Studies on neurotransmitter receptors in the locust.

Filbin, M. T.

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STUDIES ON NEUROTRANSMITTER RECEPTORS

IN THE LOCUST

Submitted by M. T. Filbin

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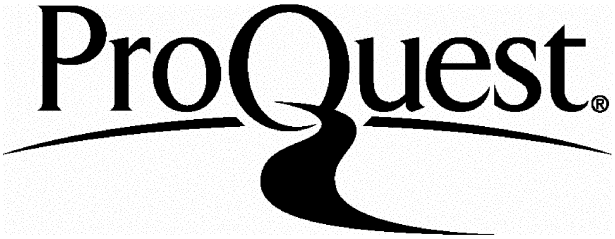

TO THE MEMORY OF MAUREEN AND JOHN

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CONTENTS

SUMMARY

1. A membranous sub-fraction has been prepared from locust (Schistocerca gregaria) muscle. The fraction shows some enrichment in the specific binding of L-glutamate compared with the total homogenate of the muscle. Electron microscopy and enzyme distribution studies suggest that the fraction is composed mainly of membrane and disrupted mitochondria. Two methods have been used to measure the specific binding of L-glutamate based on rapid filtration through Whatman GF/C filters and a rapid centrifugation procedure using a Beckman Airfuge. The latter method gave better reproducibility within any one study but both assay methods showed great variation between preparations. There appears to be no metabolism of L-glutamate by the membrane fraction during the time over which the binding studies were made. Preliminary experiments with three marine toxins - suberitine and α and β cephalotoxin suggest that these agents may prove to be useful in elucidating the nature of the glutamate binding sites on the muscle membranes. The variability of the binding activities of the muscle membranes has so far precluded any unequivocal designation of the L-glutamate binding sites as synaptic receptors.

2. The specific binding of [^{125}I]-α-bungarotoxin to a membrane fraction of locust (Schistocerca gregaria) supra-oesophageal ganglia showed the following characteristics:-

   a) binding increased linearly with protein

   b) binding saturated at concentrations of [^{125}I]-α-bungarotoxin greater than 2 nM

c) bound [^{125}I]-α-bungarotoxin dissociated in a biphasic manner

d) binding was preferentially inhibited by nicotinic ligands.

When the membrane fraction was treated with the co-valent affinity label [^{3}H]-MBTA and subjected to SDS-PAGE most of the radioactivity was recovered as a single peak, corresponding to an M_r of 58,000. The binding characteristics (K_D and B_{max}) of the membrane fraction did not change significantly on solubilisation in the non-ionic detergent Triton X-100. Up to a 1000 fold purification of the α-bungarotoxin binding component was achieved after affinity chromatography on immobilised α-bungarotoxin. The K_D of the partially purified binding component was unchanged relative to the membrane fraction, when measured from equilibrium binding studies but determination of the k_{-1} showed dissociation of [^{125}I]-α-bungarotoxin to be monophasic. The partially purified α-bungarotoxin binding component sedimented as a single component after centrifugation in a sucrose density gradient (14.2s; M_r 400,000 - 450,000) and SDS PAGE showed two major protein bands of M_r's 60,000 and 41,000. No acetylcholine esterase activity was detected in this partially purified binding component fraction.

PUBLICATIONS

(i) Briley, P.A., Filbin, M.T., Lunt, G.G. and Donnellan, J.F. (1980) In: "Insect Neurobiology and Pesticide Action (Neurotox 79)" pp. 177-184, Society of Chemical Industry, London.
The Biochemical Characterisation of Amino Acid Mediated Synaptic Transmission at the Locust Neuromuscular Junction.

(ii) Filbin, M.T., Lunt, G.G. and Donnellan, J.F. (1980) In: "Receptors for Neurotransmitters, Hormones and Pheromones in Insects" (Ed. Sattelle, D.B., Hall, L.M. & Hildebrand, J.G.) pp. 153-160, Elsevier/North Holland Press.
Glutamate Receptor Biochemistry.

