylcholine receptor) antibody population (Lindstrom *et al* 1980; Mittag *et al* 1978 (see also Introduction, page 50). The possible importance of the sub-population of antibody directed at the acetylcholine binding site was stressed in the Introduction (page 51). In the assay that was routinely used here, this antibody is not detected. This can be seen more clearly in the diagrammatic representation of

the radioimmunoassay in FIG. 7 (page 69) where it is shown that the acetylcholine binding site is occupied by  $^{125}\text{I}$ - $\alpha$  bungarotoxin thus excluding the binding of anti-(toxin site) antibody.

Several investigations have been carried out to determine the concentration of anti-(toxin site) antibodies in the serum of myasthenic patients. Here, it was found that in a study of 20 myasthenic patients, all their serum samples inhibited the binding of  $^{125}\text{I}$ - $\alpha$  bungarotoxin to solubilised acetylcholine receptor (Table 8 page 97) but the extent of the inhibition varied between 1.4% to 74%. It is interesting that two patients (1 and 15, Table 8 page 97) have relatively low antibody titres yet inhibit  $^{125}\text{I}$ - $\alpha$  bungarotoxin binding by 52% and 55% respectively. There is considerable conflict in the literature concerning anti-(toxin site) antibodies. Almon *et al* (1974) found that 30% of all myasthenic serum samples blocked the binding of  $^{125}\text{I}$ - $\alpha$  bungarotoxin to the acetylcholine receptors from denervated rat muscle whereas Lindstrom *et al* (1976) found that 38% of sera tested reduced the binding of  $^{125}\text{I}$ - $\alpha$  bungarotoxin. However, the effect was small and was seen only when the concentration of anti-(receptor) antibody exceeded the acetylcholine receptor by a factor of 200-1000. Bender *et al* (1975) using a histochemical method to demonstrate the inhibition of  $^{125}\text{I}$ - $\alpha$  bungarotoxin binding to normal muscle, showed a positive response by 75% of myasthenic sera investigated. Vincent and Newsom-Davis (1979) attempted to correlate the concentration of anti-(toxin site) antibody with disease severity and found that none of the patients they studied had a significant anti-(toxin site) antibody titre during remission although many of them still had high anti-(acetylcholine receptor) antibody titres as measured by immunoprecipitation. More recently, Fulpius *et al* (1980) have further demonstrated the presence in the serum of four

myasthenic patients of antibody directed against the cholinergic binding site. In detailed analyses of one myasthenic patient, they were able to show that the antibody binding to chicken embryo myogenic cultures was competitive with  $^{125}\text{I}$ - $\alpha$  bungarotoxin and some cholinergic ligands and that the binding of this specific antibody accelerated the rate of receptor degradation. However, one must bear in mind that this is only one observation and may not be representative of the whole myasthenic population.

The presence of anti-(toxin site) antibodies may also explain the results that were found when the effects of myasthenic serum samples on the acetylcholine sensitivity of chick muscle in culture were investigated (Results, page 95). It was shown that of the seven myasthenic sera tested, all of them showed a significant reduction in the acetylcholine sensitivity compared to control values (Table 9 page 98). In three of the samples, the anti-(acetylcholine receptor) titre was not significantly different from control values, but it is possible that the reduction in sensitivity observed resulted from an antibody directed at the toxin binding site that was not measured in the radioimmunoassay.

The suggestion that the anti-(toxin site) antibody is a major pathogenic agent has been severely challenged by the findings of both Lennon and Lambert (1980) and Richman *et al*(1980). Both groups have been successful in the production of monoclonal rat antibodies reactive with muscle acetylcholine receptor and have found that the antibodies raised are capable of causing an impairment of neuromuscular transmission in mice, rats and guinea pigs and that these same antibodies bind to an extracellular determinant of the membrane bound acetylcholine receptor which is distinct from the binding site of cholinergic ligands.



Thus, these observations suggest that the sub-population of anti-(toxin site) antibodies is not the key factor in the pathogenesis of myasthenia gravis. A more likely explanation for the results obtained by the presence of anti-(toxin site) antibody is that the inhibition of  $^{125}\text{I}$ - $\alpha$  bungarotoxin binding may be the result of steric hindrance by extensive binding of antibodies to sites on the acetylcholine receptor other than the acetylcholine site (Wonnacott *et al* 1980).

In conclusion, the detection of anti-(acetylcholine receptor) antibodies in the serum of myasthenic patients is a convenient and useful aid to diagnosis for the clinician. Prior to this recent advancement, the two most frequently used diagnostic tools for myasthenia gravis were edrophonium testing and repetitive nerve stimulation. While highly characteristic of myasthenia gravis these tests may yield positive results in disorders other than myasthenia. Therefore, a positive radioimmunoassay result can usefully serve as an additional criterion although in many cases of ocular myasthenia gravis, where frequently circulating anti-(acetylcholine receptor) antibodies are absent, the test may prove negative. The results reported here and indeed substantiated by other groups (see Lindstrom 1979) support the proposal that overall anti-(acetylcholine receptor) antibody level is not the major factor in myasthenia gravis but may well reflect a more fundamental process such as antigenic modulation. From studies of animals with EAMG, it is now becoming increasingly accepted that the aetiology of myasthenia gravis is more intimately concerned with an actual decrease in receptor concentration at the motor end-plate as a result of antigenic modulation rather than a decrease in functional receptor by the binding of antibody (see Introduction page 46). Thus it is important that now

the disease process is more fully understood, the initiating factor to the autoimmune reaction should be further investigated. As discussed in the Introduction (page 57), the most plausible explanation for the onset of myasthenia gravis is a breakdown in immunotolerance as expressed in the suppressor T cells. The question then is, whether reaction is specific to the acetylcholine receptor rather than to other host antigens. This question may be related to the known presence of  $^{125}\text{I}$ - $\alpha$  bungarotoxin binding components in the thymus gland (Heilbronn 1979).

The purification of the acetylcholine receptorAcetylcholine receptor content of human muscle

The first major problem encountered in the purification of the nicotinic acetylcholine receptor from human muscle was the initial concentration of the protein which was both variable and very low. It was found that the mean value for the acetylcholine receptor concentration was 0.7 pmol <sup>125</sup>I- $\alpha$  bungarotoxin binding sites per gram muscle with a range which varied between 0-2 pmol toxin binding sites per gram muscle. These values compared unfavourably with those for denervated mammalian muscle (with concentrations of receptor of up to 128 pmol/g Almon et al 1974) and for electric fishes, particularly *Torpedo marmorata* where reported values for the receptor concentration exceed 1700 pmol/g tissue (Eldefrawi and Eldefrawi 1973(b); Table 1 page 24). It is clearly not possible to surgically denervate human muscle to increase the endogenous receptor concentration (Introduction page 23) thus this problem is not resolvable.

There are several possible explanations for the variability of the acetylcholine receptor content of skeletal muscle. Firstly, there is the possibility that the receptor undergoes proteolytic digestion in the interval between amputation and freezing at -80°C. It was usual practice to keep this interval to a maximum of 1h.

Secondly, muscle stored at -80°C may lose acetylcholine receptor activity during the freeze-thaw cycle or during prolonged storage at -80°C. It became usual to use the muscle immediately following amputation or after a minimum storage time at -80°C and therefore the frozen muscle was never kept for more than two months.

An alternative explanation for the variability is that the endogenous acetylcholine receptor content of the muscle samples varied. The majority of limb amputations were carried out because of vascular problems. Thus, it is possible that under these circumstances, the muscles are ischaemic and this may lead to tissue autolysis. Secondly, patients undergoing amputation were, in general, over sixty years old. Tomlinson and Irving (1977) have shown that beyond sixty years, the number of motor neurones in the lumbrospinal segments decreases sometimes by up to 50% compared to control samples. Therefore, the innervation of the muscle fibres is reduced and denervation effects together with muscle atrophy may be observed. The denervation effects may be apparent in the higher concentration of acetylcholine receptor extracted from some muscle samples.

#### The purification of the acetylcholine receptor protein

The method used initially for the purification of the receptor was based on that used routinely in our laboratory for the purification of the protein from *Torpedo marmorata* (Wonnacott *et al* 1980). However, the preliminary results obtained (Table 11 page 116) showed that whilst this method was satisfactory for *Torpedo* where the initial concentration of receptor is very high, it was not wholly suitable for the purification of the receptor from innervated human muscle.

The initial investigations into the purification procedure looked to increasing the yield of the purified product. Obvious points in the purification scheme where the activity could be lost are the chromatographic steps involving the affinity beads and the ion-exchanger. It was shown that the batch elution of the receptor from the affinity beads was not 100% efficient as the use of a column elution method at

room temperature, as recommended by Karlsson *et al* 1974, increased the yield of purified product (Table 13 page 119). No loss of receptor activity during ion-exchange chromatography could be demonstrated as repeated dialysis of carbachol eluates and the measurement of their receptor activity showed that these values were always considerably lower than the activity recovered after ion-exchange chromatography. This observation may be explained by the presence of residual carbamoylcholine.

The early increases in yield of acetylcholine receptor were not accompanied by significant increases in specific activity. Silman *et al* (1978) reported that the use of protease inhibitors in the extraction of acetylcholinesterase from chicken muscle prevented degradation of the intrinsic molecular forms. Similarly, Dolly and Barnard (1975), Barnard *et al* (1979) and Lindstrom *et al* (1979a) all introduced anti-protease measures into their purification schemes for acetylcholine receptor from mammalian muscles. These included the addition of specific anti-protease reagents, an accelerated purification time and the maintenance of temperature throughout the procedure at 4°C (Lindstrom *et al* 1979a). These modifications were systematically introduced into the purification scheme.

Initial experiments included the anti-protease agents without changing any other factors. The presence of lysosomal enzymes has been demonstrated in the muscle cells of immature animals (Gutmann *et al* 1976) where the release of these enzymes is thought to stimulate the formation of the nerve-muscle junction; in adult rat diaphragm (Parsons and Pennington 1976) and in human muscle (Bury and Pennington 1975). Although the presence of lysosomes in muscle cells is not finally established Cullen *et al* (1979), on the basis of morphological and

cytochemical evidence, have proposed that with respect to lysosomal function the sarcoplasmic reticulum has an analogous role to that of the endoplasmic reticulum. Thus, it is probable that following homogenisation and extraction with Triton X100, proteolytic enzymes are liberated and a number of reagents were added to inhibit these. EDTA was used to inhibit  $\text{Ca}^{2+}$  activated muscle protease (Dayton *et al* 1976). Benzamidine has been used in the isolation of procollagen where extensive inhibition of proteolysis is needed to prevent its conversion to collagen (Byers *et al* 1975) and was accordingly included. Phenylmethylsulphonyl fluoride reacts irreversibly at the active site of serine esterases (Gold 1967), and benzethonium chloride is a bactericidal agent (Povl Elo 1963). Bacitracin has a potent effect in the prevention of the degradation of glucagon by microsomal extracts (Desbuquois *et al* 1974) while pepstatin has been shown to be effective in blocking the lysosomal proteases from rat liver (Dean 1976) and more recently the lysosomal proteases of rat skeletal muscle (Schwartz and Bird 1977). Finally,  $\text{NaN}_3$  inhibits bacterial growth.

The use of anti-protease agents alone did not increase the specific activity of the purified receptor (Table 14 page 120). It was only when both addition of anti-protease agents and an accelerated purification time were combined that the specific activity of the receptor was comparable to literature values quoted for other mammalian receptors (Table 15 page 121) viz, 0.5-3.5  $\mu\text{mol/g}$  protein.