(iii) Briley, P.A., Filbin, M.T., Lunt, G.G. and Donnellan, J.F. (1981) Ciba Foundation Symposium No. 88, Neuropharmacology of Insects (in press).
Binding and Uptake of Glutamate and γ-Aminobutyric Acid in Membrane Fractions from Locust Muscle.

(iv) Briley, P.A., Filbin, M.T., Lunt, G.G. and Turner, P.D. (1981) In: "The Biology of Glutamate and its Derivatives", Molec. and Cellular Biochem. (in press).
Glutamate Receptor Binding in Insects and Mammals.

INTRODUCTION

There are over 800,000 different species comprising the phylum Arthropoda, of which species of insects are by far the major constituents. A mere 47,000 different species belong to the phylum Chordata, which includes the whole spectrum of vertebrates. If our understanding of nervous systems is classified into the respective phyla, and considered in relation to the above figures, then what is known of the nervous systems of the Arthropoda is insignificant compared to the knowledge accumulated from the considerably fewer species of Chordata. This imbalance of knowledge is, however, rapidly changing as an economic interest, implemented in part by the insecticide industry, into insect neurochemistry and neurophysiology has shown a dramatic increase in the last twenty years.

In the past the insecticide industry has largely relied on random screening as a means of discovering new chemicals to use in pest control and, until quite recently, the belief was that this process would take care of our future requirements. At present, after screening tens of thousands of chemicals, insect control relies on only four major chemical classes : the organochlorines; the organophosphates; the carbamates; and the fast growing pyrethroid group. All four are classified as neuroactive but the fact remains that the precise mode of action of any one insecticide remains unknown. This lack of basic knowledge has only intensified the ever growing problem of insect resistance. Because the mechanism and exact site of action of these compounds is not known it is impossible to determine the precise reason for resistance and hence impossible to remedy it.

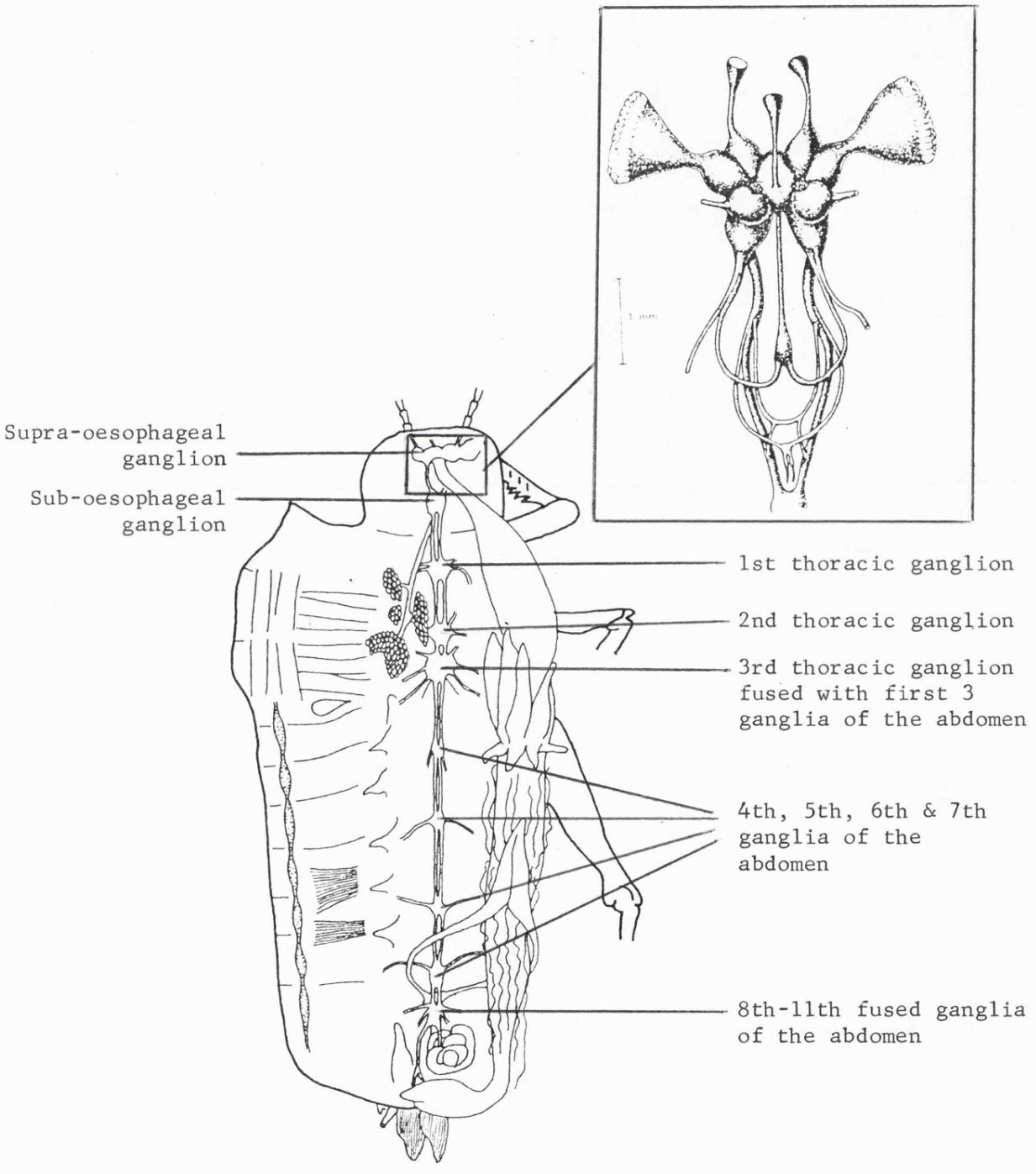
Two general conclusions follow from the results of random screening: firstly, that insects are still very difficult to control, as indicated by the availability of only a few effective compounds out of thousands tested; and secondly, it would be advantageous to know more about insect nervous systems. Such information would both add to our general knowledge within the field of investigative neurobiology and also facilitate a more rational and hopefully more successful development of toxicants. Conversely, the toxicants may prove to be valuable tools in neurobiological investigations.

The work described in this thesis has been done with tissues from the desert locust, Schistocera gregaria. The desert locust is an orthopteran, a group with the general characteristics as follows: the body is enclosed in an exoskeleton and is bilaterally symmetrical and segmented; one pair of jointed appendages may be present per body segment; coelomic cavities are present in embryos; the blood system is of the "open" type; the gut has a mouth and an anus; and the life history is of many stages, separated by moulting of old exoskeleton.

The Locust Central Nervous System:

The locust central nervous system consists of a main ganglionic mass ("brain" or supra-oesophageal ganglion) and a ventral nerve cord (Fig. 1). The supra-oesohpageal ganglion lies above the oesophagus in the head and represents a number of fused ganglia which originate in different segments of the embryo and amalgamate during development. The supra-oesophageal ganglion acts as the receptor of excitatory impulses arising from the sense organs in the head region, as well as from ascending interneurones in the

Fig. 1 Diagram Showing the Head and Ventral Ganglia of the Locust



ventral nerve cord. It is thus the chief association centre as well as the seat of long-term organised behaviour patterns (Bullock and Horridge, 1965).

The ventral nerve cord runs the length of the thorax and abdomen of the insect and consists of a series of ganglia linked by paired interganglionic connectives. As in the supra-oesophageal ganglion, fusion between ganglia may occur during development, either during embryonic growth or at different times after hatching (Pipa, 1973). In the locust there are three thoracic ganglia, two of which are distinct and a third which is fused with the first three ganglia of the abdomen. The fourth, fifth, sixth and seventh ganglia of the abdomen are distinct and the eighth through to the eleventh are fused. The degree of fusion of ganglia varies from species to species, the extreme case being the dipterans (e.g. the housefly) which display fusion of all the ganglia of the ventral nerve cord into one large thoracic ganglion from which nerves emanate to all the other parts of the body (Bullock and Horridge, 1965).