#### The criteria for purity of the acetylcholine receptor

The homogeneity of the isolated acetylcholine receptor was extensively investigated.

Because of the very low amounts of purified material the initial analyses were made using radioiodinated receptor. The only problem encountered with the iodination procedure was that both the receptor and the detergent, Triton X100 were radiolabelled. This was not unexpected as Triton X100 contains a phenolic group which is analogous to the iodinated tyrosine residue in proteins. In all experiments concerning the  $^{125}\text{I}$ -acetylcholine receptor therefore,  $^{125}\text{I}$ -Triton X100 was always included as the control.

#### Isoelectric focussing of the acetylcholine receptor

The examination of the homogeneity of the purified receptor from human muscle by radioiodination and isoelectric focussing involved very low amounts of protein (of the order of 1-2ng) and therefore it was felt that the validity of the system should be established. This was done by the radiolabelling and isoelectric focussing of comparable concentrations of acetylcholine receptor purified from *Torpedo marmorata* for which the isoelectric point is well documented in the literature. It was found that  $^{125}\text{I}$ -acetylcholine receptor purified from *Torpedo marmorata* focussed as a sharp band at pH 4.9 and this was in agreement with other reported values (Table 21). The purified receptor from *Torpedo marmorata* labelled with  $^{125}\text{I}$ - $\alpha$  bungarotoxin was found to focus as a sharp band at pH 5.65 and other values are given in Table 21 .

In preliminary measurements of the isoelectric point of the  $^{125}\text{I}$ -labelled human acetylcholine receptor, two peaks with variable intensity were observed at pH 5.6 and pH 6.6 respectively suggesting heterogeneity of the purified protein. However, a study of the time course of the changes in the isoelectric point of the receptor showed

Table 21. Isoelectric points of the acetylcholine receptor

<u>Species</u>	<u>Isoelectric point</u>	<u>Reference</u>
<i>Torpedo marmorata</i>	* R 4.8	Eldefrawi and Eldefrawi 1973
<i>Torpedo marmorata</i>	+ RT 5.3	Sobel et al 1978
<i>Torpedo californica</i>	R 4.9	Heilbronn et al 1974
<i>Electrophorus electricus</i>	R 4.7 RT 5.2	Biesecker 1973
<i>Electrophorus electricus</i>	RT 5.15	Raftery and Schmidt 1971
Cat denervated muscle	RT 5.1	Dolly and Barnard 1977
Cat denervated muscle	R 5	Shorr et al 1978
Embryonic skeletal muscle	R 6.5-6.7	Merlie et al 1978
Rat denervated muscle	RT 5.3	Brockes and Hall 1975
Rat innervated muscle	RT 5.1	Brockes and Hall 1975
Human innervated muscle	R 6.6 RT 5.1	This thesis

\* R represents native receptor

+ R-T represents receptor labelled with  $\alpha$ -bungarotoxin



this not to be the case (Results pages 131 to 136). When the isolated receptor was radiolabelled and subjected to isoelectric focussing immediately following purification, it focussed as a sharp band at pH 6.6 (FIG. 27 page 134). If the  $^{125}\text{I}$ -acetylcholine receptor was stored at  $4^{\circ}\text{C}$  and then analysed by isoelectric focussing, the receptor focussed as two distinct components with isoelectric points of 6.6 and 5.3 (FIG. 28). The formation of the second component may be a result of either proteolysis or radioautolysis. However, storage of unlabelled receptor at  $4^{\circ}\text{C}$  followed by radioiodination similarly led to a shift of pI from 6.6-5.6 over a period of three weeks, thus disproving the autoradiolytic damage proposal. Further, in support of proteolysis, an analysis of the  $^{125}\text{I}$ -acetylcholine receptor by SDS-PAGE following isoelectric focussing showed loss of subunit structure with storage of the receptor. The results could also be interpreted in terms of an induced conformational change on radioiodination followed by a slow reversion to the more stable state, and with an isoelectric point more similar to the receptors of other species. Reported values are given in Table 21. In general, the isoelectric point of the  $^{125}\text{I}$ -human acetylcholine differs markedly from those values for unlabelled receptor, the exception being that reported by Merlie *et al* (1978) for receptor extracted and purified from foetal calf skeletal muscle cells. Evidence exists that the iodination of a protein can cause an alteration of the isoelectric point (Hunter 1978). The direction of the change will presumably depend on the local Van der Waals and hydrogen bond-induced electrostatic interactions which could only be predicted by a detailed knowledge of the tertiary structure of the protein.

Purified human acetylcholine receptor labelled with  $^{125}\text{I}-\alpha$  bungarotoxin was found to focus at pH 5.1 and was more in agreement with the reported values for the receptor-toxin complex of other species (Table 21). The similarities of isoelectric points for the human receptor-toxin complex and other determinations provides supportive evidence for a conformational change of the human receptor following iodination. The shift in the isoelectric point of the acetylcholine receptor following the binding of  $^{125}\text{I}-\alpha$  bungarotoxin is usually in the direction to a higher pH consistent with the basic nature of  $\alpha$  bungarotoxin (Table 21). The fact that the isoelectric point of the human receptor moves in the opposite direction may well be a function of the anomalous pI of the  $^{125}\text{I}$ -human acetylcholine receptor as discussed above.

#### Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis under non-denaturing conditions of the purified acetylcholine receptor from human muscle labelled with  $^{125}\text{I}-\alpha$  bungarotoxin gave one broad peak of radioactivity (FIG.38 page 147). Similar results have been reported by Dolly and Barnard (1977); Meunier *et al* (1974); Eldefrawi and Eldefrawi (1973) and Klett *et al* (1973) for acetylcholine receptor purified from cat, rat and mouse denervated muscle; *Electrophorus electricus*, *Torpedo marmorata* and *Electrophorus electricus* respectively. The distribution of the radioactivity as a broad band rather than a sharp band has been suggested by Sobel *et al* (1977) as evidence for microheterogeneity.

Sucrose gradient analysis

Purified acetylcholine receptor from human muscle labelled with  $^{125}\text{I}$ - $\alpha$  bungarotoxin was shown to sediment as a single component in a sucrose density gradient with a sedimentation coefficient of  $S_{20,w} = 9.5\text{S}$ . Similar values have been reported for other species (Table 22). Also, values determined for native acetylcholine receptor are of the order  $S_{20,w} \approx 9\text{S}$  (Table 22) showing that there was no major alteration of the sedimentation coefficient following the binding of  $\alpha$  bungarotoxin. There was no evidence to indicate the presence of a second component with a higher sedimentation coefficient as has been found for both *Torpedo californica* (Reynolds and Karlin 1978) and *Torpedo marmorata* (Barnard *et al* 1978). This suggests that the purified receptor from human muscle is not associated as a dimer in dilute detergent solution.

It was estimated from the approximation of Schachman (1959) that the receptor from human muscle had a molecular weight of the order 175-2000000 (Results page 141). However, it is highly probable that this value is an under-estimation of the molecular weight of the protein. Tanford *et al* (1974) have shown that there is a significant decrease in the buoyant density of protein following the binding of either lipid material or detergents. Thus, it follows that there is an increase in the partial specific volume of the protein, and as the molecular weight of a protein is related to the sedimentation coefficient by the formula,

$$M = \frac{RTS}{D(1-\bar{v}\rho)}$$

Where  $\bar{v}$  is the partial specific volume, it follows that an apparent increase in  $\bar{v}$  would yield an erroneously low value for S hence a low

Table 22. Sedimentation coefficients of the acetylcholine receptor

Species	Sedimentation coefficient	Reference
<i>Torpedo marmorata</i>	R 9 R 9, 13	Sobel et al (1977) Barnard et al (1978)
<i>Torpedo marmorata</i>	R 8.6	Eldefrawi and Eldefrawi (1973)
<i>Torpedo californica</i>	R 8.6, 12.8	Reynolds and Karlin (1978)
<i>Torpedo nobiliana</i>	R 9	Froehner et al (1977)
<i>Electrophorus electricus</i>	R 9	Biesecker (1973)
Cat denervated muscle	R 9 RT <sup>+</sup> 9	Barnard et al (1978)
Rat denervated muscle	R 9 RT 9	Froehner et al (1977)
Embryonic skeletal muscle (calf)	R 9.5 RT 9.5	Merlie et al (1978)
Mouse muscle cell line	R 9.5 RT 9.5	Boulter and Patrick (1977)
Human	RT 9.5	This thesis

\* R native receptor

+ R-T receptor labelled with  $\alpha$  bungarotoxin

molecular weight with respect to marker enzymes that do not bind detergent. The problem can be circumvented with no knowledge of the percentage of detergent bound to a protein by adjusting the solvent density with D<sub>2</sub>O to blank out the contribution of bound detergent to the sedimentation coefficient (Reynolds and Tanford 1976). Using this technique, Reynolds and Karlin (1978) found that the molecular weight of the receptor from *Torpedo californica* with sedimentation coefficient  $s_{20,w} = 8.6S$  was 250000 with a corresponding molecular weight for the dimer of 500000. If it is assumed that the native receptor purified from human muscle behaves identically to the receptor of *Torpedo californica* in detergent solution, it will also have a molecular weight of the order 250000 (Reynolds and Karlin 1978).

#### The subunit composition of the acetylcholine receptor

The subunit composition of the purified acetylcholine receptor from human muscle was determined by two different methods. Firstly, <sup>125</sup>I-acetylcholine receptor was eluted from the peak slice of isoelectric focus gels and then examined by SDS-PAGE (Methods, page 102). The distribution of radioactivity suggested the presence of two subunits with molecular weights  $66000 \pm 3300$ ;  $42000 \pm 2100$  (FIG. 40 page 152). Secondly, the sub-unit composition was examined by application of the isolated receptor protein directly to SDS-PAGE and the subunits were identified by staining with Coomassie G250. Essentially identical results were found as for the <sup>125</sup>I-acetylcholine receptor with two major subunits of molecular weights  $68000 \pm 3400$ ;  $42000 \pm 2100$  (FIG. 41 page 153). Additional bands at  $56000 \pm 2800$ ,  $85000 \pm 4250$  and at the top of the gel were sometimes observed. The latter two bands probably resulted from the formation of aggregated material.

There is considerable debate in the literature as to the subunit composition of the well-characterised acetylcholine receptors from the electric fishes and denervated mammalian muscle. Reported subunit patterns are of two types, indicating either an oligomeric structure of identical subunits or of different molecular weight subunits. Several groups have reported that the acetylcholine receptor purified from *Torpedo* has four subunits  $\alpha$   $\beta$   $\gamma$   $\delta$  with relative stoichiometries of 2:1:1:1 (Table 23). Conversely, Sobel *et al* (1977) and Saitoh *et al* (1979) have found that acetylcholine receptor purified from *Torpedo marmorata* consists of one major subunit (Table 23). Similar differences have been found with acetylcholine receptors purified from mammalian muscle (Table 23). The data reported here, agree most closely with those reported by Kemp *et al* (1980) for denervated rat muscle with subunits of molecular weight 80000; 68000; 52000 and 40000. The absolute values determined for the subunits are found to be dependent on both the gel system (Lydiatt *et al* 1979) and the percentage of acrylamide (Nathanson and Hall 1980).

In all the reported purifications, the acetylcholine receptor is purified to similar specific activities and in some cases, the observed differences can perhaps be explained in terms of species variation. However, it has been suggested (Lindstrom 1979) that the preparations that appear to be composed of predominantly one polypeptide chain are the result of proteolytic damage during purification. But, Barnard *et al* (1979), Merlie *et al* (1978) and Sobel *et al* (1977) all include anti-proteolytic measures in their purification schemes. Alternatively, Lindstrom (1979) suggests that on solubilisation from the membrane, the acetylcholine receptor is

Table 23. The subunit composition of the acetylcholine receptor

Species	Subunit composition			Reference
<i>Torpedo marmorata</i>	40000			Sobel et al (1977)
<i>Torpedo marmorata</i>	38-40000	50000	57000 64000	Barnard et al (1979)
<i>Torpedo californica</i>	38-40000	50000	57000 64000	Lindstrom et al (1979a)
<i>Electrophorus electricus</i>	38-40000	50000	57000	Lindstrom et al (1979a)
Cat denervated muscle	41000			Shorr et al (1978)
Rat denervated muscle	45000	49000	51000 56000 62000 110000	Froehner et al (1977)
rat innervated muscle	45000	49000	51000 56000 66000	Nathanson and Hall (1979)
rat denervated muscle	42000	66000		Kemp et al (1980)
Embryonic skeletal muscle (culture)	41000			Merlie et al (1978)
Mouse muscle cell line	44000	53000	65000 72000	Boulter and Patrick (1977)
Embryonic skeletal muscle (calf)	38000	50000	57000 64000	Lindstrom et al (1979a)
Chicken muscle	42000	52000	62000	Barnard (personal communication)
Human innervated muscle	42000	66-68000		This thesis

irreversibly associated in mixed detergent micelles with contaminating proteins. What is conserved between all species is the homology of the 40000 subunit which carries the acetylcholine binding site (for further discussion, page 215).

#### The purity of the acetylcholine receptor

The reported specific activities for the purified acetylcholine receptor from mammalian species vary between  $0.19\mu\text{mol/g}$  protein to the highest reported value of  $11\mu\text{mol/g}$  protein (Table 24). The data that are reported here are in the range of the published literature values.

Based on the determination of the subunit structure and the estimated molecular weight of the purified human receptor, it is possible to propose that the protein consists of one 42000 subunit that carries the acetylcholine binding site and three 66000 subunits, in which case the purified protein would have a theoretical specific activity of  $4.16\mu\text{mol/g}$  protein. A second model is one where there are two of each subunit with a theoretical specific activity of  $9.26\mu\text{mol/g}$  protein. The highest specific activities reported here approach the former value however no conclusions can be drawn until further information is available regarding both the stoichiometry of the subunits and the binding of the cholinergic ligands. The lower values obtained in this work could be the result of non-receptor protein contamination or because some of the receptor molecules are functionally altered during the purification procedure such that they no longer



Table 24. The specific activity of purified acetylcholine receptors of mammalian species

Species	Specific activity $\mu\text{mol/g}$ protein	Reference
Cat denervated muscle	10-11	Shorr et al (1978)
Rat denervated muscle	0.53 2.8 - 7.5	Brockes and Hall (1975) Kemp et al (1980)
Rat innervated muscle	0.19 2.1 ; 6.4	Brockes and Hall (1975) Kemp et al (1980)
Embryonic skeletal muscle (calf)	Value not expressed as /g protein	Merlie et al (1978)
Mouse muscle cell line	2.6	Boulter and Patrick (1977)
Human innervated muscle	0.5 - 3.5	This thesis

bind  $\alpha$  bungarotoxin. The copurification of receptor and non-receptor proteins through the scheme employed is possible although it is probably more likely that the relatively low specific activity of the final product could result from an over-estimation of the protein concentration of the purified receptor. The protein assay used was the Coomassie G250 microassay (Methods page 109) and it was reproducible within the range 0.5-10 $\mu$ g. The standard protein was always bovine serum albumin and it was always dissolved in the same buffer that was used for the elution of the purified receptor from the ion-exchange column to eliminate any solvent effects. However, the receptor is an integral membrane protein and contains bound molecules of detergent (Meunier *et al* 1972; Karlin *et al* 1979) and therefore may behave differently from the standard protein.

With regard to the possibility that some of the receptor molecules may lose their ability to bind  $\alpha$  bungarotoxin during the purification process it is known that Triton X100 inhibits receptor function (Fischbach and Lass 1978) and so solubilisation may contribute to a decrease in  $\alpha$  bungarotoxin binding activity. Secondly, during the purification procedure, the receptor is exposed to a high concentration of agonist, carbamoylcholine and it has been suggested that this may desensitise some or all of the receptor molecules. The desensitisation would take the form of a slow change in conformation and could lead to a reduction in the ability to bind  $\alpha$  bungarotoxin (Heidmann and Changeux 1979). A desensitisation effect would partly explain the low recovery of purified acetylcholine receptor from the detergent extract. In all preparations, the yield of purified protein never exceeded 16% expressed as the recovery of  $^{125}$ I- $\alpha$  bungarotoxin binding activity. Thus a decrease in binding ability following

desensitisation may lead to an overall underestimation of receptor recovery. Further, the subunit composition determined by analysis of the purified  $^{125}\text{I}$ -acetylcholine receptor was the same irrespective of the specific activity of the particular preparation. In initial purifications of the receptor, the exposure to high concentrations of carbamoylcholine was prolonged (48h as compared to 11h in the modified procedure). Colquhoun and Rang (1976) found that the inhibition of toxin binding to both membrane-bound and detergent-solubilised rat denervated receptor, by agonists developed more slowly than for antagonists. They proposed that the observed slow rate of inhibition was not accounted for in terms of receptor occupancy and that desensitisation of the receptors was a more plausible explanation.

By far the most important factor in the purification of the human acetylcholine receptor seemed to be the elimination of proteolysis. However, one observation contradictory to this is that detergent solubilised receptor extracts retain  $^{125}\text{I}$ - $\alpha$  bungarotoxin binding activity after storage of up to two months at  $4^{\circ}\text{C}$ . Further, it was found that storage of the detergent extract containing solubilised acetylcholine receptor for up to one week at  $4^{\circ}\text{C}$  retained 93%  $^{125}\text{I}$ - $\alpha$  bungarotoxin binding capacity with no apparent increase of low molecular weight species as observed by SDS-PAGE (Results, page 122). In this context it is relevant that Bartfeld and Fuchs (1979) reported that tryptic digestion of the acetylcholine receptor from *Torpedo californica* did not destroy the  $\alpha$  bungarotoxin binding activity of the receptor but did reduce all the subunits to a uniform polypeptide of molecular weight 27000. However it was found in our laboratory that digestion of the receptor from *Torpedo marmorata* with either chymotrypsin or trypsin completely abolished its ability

to bind  $^{125}\text{I}$ - $\alpha$  bungarotoxin and decreased the antigenicity by 60% (H. Morris and S. Wonnacott, personal communication).

### The affinity labelling of the acetylcholine receptor

The labelling procedure with the affinity reagents enables the acetylcholine binding site to be identified. It was found that one component with molecular weight  $40000 \pm 2000$  was labelled with the affinity reagent  $[\text{}^3\text{H}]\text{-MBTA}$  in a preparation of membranes from human muscle. Similarly, the  $42000 \pm 2100$  subunit of purified acetylcholine receptor was specifically labelled with  $[\text{}^3\text{H}]\text{-MBTA}$  and the components were assumed to be identical. The results agree with affinity labelling studies of other groups (Table 25) with the exception of Nathanson and Hall (1979) who found that in both innervated and denervated rat, two polypeptides were labelled (Table 25).

Table 25. The affinity labelling of the acetylcholine receptor

Species	Affinity ligand	Labelled subunit	Reference
<i>Torpedo marmorata</i>	$[\text{}^3\text{H}]\text{-MPTA}$	40000	Sobel et al (1977)
<i>Torpedo californica</i>	$[\text{}^3\text{H}]\text{-MBTA}$	40000	Weill et al (1974)
<i>Torpedo ocellata</i>	$[\text{}^3\text{H}]\text{-MBTA}$	40000	Rubsamen et al (1978)
Cat denervated muscle	Bromo $[\text{}^3\text{H}]\text{-acetylcholine}$	43000	Lyddiatt et al (1979)
Rat denervated muscle	$[\text{}^3\text{H}]\text{-MBTA}$	45000 49000	Froehner et al (1977)
Rat innervated muscle	$[\text{}^3\text{H}]\text{-MBTA}$	45000 49000	Nathanson and Hall (1979)
Human innervated muscle	$[\text{}^3\text{H}]\text{-MBTA}$	42000	This thesis

The glycoprotein properties of the human acetylcholine receptor

The human acetylcholine receptor was found to contain carbohydrate as measured by its reaction with immobilised lectins (Results, page 160). It was found that concanavalin A and lens culinaris immobilised lectins adsorbed 37% and 46% of the acetylcholine receptor activity respectively from a detergent extract containing solubilised acetylcholine receptor. Conversely, immobilised soybean lectin (Type VI) adsorbed only 3% of the acetylcholine receptor activity relative to the control value. These results suggest the presence of mannose and/or glucose in the carbohydrate moiety of the glycoprotein and are consistent with those found by other workers for receptor from other sources (Introduction page 31). Shorr *et al* (1978) and Brockes and Hall (1975) have shown that purified receptor from denervated cat muscle and denervated rat muscle binds to both immobilised lens culinaris lectin and concanavalin A and have used this property in the purification of the receptor protein. Further, in agreement with the results found for the human receptor, Boulter and Patrick (1977) showed that the acetylcholine receptor from a non-fusing mouse muscle cell line was retained by a concanavalin A agarose conjugate but not by soybean agglutinin. These observations are interpreted to mean that the receptor contains no accessible D-galactose or N-acetyl-D-galactosamine and therefore apparently differs from that of *Electrophorus electricus* where the presence of N-acetyl-D-galactosamine has been demonstrated (Meunier *et al* 1974).

The immobilised lectins adsorbed 37% and 46% of the total soluble receptor activity of human muscle despite being present in at least a hundred fold excess. It has been suggested that there may be a heterogeneous population of acetylcholine receptors and that the

microheterogeneity may be related to the carbohydrate moiety (Mittag et al 1978, Wonnacott et al 1980).. This may explain the results obtained if only approximately 40% of the population of receptors contain either glucosyl or mannosyl residues in arrangements with affinity for concanavalin A and lens culinaris lectin. The function of the carbohydrate of the receptor is not known. In general, it is thought that the carbohydrate portion of membrane glycoproteins is important in cell surface recognition (Harrison and Lunt 1980). Thus, it is conceivable that it may be instrumental in the reception of acetylcholine. However, concanavalin A treatment of cultured muscle cells (Boulter and Patrick 1977) or a frog nerve-muscle preparation (Dolly 1979) did not affect the response to iontophoretically applied acetylcholine or carbamoylcholine induced contraction. Moreover, removal of the carbohydrate from purified receptor of *Torpedo marmorata* has no effect on the antigenicity of the protein (Wonnacott et al 1980) or on toxin binding (Wonnacott et al in press). It may have a more general role involved in synthesis and insertion in the membrane (Harrison and Lunt 1980).