Ganglia, in insects that have been studied to date, be they fused ganglia of the oesophagus or the ganglia of the ventral nerve cord, all have the following common structural details: they are surrounded by an acellular neural lamella beneath which lies a cellular perineurium. Under this are found nerve cell bodies enveloped by glial cells and a central neuropile within (Fig. 2). The nerve cell bodies are situated in the cortical region, peripheral to the central neuropile. The neuropile, therefore, lacks neurones and is transversed by axons of different diameters which may originate in nerve cell bodies within the same or a more distant ganglion or may come into the neuropilar mass from a peripheral nerve.

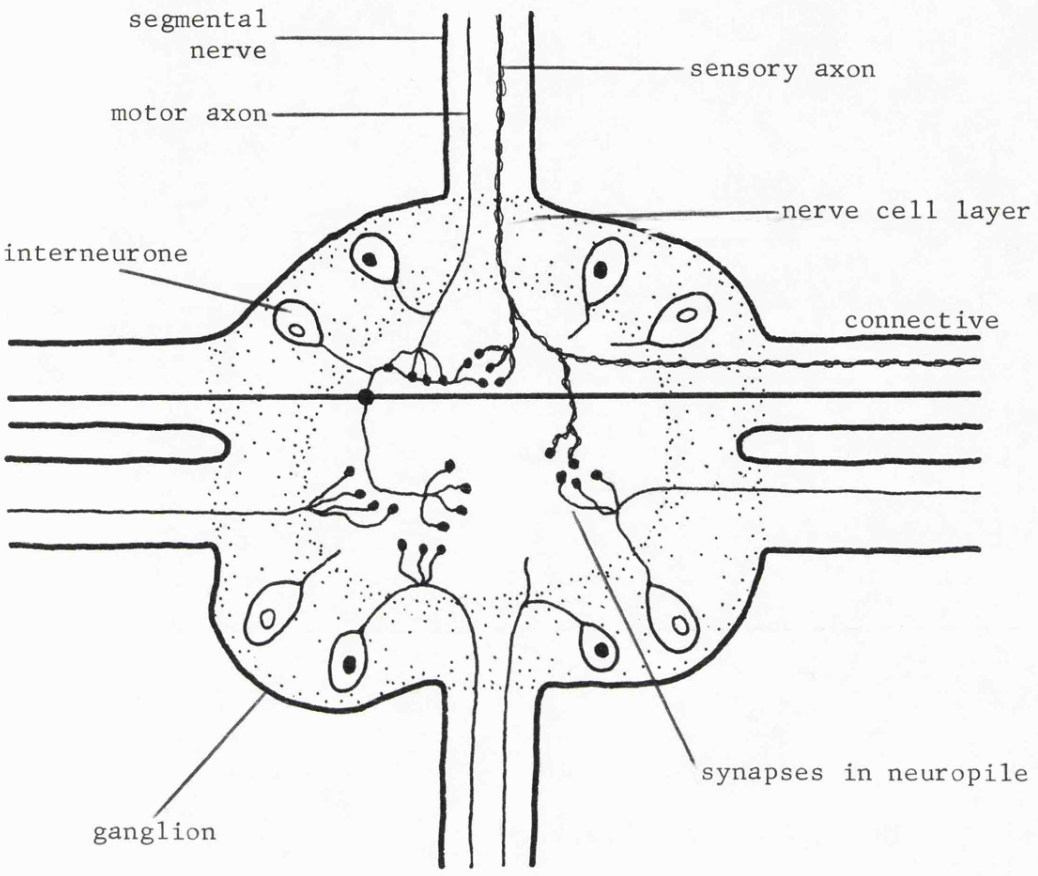


FIG 2 Diagram of an idealised insect ganglion to show arrangement of nerve cells and neuronal processes

It is in this central neuropilar region that synaptic contacts between nerve cells take place. Because in insects the cell bodies are completely ensheathed by a glial investment, synapses between axon terminals and nerve cell bodies do not occur and in this respect insects differ from vertebrates.