#### The binding properties of the human acetylcholine receptor

##### The reaction with $^{125}\text{I}$ - $\alpha$ bungarotoxin

When the binding of  $^{125}\text{I}$ - $\alpha$  bungarotoxin to purified human receptor was studied, regular second order kinetics were followed and a value of  $3.46 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$  was determined for the rate constant  $k_{\text{on}}$  (Results page 163). The dissociation of the receptor-toxin complex was a much slower process, with a value for the dissociation rate constant of  $k_{\text{off}} 2.4 \times 10^{-6} \text{ s}^{-1}$  (Results page 164). The results obtained are compatible with the known almost irreversible

nature of  $\alpha$  bungarotoxin binding and when combined give a value of  $6.9 \times 10^{-10}$  M for the dissociation constant  $K_D$ . When the dissociation constant was determined directly, a value of  $5 \times 10^{-10}$  M was found for purified receptor and  $6 \times 10^{-10}$  M for detergent solubilised receptor (Results page 165). The values determined for  $K_{on}$  are of the same order of magnitude as those found in the literature for acetylcholine receptors from mammalian muscle (Table 26) and agree particularly well with the measurement by Vincent and Newsom-Davis (1979) for crude receptor-containing extracts of human muscle. The reported rate of association constants are similar to those seen *in vivo*. Lester (1972) found that the rate constant for the blockade of frog muscle by  $\alpha$  toxin from *Naja naja siamensis* was  $1.5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ . Brockes and Hall (1975) showed that the acetylcholine receptor-toxin complexes from both denervated and innervated rat muscle dissociated in a biphasic manner and that this observation was consistent with two toxin binding components. Vincent and Newsom-Davis (1979) reported similar results for crude human acetylcholine receptor. However, the results reported here for the dissociation of the purified human receptor-toxin complex showed no evidence of a biphasic dissociation.

The dissociation constant of the complex between acetylcholine receptor of human muscle and  $^{125}\text{I}$ - $\alpha$  bungarotoxin is of the same order of magnitude as for the corresponding receptor-toxin complex from other mammalian species (Table 26). Also, the dissociation constants found for the crude and purified acetylcholine receptor are very similar thus it is apparent that there has been no appreciable alteration in the affinity of the receptor for  $^{125}\text{I}$ - $\alpha$  bungarotoxin following purification. A Hill plot for the binding of  $^{125}\text{I}$ - $\alpha$  bungarotoxin to the purified receptor suggested the presence of

Key to Table 26

- 1 Brockes and Hall (1975 )
- 2 Colquhoun and Rang (1976)
- 3 Almon and Appel (1976)
- 4 Dolly (1979)
- 5 Barnard et al (1977)
- 6 Lindstrom (1977)
- 7 Vincent and Newsom-Davis (1979)



Table 26. Kinetic constants for the reaction of  $\alpha$  bungarotoxin with the acetylcholine receptor of mammalian muscle

Muscle	Preparation	Temperature (°C)	Kinetic Constants			Reference
			$K_{on}$ ( $10^5 M^{-1} s^{-1}$ )	$k_{off}$ ( $10^{-6} s^{-1}$ )	$K_D$ ( $10^{-10} M$ )	
Rat innervated diaphragm	Partly purified	35	1.3 0.5	48 1.9	3.7 0.4	1
Rat denervated diaphragm	Partly purified		3.2 0.9 0.3 0.8	55 1.9 0.3 -	1.7 0.2 0.1 -	1 1 2 2
Rat innervated leg	Crude extract Purified by chromatography on a concanavalin A Sepharose column	14 4	-	-	23	3
Rat denervated leg	Crude extract Purified by chromatography on a concanavalin A Sepharose column	4	-	-	12.2	3
Cat denervated leg	Partly purified Membrane bound	25	0.6 0.3	4.7 -	0.8 -	4 5
Human innervated leg	Crude extract	4	-	-	2.09	6
Human ischaemic calf muscle	Crude extract	23	1.9	32	1.68	7
Extra ocular muscle	Crude extract	23	1.3	32	.051 2.46	7 7
Human ischaemic muscle - denervated	Crude extract	23	2.4	48 1.2	.06 2.0 .05	7 7 7

negative cooperativity since the gradient of the analysis was 0.84. The apparent negative cooperativity may be a true effect or may be an artefact of the assay conditions. This could arise from either the "retention effect" (Silhavy et al 1975) where diffusion of dissociated radio-ligand away from the free receptor site is hampered because of the high concentration of unoccupied sites, or from a heterogeneity of binding sites that was not detected in the determination of the dissociation constant by the Scatchard analysis (De Lean and Rodbard 1979). Further experimentation is clearly required to resolve this question. Eldefrawi and Eldefrawi (1973) and Weber and Changeux (1974) both demonstrated the presence of a positive cooperative interaction between [<sup>3</sup>H]-acetylcholine and acetylcholine receptors from *Torpedo*. Further, at high ligand concentrations, Eldefrawi and Eldefrawi (1973) showed that this interaction showed opposite effects and tentatively related the phenomenon to that of desensitisation.

#### The reaction with d-tubocurarine

It was found that the dissociation constant between the purified receptor and d-tubocurarine is 0.25 $\mu$ M (Results page 168). when measured by the displacement of <sup>125</sup>I- $\alpha$  bungarotoxin binding at equilibrium and 0.18 $\mu$ M (Results, page 168). when measured by the retardation of the initial velocity of the reaction between receptor and <sup>125</sup>I- $\alpha$  bungarotoxin. There are certain caveats associated with both methods of determination. In the method where the displacement of <sup>125</sup>I- $\alpha$  bungarotoxin is measured at equilibrium, it is assumed that the toxin and ligand sites are mutually exclusive and that true

equilibrium is attained. This is usually ensured by approaching the equilibrium position from both directions. In the second method, again it must be assumed that toxin and cholinergic effector occupy the same site and that the rate of  $^{125}\text{I}$ - $\alpha$  bungarotoxin binding is not limited by a diffusion factor. Despite the limitations of each method, good agreement was obtained between the two evaluations. Similarly, Weber and Changeux (1974) obtained close agreement between the dissociation constants measured by both methods for several cholinergic ligands. In fact the derivation of  $K_D$  and  $K_P$  (Introduction page 33) shows that indeed they are identical if it is assumed that the rate of dissociation of the receptor-toxin is negligible with respect to the rate of association. The values obtained for the respective rate constants for the human acetylcholine receptor (Results, page 163) indicate that this assumption is valid.

The values obtained for the  $K_D$  between human receptor and d-tubocurarine are in agreement with the values in the literature for the acetylcholine receptors of other species (Table 27) and is an order of magnitude lower than the value found *in vivo* for the  $K_D$  of receptor from frog muscle,  $2.4\mu\text{M}$  (Jenkinson 1960). The differences between values may be explained in terms of species differentiation. Brockes and Hall (1975) were able to distinguish between junctional and extra-junctional receptors by differences in their binding properties with d-tubocurarine. However, their findings were not confirmed by Colquhoun and Rang (1976) nor did I find any evidence of heterogeneity of binding sites using purified human receptor.

Table 27. The dissociation constants for the binding of d-tubocurarine to the acetylcholine receptor

Species	Preparation	$K_D$ $\mu$ M Method *	Reference
<i>Electrophorus electricus</i>	Membrane	0.17R (naja $\alpha$ toxin)	Weber & Changeux (1974)
<i>Electrophorus electricus</i>	Purified	0.39I <sup>+</sup> <sub>S</sub> (C10)	Meunier & Changeux (1973)
<i>Torpedo marmorata</i>	Membrane	0.17R (naja $\alpha$ toxin)	Weber & Changeux (1974)
<i>Torpedo marmorata</i>	Membrane	0.17I (C10)	Weber & Changeux (1974)
Rat diaphragm (denervated)	Homogenate	0.4R ( $\alpha$ bt)	Colquhoun et al (1974)
Rat diaphragm (denervated)	Homogenate	0.22R ( $\alpha$ bt)	Colquhoun & Rang (1976)
Rat diaphragm (denervated)	Triton extract	0.38R ( $\alpha$ bt)	Colquhoun & Rang (1976)
Rat diaphragm (innervated)	Homogenate	0.24R ( $\alpha$ bt)	Colquhoun & Rang (1976)
Cat leg muscle (denervated)	Membrane	0.04R ( $\alpha$ bt)	Dolly (1979)
Rat diaphragm (innervated)	Purified	0.045R ( $\alpha$ bt)	Brockes & Hall (1975)
Rat diaphragm (denervated)	Purified	0.055R ( $\alpha$ bt)	Brockes & Hall (1975)

\* R (ligand)  $K_D$  determined by the retardation of binding of the specified ligand

+ I (ligand)  $K_D$  determined by the inhibition of binding at equilibrium of the specified ligand

§ C10 decamethonium

The immunological properties of the human acetylcholine receptor

It was found that the antigenicity of the purified human acetylcholine receptor was the same as that of the solubilised receptor of the crude detergent extract as judged by its ability to interact with anti-(acetylcholine receptor) antibodies from myasthenic sera (Results, page 176).

The purified receptor was found to produce an immune response in experimental animals with the production of anti-(human acetylcholine receptor) antibodies. However, in all three rabbits, despite an antibody titre of up to  $350 \times 10^{-10}$  M, no physical signs of EAMG were observed. There are several possible reasons for this result. Firstly, it is possible that the rabbits did develop a mild form of EAMG in which the physical signs of paralysis are not apparent and unfortunately, electrophysiological data were not available. Secondly, it was found that there was no cross-reactivity between the anti-(human acetylcholine receptor) antibody and the host rabbit receptor (Results page 178) even in the sera of highest antibody titre. In a converse experiment, a series of myasthenic serum samples were assayed for anti-(acetylcholine receptor) antibody with solubilised receptor from rabbit muscle as the antigen. Again, the cross-reactivity was very low (less than 1%) and if the initiation of EAMG is the result of the binding between the antibody produced to the immunogen, and the host acetylcholine receptor (Introduction page 45) then if there is no cross-reactivity between species, EAMG will not be induced.

Thirdly, although the immune serum raised did have a maximum anti-(human acetylcholine receptor) antibody titre of  $350 \times 10^{-10}$  M, the value is much less than an anti-(*Torpedo* acetylcholine receptor)

antibody titre of 3-30 $\mu$ M (Barkas *et al* 1978) produced in both rabbits paralysed by EAMG and chickens which developed transient symptoms of EAMG.

### The acetylcholine receptor: structure and function

Thus in summary, the nicotinic acetylcholine receptor from human muscle has been purified to homogeneity. It has an isoelectric point of pH 6.6 when radioiodinated and pH 5.1 when bound to  $^{125}\text{I}$ - $\alpha$  bungarotoxin. The purified receptor-toxin complex behaves as a single species upon analysis by polyacrylamide gel electrophoresis and sediments as a single component of  $S_{20,w} = 9.5\text{S}$  from which an approximate value for its molecular weight of 250000 has been calculated. The purified protein consists of two major subunits with molecular weights 42000 and 66000 and the 42000 subunit alone is labelled with the affinity ligand [ $^3\text{H}$ ]-MBTA. The receptor is a glycoprotein as shown by its reaction with immobilised lectins. The purified receptor resembles the crude detergent extracted receptor in that it possesses similar properties with respect to both the binding of cholinergic ligands and antigenicity.

Now that the receptor has been purified to homogeneity, further work should concentrate on a comparison of the properties of the isolated protein with those of receptors from other species that are already well characterised. The studies should include further investigation into the binding of cholinergic ligands; a detailed study of the stoichiometry of the subunit structure and precise measurements of the molecular weight of the protein and finally the amino acid composition and sequence of the isolated protein. Further, it would be interesting to examine the relationship between the isolated receptor and histri-

icotoxin to gain an insight into the ionophore structure, whether it is an integral part of the receptor molecule or a discrete entity. More intimately concerned with the early part of this thesis, is the fact that a pure antigen is now available and can be used in the radio-immunoassay for the detection of anti-(acetylcholine receptor) antibodies. Secondly, in relation to the antibody in myasthenic serum, the possibility of an alternative purification method is appealing. A preliminary report is given in this thesis (Results page 127) concerning the use of an antibody affinity column in the purification of the acetylcholine receptor.

However, more importantly, the purification of the nicotinic acetylcholine receptor from human muscle represents the isolation of the entity that transduces the chemical message carried by acetylcholine into muscle contraction. The 42000 subunit carries the acetylcholine binding site and the question arises as to the function of the second subunit.

The acetylcholine receptor has been shown to be multifunctional in that it is responsible for the binding of cholinergic agonists and antagonists which include the  $\alpha$  toxins. It has been suggested that the isolated protein also constitutes the cation translocation channel (Kasai and Changeux 1971) and carries binding sites for inhibitors of this process such as histrionicotoxin (Eldefrawi *et al* 1977) and local anaesthetics (see Heidmann and Changeux 1978). Further, the receptor of *Torpedo marmorata* and *Torpedo californica* has been shown to be highly immobilised in the subsynaptic membrane with limited rotational and translational movement. Thus, it is possible to propose a structural role for the 66000 subunit where it maintains the receptor in a stabilised form in the muscle membrane.

Alternatively, Saitoh *et al* (1980) have shown that a subunit of molecular weight 66000 from *Torpedo* electric organ is specifically labelled with the local anaesthetic 5-azido- $[\text{}^3\text{H}]$  trimethisoquin and thus is functionally and/or topographically related to the molecular device involved in the regulation of ion translocation.

The acetylcholine receptor of *Torpedo* has been visualised by the observation of receptor-rich membrane fragments in a scanning transmission electron microscope, as an asymmetric rosette like structure with a central pore (Zingsheim *et al* 1980). Zingsheim *et al* (1980) find components of 3 fold symmetry whilst Barnard *et al* (1980) find that the rosettes possess a five fold axis of symmetry and propose that the rotational symmetrical unit is a receptor subunit. Thus, a picture of the receptor emerges with the binding of acetylcholine to an  $\alpha$  subunit. The binding of the cholinergic ligand induces a conformational change through all the subunits which ultimately leads to the opening of the ionophore which is represented as the central portion of the rosette.

The *Torpedo* receptor has been shown to be a transmembrane protein by both histochemical techniques (Tarrab-Hazdai *et al* 1978) and by protease digestion of acetylcholine receptor-containing membrane vesicles (Huang 1979). Further, it is known to protrude 5-6nm above the extracellular surface in both muscle (Rosenbluth 1974) and in electric organ (Klymkowsky and Stroud 1979) and 1.3nm from the inner surface. It has a diameter as viewed perpendicular to the plane of the membrane of 8.6nm with a central hole of 2.5nm which narrows to a 0.65nm ion translocating pore. But, no data are yet available concerning the transmembrane properties of the individual polypeptides.



Thus, the "receptive substance" as visualised by Langley has been identified and extensively characterised. Although species differences are apparent, it is notable that the acetylcholine binding subunit has been conserved. Recent observations by Raftery *et al* (1980) have shown that the four subunits of *Torpedo californica* have distinct but homologous amino acid sequences. The reported results suggest that the genes encoding each of the four subunits descended from a single ancestral coding sequence and they propose that each subunit has evolved to perform a discrete function in the receptor complex. We can anticipate that in the near future these functions will be elucidated and we can look forward to a complete understanding of the total function of the acetylcholine receptor protein.

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## Chapter Sixteen

### ACETYLCHOLINE RECEPTOR ANTIBODY TITRES IN MYASTHENIA GRAVIS

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One of us first suggested in 1960 that myasthenia gravis was an autoimmune disease [1]. The suggestion was based on the association between myasthenia and other disorders subsequently shown to be autoimmune. The hypothesis was that a breakdown of immunological tolerance in myasthenic patients led to the development of antibodies directed against end plate receptors of skeletal muscle. It is only in the last four years, however, that the presence of such antibodies has been demonstrated in the sera of patients suffering from myasthenia gravis [2-4].

The end plate receptor of skeletal muscle is a nicotinic acetylcholine receptor and the study of its immunology has been facilitated by a combination of two discoveries. First, it was found that the electric organ of various species of electric fish contained a rich source of nicotinic acetylcholine receptors. These are pharmacologically identical to the receptor of the mammalian neuromuscular junction [5]. In contrast to a muscle fibre, which has a single synapse containing approximately  $4 \times 10^7$  acetylcholine receptors [6], a single electroplax cell from the electric organ of the eel, *Electrophorus electricus*, contains some  $2 \times 10^{11}$  receptors [7]. The cells of the electric ray *Torpedo* contain even higher concentrations of receptor [8]. Secondly, certain snake venom  $\alpha$ -toxins have been found to bind with high affinity and great specificity to the acetylcholine binding site of these receptors [9]. The  $\alpha$ -toxins have accordingly been used not only to purify the receptor, using affinity chromatography, but also, after radioactive labelling, to monitor the purification procedure in terms of acetylcholine binding sites. In this way the isolation of milligram quantities of receptor protein has been achieved [10] and the techniques so developed have been applied to the very much less readily available mammalian receptor [11]. In 1972, Patrick and Lindstrom [12] injected purified eel acetylcholine receptor into rabbits which subsequently developed myasthenia-like signs. The occurrence of this experimental model, experimental autoimmune myasthenia gravis (EAMG), suggested

that an immune response to foreign (eel) acetylcholine receptor could be directed against self-receptor so causing the observed myasthenic signs. This clearly supported the earlier suggestion [1] of an autoimmune basis for myasthenia gravis and caused an explosion of scientific interest in this disease.

The availability of purified eel receptor that could be radioactively labelled with  $^{125}\text{I}$ - $\alpha$ -bungarotoxin (the  $\alpha$ -toxin from the Taiwan banded krait) led to the development of an assay for eel acetylcholine receptor antibodies in the sera of experimental animals with EAMG [13]. The assay involved precipitation of antigen-antibody complexes with sheep (or goat) anti-rabbit IgG. We have recently found that protein A from the cell walls of *Staphylococcus aureus* is equally effective in precipitating the initial antigen-antibody complex [14]. Use of the above assays has shown that levels of circulating antibodies are closely related to clinical signs of weakness in EAMG [4,15,16].

Lindstrom [17] modified the assay for acetylcholine receptor antibodies to detect autologous antibodies in the sera of patients with myasthenia gravis. In the absence of purified human acetylcholine receptor he exploited the specificity of  $\alpha$ -bungarotoxin for the acetylcholine-binding sites of the receptor by using the  $\alpha$ -toxin to label directly the receptor in a detergent extract of whole human muscle without the need for purification. The detergent extract containing radio-labelled receptor was then allowed to react directly with human serum, and specific antigen-antibody complexes were precipitated with sheep anti-human IgG. In this way human acetylcholine receptor antibodies were detected in the sera of over 90 per cent of myasthenic patients [17,18].

We have assayed human acetylcholine receptor antibodies in 39 myasthenic patients using a modification [19] of the Lindstrom assay. Patients were classified according to severity of both cranial and generalised symptoms on a scale of 0 to 3, where 3 represents the most severely affected state. We find that the means of antibody titres show poor correlation with clinical signs. This is in agreement with the results of Lindstrom et al [4] and Ito et al [20]. The lack of correlation in our data can be largely attributed to the occurrence of occasional extreme titres in all groups of patients. Median titres accordingly show a much better correlation with severity of signs (Table I). The unexplained exceptionally high and low titres preclude unequivocal statements concerning the role of acetylcholine receptor antibodies in myasthenia gravis.

The most dramatic evidence for the involvement of a humoral factor in the aetiology of this disease is the striking clinical improvement in myasthenic patients following plasma-exchange [21,22]. We have monitored the serum levels of human acetylcholine receptor antibodies in eight myasthenic patients during the course of a series of plasma-exchanges. In each exchange four litres of the patient's plasma were replaced and the process repeated on up to six occasions at two to four day intervals. After the third plasma exchange each patient received 100 mg of prednisolone and 150 mg azathioprine for one month. These drugs were then reduced gradually over the next month.

TABLE I Antibody Titres of Sera from Myasthenic Patients Undergoing Successive Plasmaphoreses

Patient No.	Age	Sex	Clinical State		Antibody Titre $10^{-10}M$ ( $\alpha$ -bungarotoxin binding sites)													
					1st Exchange		2nd Exchange		3rd Exchange		4th Exchange		5th Exchange		6th Exchange			
					Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post		
1	52	F	3	0	25.7	7.1	16.3	3.0	6.1	0.75	0.88	0.53						
2	56	F	3	3	37.8	22.7	32.8	2.9	5.6	3.9	7.7	4.6	8.6	2.6				
3	66	M	3	2	29.8	19.6	25.9	5.5	28.3	10.2	8.9	9.2						
4	35	F	3	3	9.6	1.4		0.8	7.8	3.2								
5	68	F	3	3	46.8	8.6	7.1	3.2	44.1	5.3								
6	62	M	2	1	14.2	10.8	19.0	11.8	12.0	5.2	15.9	6.1	2.4	29.8	1.7	6.7		
7	29	F	3	3	1.4	0.4	0.1	0.1	0.2	0.3	0.1	0.0	0.1	0.3	0.5			
8	44	F	2	2	0.3	2.3	0.5	0.4	0.5	0.2	0.0	0.2	0.7	0.1				

Seven out of eight patients showed apparently complete remission over time periods of up to six months after the series of plasma-exchanges [23]. Serum samples were taken immediately before and after each plasma exchange and were assayed for acetylcholine receptor antibodies as previously described. Patients 1 to 5 showed similar behaviour in that their antibody titres fell after each exchange but then rose again, not quite, in general, attaining the previous higher value. The overall trend was accordingly down, and all patients showed marked clinical improvement [23]. Patient 6 showed an apparently similar trend except that antibody titres surprisingly rose after each of the fifth and sixth plasma-exchanges and, more importantly, the patient showed little clinical improvement. This patient did not respond to 10 mg of edrophonium chloride given intravenously and had no clinical improvement when treated with up to 900 mg of pyridostigmine daily. Prednisolone given in doses from 60 to 100 mg daily for two months was likewise without any improvement. However, after plasma exchange he showed marked and exquisite sensitivity to edrophonium and to other anticholinesterases. Patients 7 and 8, on the other hand, both achieved complete remission of symptoms after five plasma exchanges, behaviour difficult to explain in terms of their antibody titres which, both initially and throughout, differed little from zero.

Serum from myasthenic patients has been shown to block the electrophysiological response to iontophoretically-applied acetylcholine in cultured human [24], rat [25] and chicken [26] muscle cells. In the case of chicken cells, some myasthenic sera that gave greater than 90 per cent block of acetylcholine-induced response nonetheless had human acetylcholine receptor antibody titres that did not differ significantly from zero.