The connectives of the ventral nerve cord consist of axonal processes only, invested by glial cells. The glial cells that surround the nerve cord are present in the so-called tunicated condition (Smith and Treherne, 1963), not in the myelinated form that occurs in vertebrates; that is to say attenuated glial cell processes spiral around the axons. Other glial cells also form the perineurium, invest the neuropile, and giant glial cells send invaginations into the peripheral cytoplasm of the large nerve cells (Wrigglesworth, 1959 b; Sohal et al , 1972).

The ventral nerve cord and the brain of insects have no vascular system, so that substances have access to the nervous system only by diffusion from the haemolymph through the various layers. This is a very important consideration with respect to how insecticides may reach their site of action.

Locust Muscle:

Morphologically, skeletal muscle of the locust and other insects closely resembles vertebrate striated muscle in that there are comparable striation patterns with A-, I- and H-bands and Z- and M-lines (See, Elder, 1975; Piek and Njio, 1979). The presence of both thick and thin myofilaments (Huxley and Hanson, 1956) has been demonstrated. Basic biochemical similarities also occur in that the thick filaments are myosin-containing and thin filaments are actin-containing (Maruyama, 1965). This biochemical evidence in turn lent support

to the assumption (Hanson, 1956) that in insect, as in vertebrate striated muscle, active muscle shortening is achieved in the way proposed in the sliding filament theory of Huxley and Hanson (1954) and Huxley and Niedergerke (1954). Apart from a few cases which do not appear to conform (Gilmour and Robinson, 1964) the sliding filament theory is now generally accepted.

Large mitochondria (sarcosomes) are typically found in columns, longitudinally aligned between the fibrils. Usually there is no alignment with the striation pattern of the myofibrils.

In addition to the mitochondria and the contractile fibres, a system of tubules and vesicles can be discerned within the muscle fibre. As early as 1890 Ramon Y Cajal, using the light microscope and the Golgi-silver impregnation method, observed the presence of fine filaments forming longitudinal and transverse reticulae, in both vertebrate and arthropod skeletal muscle. Verratti (1902) noted that the arrangements of the internal reticular apparatus differed characteristically between different muscles. This sarcotubular system is now defined as consisting of a transversely orientated tubular system (T-system) which is formed by invaginations of the plasma membrane and a longitudinally orientated sarcoplasmic reticulum (Anderson-Cedergren, 1959) which has no connection with the outer plasma membrane. These systems are involved in the generation of membrane potentials, and in the mechanism of muscle excitation, contraction and relaxation.

Insect muscle fibres are surrounded by a connective tissue sheath called the basement membrane, which has predominantly a supportive role, binding fibres together. However, Clements and May (1974) have suggested that in intact locust muscle the basement

membrane could serve also as a barrier to the putative neurotransmitter, glutamate. They observed that in muscles fixed by perfusion, the basement membrane is more compact than in muscles that are dissected out and then fixed by immersion, indicating that this membrane is probably damaged by the dissection procedure and therefore may not demonstrate the same properties as the intact muscle that is fixed *in vivo*.

Light microscopy reveals three broad categories of insect flight muscle: tubular; close-packed; and fibrillar which functionally can be divided into two groups:

1. Synchronous muscle which is nonfibrillar and in which there is a 1:1 ratio of the frequency of motor nerve impulses and the frequency of the mechanical responses. There is also a correlation between the speed of contraction and the extent of sarcoplasmic reticulum (Cochrane *et al.*, 1972).
2. Asynchronous muscle which is fibrillar and in which the frequency of contraction greatly exceeds and is relatively independent of the motor nerve impulse frequency (Pringle, 1967; 1972). In asynchronous muscle there is no correlation between the extent of sarcoplasmic reticulum, which is very much reduced (Smith, 1965; Smith and Sacktor, 1970) and the speed of contraction.