It is clear from the results described here that, whereas some humoral factor is involved in the aetiology of myasthenia gravis, its effect is not fully reflected in serum human acetylcholine receptor antibody titres assayed by the method of Lindstrom. This method determines antibodies directed at all antigenic sites other than the acetylcholine binding site. It may be that assays of antibodies directed specifically against the acetylcholine binding site of the receptor would give a better correlation with clinical symptoms. This has been found by Zurn and Fulpius [27] to be the case in a rabbit injected with purified Torpedo receptor although Lindstrom [28] dismisses the importance of such antibodies. It is also possible that antibodies directed against other antigenic sites exposed on the membrane-bound receptor could block neuromuscular transmission. The Lindstrom assay includes all such subpopulations and specific assays might prove to be more significant. A recent report from Sobel et al [29] shows that the ionophore responsible for ion-translocation in the post-synaptic membrane is a protein distinct from the acetylcholine receptor protein. Antibodies directed against the ionophore protein could also block neuromuscular transmission and it is doubtful whether such antibodies would be detected in the Lindstrom assay.

Whereas it appears that acetylcholine receptor antibody titres may be useful

TABLE II Antibody Titres of Sera from 39 Myasthenic Patients

Clinical State		Antibody Titre $10^{-10}M$ ( $\alpha$ -bungarotoxin binding sites)		
Cranial	General	Mean	Median	Range
0	1	19.3	1.2	0.4 - 108.0
1	0			
2	0	7.1	3.0	0.5 - 16.5
0	2			
1	2	90.6	7.1	0.0 - 480.0
2	1			
2	2	29.4	12.6	0.8 - 106.0
2	3	26.1	22.0	10.6 - 45.7
3	2			

in following the progress of individual patients during therapy the significance of such titres as currently assayed is not clear and development of assays for specific antibody subpopulations may provide a better aid in understanding and monitoring myasthenia gravis.

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## The Isolation of the Nicotinic Acetylcholine-Receptor Protein from Human Muscle

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It is now established that the human neuromuscular disease myasthenia gravis is an autoimmune disorder in which the nicotinic acetylcholine-receptor protein is the major auto-antigen. Although it is clear that the receptors from electric fish, vertebrate skeletal muscle and human muscle are pharmacologically very similar, the proteins differ immunochemically (Savage-Marengo *et al.*, 1979). Characterization of the receptor protein will clearly help to understand its role in the pathogenesis of myasthenia gravis, and we now report the isolation of the human receptor in a highly purified state.

Isolation of the receptor protein from human skeletal muscle (from limb amputation) was essentially as previously described for the isolation of receptor from the electric organs of *Torpedo marmorata* (Harvey *et al.*, 1978). Muscle (300 g) was homogenized in 1.2 litres of 10 mM-potassium phosphate buffer, pH 7.4, containing 1 mM-EDTA, 0.1 M-NaCl and 0.02% (w/v)  $\text{NaN}_3$ .

The homogenate was centrifuged at 20 000  $g_{av}$  for 60 min and the pellet was extracted with 600 ml of 10 mM-potassium phosphate buffer, pH 7.4, containing 1 mM-EDTA, 0.1 M-NaCl and 1% (v/v) Triton X-100 for 2 h at 4°C followed by centrifugation at 20 000  $g_{av}$  for 60 min. The supernatant was applied to an affinity column prepared by coupling the  $\alpha$ -toxin from *Naja naja siamensis* to Sepharose 4B (Cooper & Reich, 1972). Bound receptor was eluted with 1 M-carbamoylcholine (20 ml). The eluate was dialysed overnight at 4°C against 2 litres of 10 mM-potassium phosphate buffer, pH 7.4, containing 0.1% (w/v) Triton X-100. Residual carbamoylcholine was removed on a column (2 cm  $\times$  1 cm) of DEAE-cellulose, and the protein was eluted with 10 mM-potassium phosphate buffer, pH 7.4, containing 0.5 M-NaCl, 1 mM-EDTA and 0.1% (w/v) Triton X-100. Receptor activity was assayed by measuring the binding of  $^{125}\text{I}$ -labelled  $\alpha$ -bungarotoxin (Harvey *et al.*, 1978). The purified protein bound 0.1–0.6 pmol of  $\alpha$ -bungarotoxin/g of original muscle, representing a recovery of activity of 2–5% with respect to the starting homogenate. The binding of  $\alpha$ -bungarotoxin to the purified protein was completely inhibited by 0.1 mM-tubocurarine or 0.1 mM-bunzoquinonium.

The purified protein was directly radiolabelled with  $^{125}\text{I}$  by the method of Urbaniak *et al.* (1973), and its homogeneity was examined by isoelectric focusing in polyacrylamide gels (prepared from 4% acrylamide) with 3% Ampholines (pH 4–6 and 3.5–10; LKB Produkter). Gels were pre-run at 350 V for 1 h with 0.4% (v/v)  $\text{H}_2\text{SO}_4$ , pH 2.0, containing 1% (v/v) Triton X-100 in the anode chamber and 0.2% (v/v) ethanolamine, pH 10.6, containing 1% (v/v) Triton X-100 in the cathode chamber.

Receptor protein (23–50 fmol of toxin-binding sites/gel) was focused for 1½ h under the conditions described above. Gels were removed, and slices (1 mm) were suspended in double-distilled water (200  $\mu\text{l}$ ) for determination of radioactivity and of pH.

As shown in Fig. 1(a), the  $^{125}\text{I}$ -labelled receptor focused as a single sharp peak at pH 5.6. Receptor labelled with  $^{125}\text{I}$ -labelled  $\alpha$ -bungarotoxin was similarly analysed and found to focus at pH 5.2, a value close to that described for toxin-labelled receptor from *Electrophorus electricus* by Raftery *et al.* (1971) (pI 5.15) and for that from denervated cat and rat muscle by Dolly & Barnard (1977) (pI 5.3).

In a further series of experiments the gel slice corresponding to the peak centre of focused  $^{125}\text{I}$ -labelled receptor was extracted overnight at 4°C with 100  $\mu\text{l}$  of 10 mM-potassium phosphate buffer, pH 7.4, containing 0.1% (v/v) Triton X-100 and 0.1% (w/v) sodium dodecyl sulphate. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was carried out under denaturing conditions. Gels were sliced at 1 mm intervals and the radioactivity of the slices was measured. Fig. 1(b) shows the distribution of radioactivity; two sharp peaks corresponding to mol.wts. of 42 000 and 66 000 were

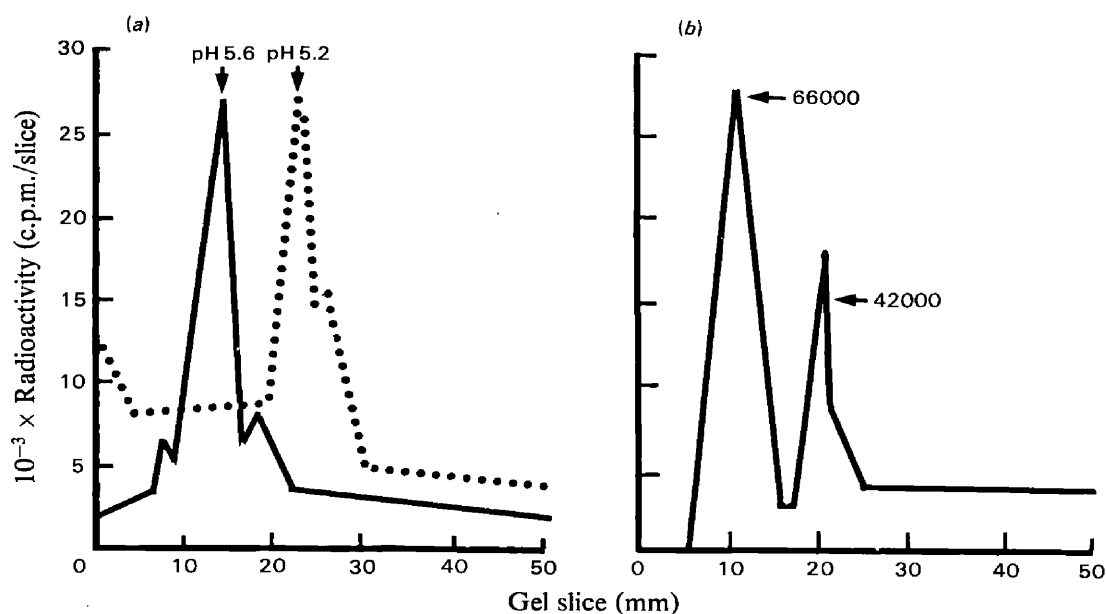


Fig. 1. Isoelectric focusing and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of human acetylcholine-receptor protein

Experimental conditions are indicated in the text. (a) Distribution of radioactivity in isoelectric-focusing gels. —,  $^{125}\text{I}$ -labelled receptor; . . . receptor labelled with  $^{125}\text{I}$ -labelled  $\alpha$ -bungarotoxin. (b) Distribution of radioactivity in sodium dodecyl sulphate/polyacrylamide-gel-electrophoresis gels of  $^{125}\text{I}$ -labelled receptor from peak of isoelectric-focusing gels.

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These preliminary results show that the purified human receptor protein is similar to the receptor proteins from other species. It is hoped that the further characterization of the protein, particularly if its immunochemical properties, will aid our understanding of its involvement in myasthenia gravis.

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## The Isolation of the Nicotinic Acetylcholine-Receptor Protein from Human Muscle

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It is now established that the human neuromuscular disease myasthenia gravis is an autoimmune disorder in which the nicotinic acetylcholine-receptor protein is the major auto-antigen. Although it is clear that the receptors from electric fish, vertebrate skeletal muscle and human muscle are pharmacologically very similar, the proteins differ immunochemically (Savage-Marengo *et al.*, 1979). Characterization of the receptor protein will clearly help to understand its role in the pathogenesis of myasthenia gravis, and we now report the isolation of the human receptor in a highly purified state.

Isolation of the receptor protein from human skeletal muscle (from limb amputation) was essentially as previously described for the isolation of receptor from the electric organs of *Torpedo marmorata* (Harvey *et al.*, 1978). Muscle (300 g) was homogenized in 1.2 litres of 10 mM-potassium phosphate buffer, pH 7.4, containing 1 mM-EDTA, 0.1 M-NaCl and 0.02% (w/v) NaN<sub>3</sub>.

The homogenate was centrifuged at 20 000 *g*<sub>av</sub> for 60 min and the pellet was extracted with 600 ml of 10 mM-potassium phosphate buffer, pH 7.4, containing 1 mM-EDTA, 0.1 M-NaCl and 1% (v/v) Triton X-100 for 2 h at 4°C followed by centrifugation at 20 000 *g*<sub>av</sub> for 60 min. The supernatant was applied to an affinity column prepared by coupling the  $\alpha$ -toxin from *Naja naja siamensis* to Sepharose 4B (Cooper & Reich, 1972). Bound receptor was eluted with 1 M-carbamoylcholine (20 ml). The eluate was dialysed overnight at 4°C against 2 litres of 10 mM-potassium phosphate buffer, pH 7.4, containing 0.1% (w/v) Triton X-100. Residual carbamoylcholine was removed on a column (2 cm  $\times$  1 cm) of DEAE-cellulose, and the protein was eluted with 10 mM-potassium phosphate buffer, pH 7.4, containing 0.5 M-NaCl, 1 mM-EDTA and 0.1% (w/v) Triton X-100. Receptor activity was assayed by measuring the binding of <sup>125</sup>I-labelled  $\alpha$ -bungarotoxin (Harvey *et al.*, 1978). The purified protein bound 0.1–0.6 pmol of  $\alpha$ -bungarotoxin/g of original muscle, representing a recovery of activity of 2–5% with respect to the starting homogenate. The binding of  $\alpha$ -bungarotoxin to the purified protein was completely inhibited by 0.1 mM-tubocurarine or 0.1 mM-bunzoquinonium.

The purified protein was directly radiolabelled with <sup>125</sup>I by the method of Urbaniak *et al.* (1973), and its homogeneity was examined by isoelectric focusing in polyacrylamide gels (prepared from 4% acrylamide) with 3% Ampholines (pH 4–6 and 3.5–10; LKB Produkter). Gels were pre-run at 350 V for 1 h with 0.4% (v/v) H<sub>2</sub>SO<sub>4</sub>, pH 2.0, containing 1% (v/v) Triton X-100 in the anode chamber and 0.2% (v/v) ethanolamine, pH 10.6, containing 1% (v/v) Triton X-100 in the cathode chamber.

Receptor protein (23–50 fmol of toxin-binding sites/gel) was focused for 1½ h under the conditions described above. Gels were removed, and slices (1 mm) were suspended in double-distilled water (200  $\mu$ l) for determination of radioactivity and of pH.

As shown in Fig. 1(a), the <sup>125</sup>I-labelled receptor focused as a single sharp peak at pH 5.6. Receptor labelled with <sup>125</sup>I-labelled  $\alpha$ -bungarotoxin was similarly analysed and found to focus at pH 5.2, a value close to that described for toxin-labelled receptor from *Electrophorus electricus* by Raftery *et al.* (1971) (pI 5.15) and for that from denervated cat and rat muscle by Dolly & Barnard (1977) (pI 5.3).

In a further series of experiments the gel slice corresponding to the peak centre of focused <sup>125</sup>I-labelled receptor was extracted overnight at 4°C with 100  $\mu$ l of 10 mM-potassium phosphate buffer, pH 7.4, containing 0.1% (v/v) Triton X-100 and 0.1% (w/v) sodium dodecyl sulphate. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was carried out under denaturing conditions. Gels were sliced at 1 mm intervals and the radioactivity of the slices was measured. Fig. 1(b) shows the distribution of radioactivity; two sharp peaks corresponding to mol.wts. of 42 000 and 66 000 were

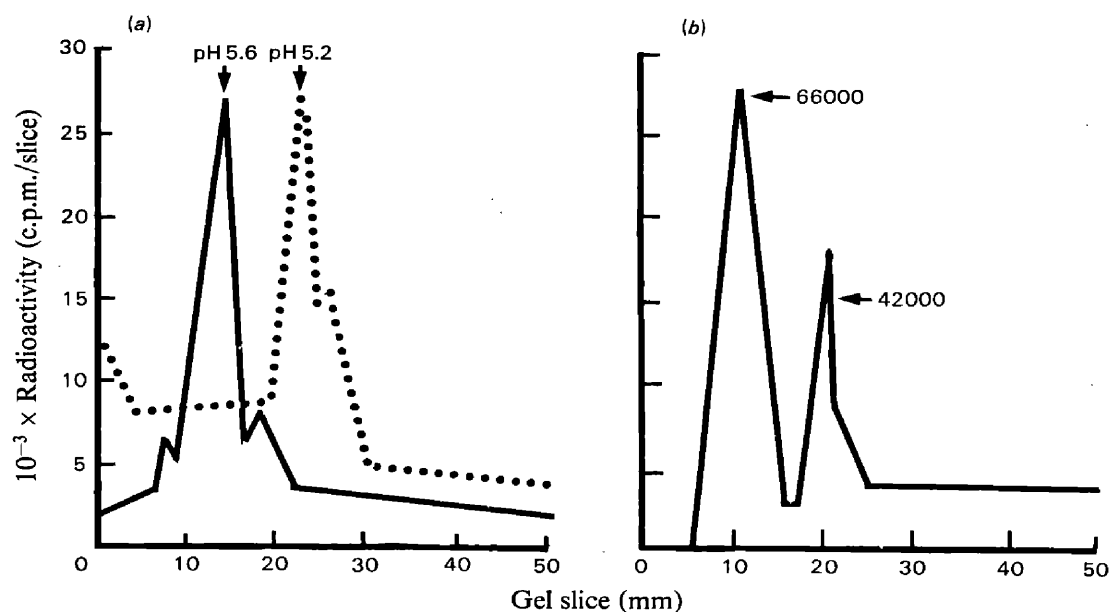


Fig. 1. Isoelectric focusing and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of human acetylcholine-receptor protein

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These preliminary results show that the purified human receptor protein is similar to the receptor proteins from other species. It is hoped that the further characterization of the protein, particularly if its immunochemical properties, will aid our understanding of its involvement in myasthenia gravis.

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## Reduction of acetylcholine sensitivity of chick muscle in culture by myasthenia gravis serum

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### SUMMARY

Sera from seven patients with myasthenia gravis and from six control subjects were tested for the presence of anti-acetylcholine receptor antibodies and for the ability to reduce acetylcholine sensitivity of embryonic chick skeletal muscle cells in culture. Anti-receptor antibodies were found in all but one of the myasthenic samples and in none of the control samples. The average reduction in sensitivity caused by the myasthenic sera was  $89 \pm 4\%$ , compared with an average reduction of  $15 \pm 7\%$  caused by the control sera. A sample taken from a patient during myasthenic crisis was found to have less blocking activity and less anti-receptor antibody than a sample from the same patient after recovery.

### INTRODUCTION

Recent studies have established the presence of elevated levels of antibodies to skeletal muscle acetylcholine receptor in the sera of myasthenic patients (for review see Lindstrom, 1977a), and attention has consequently been focussed on the possible role of such antibodies in the aetiology of the disease.

Muscle cells in culture, which have a relatively high density of acetylcholine receptor distributed over their membrane surface, constitute an attractive experimental system for the study of the interaction of specific antibodies with the receptor in its physiological environment. We have accordingly studied the effects of myasthenic sera on the response of chick embryo muscle cells in culture to iontophoretically-applied acetylcholine. Initial results, in which the sensitivity of the cells was found to be diminished by sera from animals with experimental autoimmune myasthenia gravis and by some myasthenic sera, have already been reported (Harvey *et al.*, 1978). We now report on an extended series of studies using sera from a further seven patients in various clinical states and undergoing different therapies.

### MATERIALS AND METHODS

*Subjects.* Blood was obtained from seven patients with myasthenia gravis and from six normal control subjects. Sera were used without heat inactivation in the antibody assays and were heat-inactivated at  $56^\circ\text{C}$  for 60 min before testing on cultured cells. All sera were stored at  $-20^\circ\text{C}$ .

*Muscle culture.* Cell cultures were established from 10 to 11 day old chick embryo leg muscle as described previously (Harvey & Dryden, 1974), except that the initial cell density was  $5 \times 10^5/\text{ml}$ . Myoblast fusion occurred after 2–3 days in culture and myotubes in cultures between 5–10 days were used for the electrophysiological studies.

*Electrophysiology.* Cultures were mounted on an inverted phase contrast microscope and maintained at  $37^\circ\text{C}$  on a heated stage over which a stream of  $\text{CO}_2$  was passed in order to buffer the culture medium at pH 7.4. Membrane potentials were measured with conventional intracellular recording techniques using glass microelectrodes filled with 3 M potassium chloride (electrode resistance 10–20 megohm). Responses were obtained to the application of 0.5 M acetylcholine chloride from an

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iontophoretic electrode (resistance 100–200 megohm). Dose-response curves to acetylcholine were established, following which the cultures were incubated in a 50% solution of the heat-inactivated test serum for 2 hr at 37°C. The serum was then removed and the sensitivity to acetylcholine reassessed.

*Antibody titres.* Antibodies to human nicotinic acetylcholine receptor were assayed by a modification of the method of Lindstrom (1977b). Acetylcholine receptor was prepared from human muscle amputation; placed on ice within 5 min and subsequently stored at  $-30^{\circ}\text{C}$ , as described by Lindstrom (1977b) except that centrifugations were carried out at 20,000 *g* for 1 hr. The concentration of acetylcholine receptor in the resulting supernatant was determined by binding [ $^{125}\text{I}$ ]- $\alpha$ -bungarotoxin. Aliquots of supernatant (100  $\mu\text{l}$ ) were incubated for 16 h at  $4^{\circ}\text{C}$  with [ $^{125}\text{I}$ ]- $\alpha$ -bungarotoxin (50  $\mu\text{l}$ ), prepared according to the method of Urbaniak, Penhale & Irvine (1973) to a specific activity of 0.58–0.7 mC/nmol and diluted 100 times. Bound toxin was precipitated with 30% w/v ammonium sulphate and allowed to stand for 5 hr at  $4^{\circ}\text{C}$ . The precipitate was collected on a Whatman glass fibre filter, washed with 30% w/v ammonium sulphate and counted in a gamma counter. Corrections for non-specifically bound [ $^{125}\text{I}$ ]- $\alpha$ -bungarotoxin were made by repeating the incubations in the presence of  $10^{-4}$  M d-tubocurarine and subtracting the resulting counts from the total in the absence of d-tubocurarine.

Antibody titre was determined by addition of serum (5  $\mu\text{l}$ ) to a pre-incubated mixture of soluble acetylcholine receptor ( $2.5\text{--}3.5 \times 10^{-10}$  M) and [ $^{125}\text{I}$ ]- $\alpha$ -bungarotoxin ( $4.6\text{--}5.8 \times 10^{-10}$  M) and incubation for 16 hr at  $4^{\circ}\text{C}$ . Sheep anti-human  $\gamma$ -globulin was added to give complete precipitation in 5 hr of [ $^{125}\text{I}$ ]- $\alpha$ -bungarotoxin-acetylcholine receptor-antibody complexes. The mixture was centrifuged (2000 *g*, 10 min) and the pellet was washed with physiological saline buffer (0.9 ml) and counted in a gamma counter. Control samples were incubated as above but in the presence of  $10^{-4}$  M d-tubocurarine and the resulting counts were subtracted from the total in the absence of tubocurarine. Titres were expressed as moles [ $^{125}\text{I}$ ]- $\alpha$ -bungarotoxin binding sites precipitated per litre of serum. Sera with titres greater than  $30 \times 10^{-10}$  M were diluted four times, and ten times with normal human serum, and the assay repeated.

## RESULTS

None of the sera tested, either control or myasthenic, produced any visible change in the morphology of the cultures after 2 hr incubation. Similarly, there was no effect on the average resting membrane potentials. The average potential in cultures before incubation with normal serum was  $-48.7 \pm 3.9$  mV (mean  $\pm$  s.d. of at least ten cells in each of six cultures), and  $-45.2 \pm 4.4$  mV after 2 hr incubation with serum. The corresponding values for cultures incubated with myasthenic serum were  $-38.2 \pm 7.8$  mV (mean  $\pm$  s.d. of at least ten cells in each of eleven cultures) before incubation, and  $-45.6 \pm 9.6$  mV after incubation.

Iontophoretic application of acetylcholine produced a dose-dependent depolarization of the cultured muscle membrane potential. The maximum depolarization was about 70% of the control resting potential and was generally produced by application of 50–100 nC of charge through the acetylcholine-containing electrode (Figs 1, 2).

The shape of the dose-response curve did not alter markedly after incubation with serum from normal patients. Fig. 1a shows the result of an experiment with the sample from control subject 9. With samples from control subjects 12 and 13 there was some depression of the sensitivity to acetylcholine, although the maximum responses were reduced by only 10–25%. For comparison, Table 1 shows the average reduction of sensitivity at the level of 50% of the control maximum response. The average depression induced by the samples from the six normal subjects was  $15 \pm 7\%$  (mean  $\pm$  s.e.m.,  $n = 6$ ).