From these observations it can be said that the presence of a well developed sarcoplasmic reticulum (synchronous muscle) is associated with a high frequency excitation-contraction coupling system rather than with a high frequency oscillation (asynchronous

muscle) of the myofibrils. Locust flight muscle is synchronous and therefore has a well developed sarcoplasmic reticulum which presents many problems in fractionation procedures.

A major difference between vertebrate and invertebrate muscle is that the latter has a multiterminal and polyneuronal innervation (Hoyle, 1957; Edwards, 1959) i.e. a single muscle fibre can be innervated by more than one axon of different kinds (excitatory and inhibitory) and each axon makes multiple contacts with each fibre (Fig. 3). The axon can also make contact with the muscle in a number of ways; the axon may lie in a groove in the surface of the muscle fibre (Edwards et al., 1958), or it may make contact with projections from the surface of the muscle which take the form of pillars or sheets as in the locust retractor unguis muscle, or finally, the axon can penetrate into the muscle where it is completely surrounded by the muscle cell (Shafiq, 1964; Smith, 1960). This last type of axonal contact usually has no glial investment (See, Osborne, 1970). Axon-muscle contacts are termed neuromuscular junctions and it is there that information is transmitted from axon to muscle.

Synaptic Transmission:

Where an axon makes contact with either another axon as in the neuropilar region of the ganglia, or with a muscle fibre at the neuromuscular junction, the axon terminal is called a synapse. The word was coined by Sherrington (1925) whose eletrophysiological studies confirmed the earlier hypothesis of Raman Y Cajal (1888) that the nervous system was not a continuous network, as was the general opinion at that time, but consisted of a number of discrete cells or neurones, which only made contact at specialised areas.

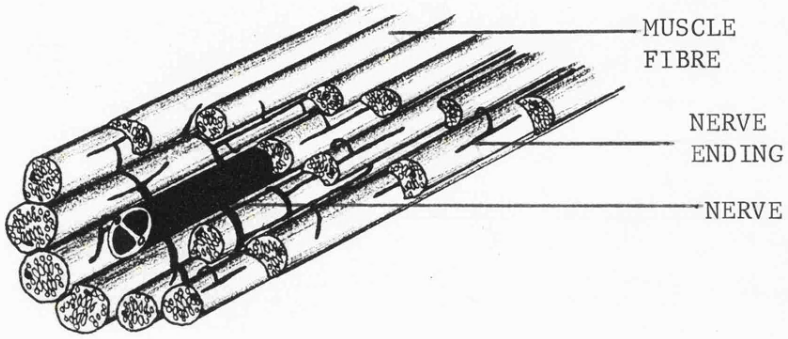


FIG 3 Diagramatic representation of insect muscle showing multiple innervation

It is at the synapse that information is transferred between cells. The synaptic area consists of a pre-synaptic membrane (the axon terminal), a post-synaptic membrane (either another axon or a muscle fibre) and a synpatic gap or cleft between (Fig. 4). The axon terminal is characterised by the presence of numerous synaptic vesicles about 20-40 nm in diameter. At the junction these vesicles are usually clustered in groups, lying close to the axon membrane. It is generally accepted that the synaptic vesicles contain transmitter substance.

As in vertebrates, at the insect neuromuscular junction, upon arrival of the nerve impulse at the synapse, an influx of Ca^{++} occurs (Kerkut et al., 1965 b; Usherwood et al., 1968) whereupon the transmitter substance 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 released neurotransmitter then diffuses across the synaptic gap and interacts with the post-synaptic membrane causing a permeability change to ions in that membrane. The specificity of the permeability change is dependent on the neurotransmitter released and on the resting state of the membrane; if an excitatory neurotransmitter is released a permeability change to Na^{+} and K^{+} ions (and possibly Ca^{++} and Mg^{++}) is the result but if an inhibitory neurotransmitter is released then there is a permeability change to Cl^{-} and/or K^{+} ions. In general the release of an excitatory neurotransmitter causes a depolarisation of the post-synaptic