In contrast, incubation with myasthenic sera markedly depressed acetylcholine sensitivity. In many cases responses could only be detected at the highest doses of acetylcholine (Figs 1b, 2b), and in others the overall sensitivity was lower and the maximum response depressed (Fig. 2a).

The reduction in sensitivity induced by the myasthenic sera is shown in Table 1. The range is from 67% with subject 7 to 98% with subjects 1 and 4. The average decrease in sensitivity with samples from the seven myasthenic patients was  $89 \pm 4\%$  (mean  $\pm$  s.e.m.,  $n = 7$ ).

Anti-acetylcholine receptor antibody titres were determined in all serum samples except for two control subjects (Table 1). Titres from normal human controls did not differ significantly from zero. Patients having ocular myasthenia only (Osserman Group I) also showed zero or low ( $< 1.0$  nM) titres, whereas four out of five samples from generalized myasthenics (Osserman Groups II–IV) had titres greater than 1 nM. However, one sample from a patient with generalized myasthenia had a titre which did not differ significantly from zero.

Two blood samples were obtained from subject 7, one during an episode of myasthenic crisis and another following recovery. The blocking activity in the crisis sample was found to be lower than

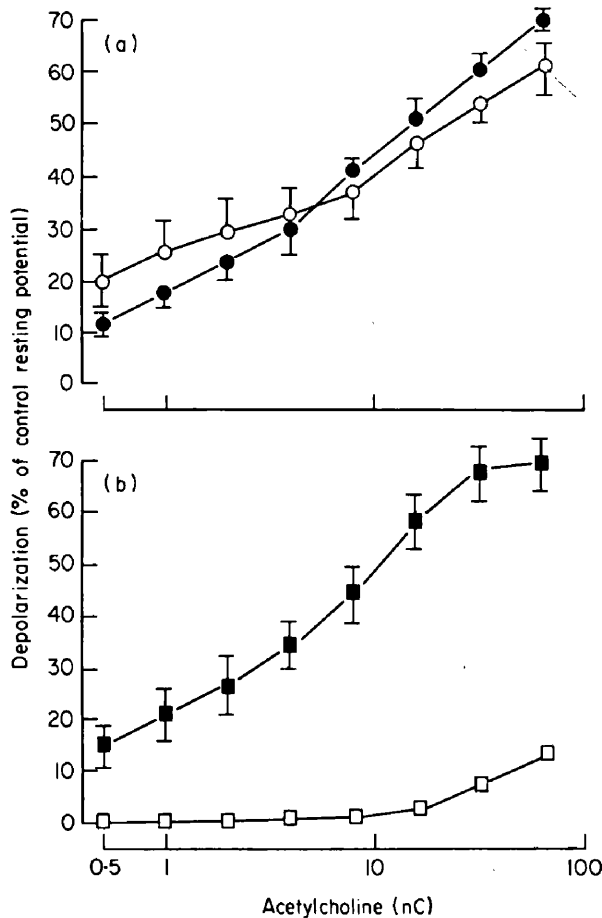


FIG. 1. The effect of normal and myasthenic sera on the sensitivity of cultured muscle fibres to acetylcholine. (a) Control pre-incubation dose-response curve (●—●) and dose-response curve constructed after 2 hr incubation with 1.0 ml of serum from normal subject 9 (○—○). (b) Control pre-incubation dose-response curve (■—■) and dose-response curve constructed after 2 hr incubation with 1.0 ml of serum from myasthenic patient 1 (□—□). The depolarization response is expressed as percentage depolarization from the original resting membrane potential, and the acetylcholine dose is expressed as the charge in nanocoulombs (nC) passed through the iontophoretic electrode. Each point represents the mean of responses in at least ten fibres, standard error bars are indicated unless smaller than the symbols.

that of the recovery sample (Fig. 2, Table 1). Correspondingly, the level of antibody increased from 1.47 nM during crisis to 2.63 nM during the recovery phase.

## DISCUSSION

The marked effect of all myasthenic sera tested on the acetylcholine response of cultured chicken cells supports the data published recently by Bevan, Kullberg & Heinemann (1977) and Anwyl, Appel & Narahashi (1977), who reported similar effects of selected samples of myasthenic sera on cultured human and rat muscle cells respectively. These results contrast with studies using conventional nerve-muscle preparations in which it has been difficult to demonstrate a reduction in acetylcholine sensitivity following *in vitro* application of myasthenic sera (Albuquerque *et al.*, 1976). An explanation (Bevan *et al.*, 1977) may be that endplate receptors are more stable and, therefore, less susceptible to antibodies than receptors of cultured cells.

Anti-acetylcholine receptor antibodies, as assayed by the method of Lindstrom (1977b), have been reported to be present in approximately 90% of patients with myasthenia gravis, but the correlation between titres and severity of symptoms is generally poor (Lindstrom, 1977a; Ito *et al.*, 1978). In a recent study of thirty-nine myasthenics (Barkas *et al.*, 1978a) those patients more severely affected were

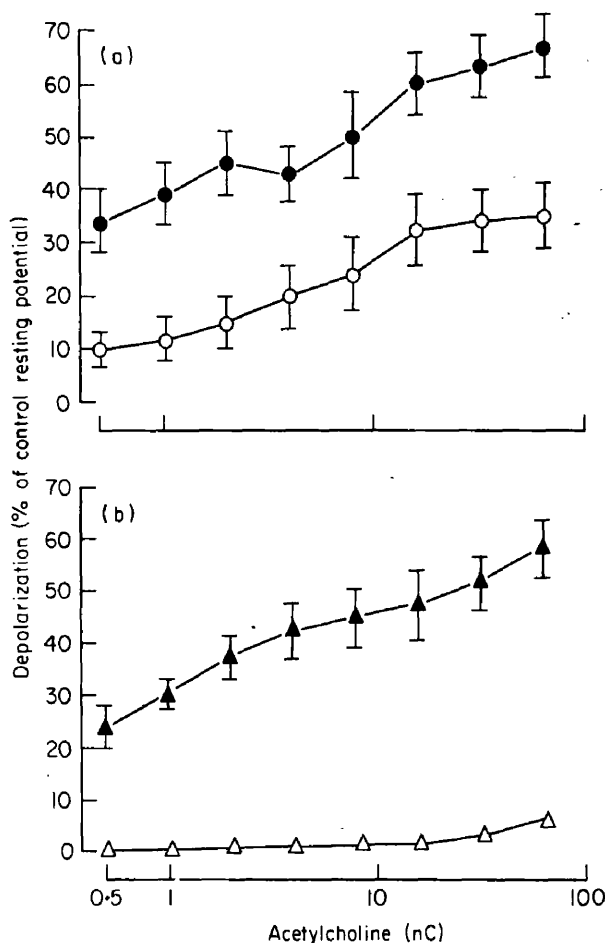


FIG. 2. The effect of serum samples taken during myasthenic crisis and following recovery on the sensitivity of cultured muscle fibres to acetylcholine. (a) Control pre-incubation dose-response curve (●—●) and dose-response curve constructed after 2 hr incubation with 1.0 ml of serum taken from myasthenic 7 during myasthenic crisis (○—○). (b) Control pre-incubation dose-response curve (▲—▲) and dose-response curve constructed after 2 hr incubation with 1.0 ml of serum taken from myasthenic 7 following recovery from myasthenic crisis (△—△). Other conditions as in Fig. 1.

found to have generally higher antibody titres, although occasional extremely high or low titres occurred in all groups. This resulted in a good correlation of the median, but not of the mean titre, with clinical state. A similar tendency can be seen in the seven patients discussed here. Consistent with previous reports (Lindstrom, 1977a), those patients with ocular myasthenia had only relatively low antibody titres but patient 6, with generalized symptoms, also had a very low titre. The assay method of Lindstrom (1977b) determines antibodies directed at all antigenic sites of the receptor, other than the acetylcholine-binding site, and it is possible that assays of specific subpopulations of these antibodies or of antibodies directed at the acetylcholine-binding site itself may be clinically more significant.

Whereas the occurrence of myasthenia gravis in a patient is not always reflected in the anti-acetylcholine receptor antibody titre of his serum, all serum samples from the myasthenic patients examined in the present study showed greater than 67% reduction of acetylcholine sensitivity of cultured cells compared with a maximum of 39% in the non-myasthenic controls. It may be that an electrophysiological approach such as that described here could provide a more reliable diagnosis of myasthenic gravis than the anti-acetylcholine receptor antibody titres as currently assayed. The method is, however, time consuming and would not be generally applicable for routine screening. The degree of blockade of acetylcholine response in chicken cells showed little correlation with the severity of the disease. It is conceivable that cultured human muscle cells might show both a greater sensitivity to myasthenic serum and a better correlation of blockade with clinical state.



TABLE 1. Reduction of acetylcholine sensitivity of cultured cells caused by addition of myasthenic and of control sera

Case number	Age	Sex	Duration of myasthenia (years)	Severity of myasthenia*	Steroid therapy	Thymectomy†	Percentage reduction‡	Antibody titres (nM $\alpha$ -bungarotoxin sites)
1	13	F	0.5	I	None	No	98±1	0.06
2	76	F	6	I	None	No	94±3	0.98
3	55	M	4	I	None	No	80±7	0
4	52	M	10	IV	None	1969	98±1	6.93
5	31	F	12	IIb	15 mg prednisolone/ alternate day	1968	91±7	1.28
6	39	M	3	IIa	None	No	90±3	0.03
7	75	F	2	III	None	No	67±11	1.47
7	75	F	2	III in crisis recovery phase following crisis	60 mg prednisolone/ day	No	99±1	2.63
Normal controls								
8	31	F	—	—	—	—	0±16	0.03
9	27	F	—	—	—	—	0±20	—
10	31	F	—	—	—	—	0±11	0
11	39	F	—	—	—	—	15±11	—
12	29	F	—	—	—	—	37±11	0.03
13	32	F	—	—	—	—	39±5	0.1

\* Grading scale according to Osserman (1958).

† Patients with no thymectomy showed no evidence of thymoma.

‡ Percentage reduction of acetylcholine response, at 50% level of control dose-response curve, shown by addition of human sera. Values are given as mean±s.e.m. determined in at least ten fibres in a single culture.

The effectiveness of myasthenic sera in blocking the acetylcholine response of muscle cells provides further evidence for the involvement of a humoral factor in the aetiology of the disease. It is of interest that serum taken from patient 7 during myasthenic crisis showed less inhibition of acetylcholine response and had lower antibody levels than a sample taken from the same patient after clinical improvement. Similar results have been obtained by us in chickens with experimental myasthenia gravis (Barkas *et al.*, 1978b), and these findings are apparently complemented by a recent report from de Crousaz & Fulpius (1978) that the anti-acetylcholine receptor antibody titres of two patients fell during myasthenic exacerbation. It may be, as suggested in the latter report, that circulating antibodies associate with the muscle endplate during the onset of symptoms thus leading to an apparent fall in serum titre. Decrease in free receptor at the neuromuscular junction has, in fact, been recently correlated with increased severity of myasthenic symptoms (Lindstrom & Lambert, 1978). Information on such a possible mechanism is clearly relevant to the significance of circulating antibody titres in myasthenia gravis and further evidence from serial determinations on individual patients during remission and exacerbation of the disease is required.

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