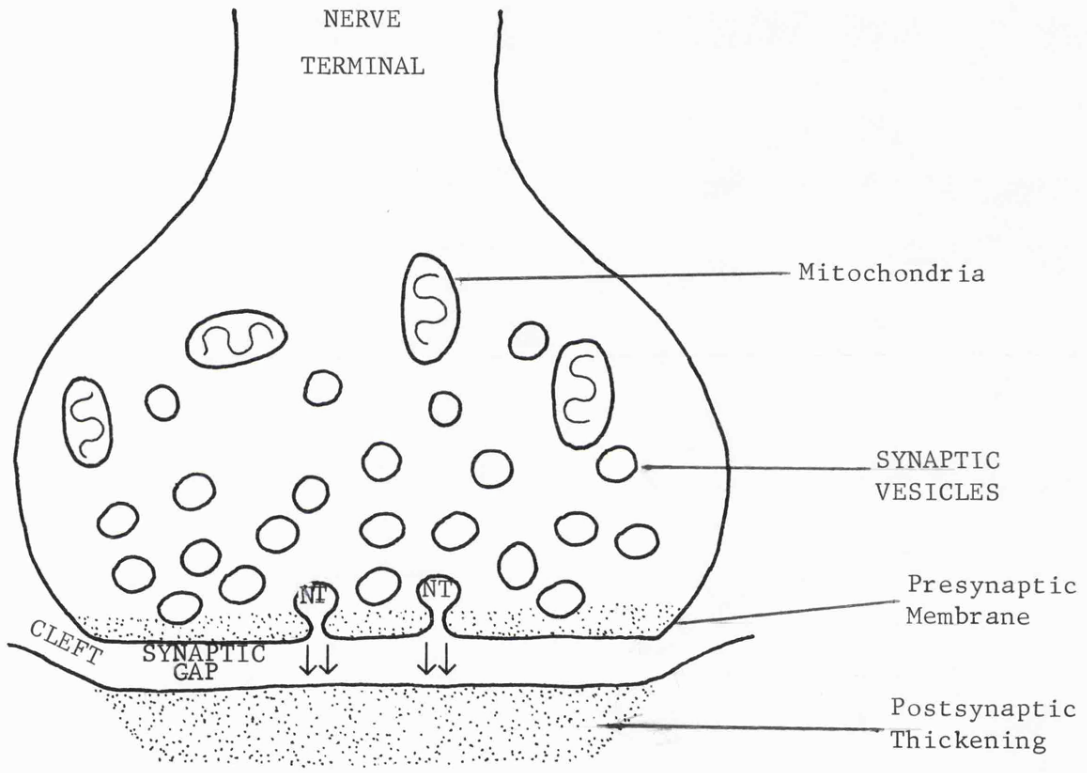


FIG. 4 Diagramatic Representation of a Typical Synapse

membrane by decreasing the resting potential of the membrane, whereas release of an inhibitory neurotransmitter usually causes hyperpolarisation by increasing the membrane resting potential.

In the case of axonal-axonal contact the extent of depolarisation must reach a threshold value before an action potential is achieved and the impulse propagated along the post-synaptic axon. Hyperpolarisation acts to reduce the extent of depolarisation and so prevent the threshold depolarisation being reached. This can be achieved in one of two ways; firstly, an inhibitory axon may synapse pre-synaptically onto an excitatory axon and so inhibit the release of excitatory neurotransmitter, or secondly, if two axons, one excitatory and one inhibitory, synapse very close to each other onto a third axon, then the hyperpolarisation caused by the inhibitory axon will diminish the depolarisation caused by the excitatory axon and threshold depolarisation will not be reached in the post-synaptic axon.

With insect muscle, because innervation of each fibre is usually multiterminal, it is not necessary to generate a full action-potential, unlike vertebrate skeletal muscle. This is because in the insect, the muscle fibre is made to contract by excitation of a series of synapses. The post-synaptic depolarisation arising in each synapse spreads to give a local depolarisation in the immediate non-synaptic membranes (a graded response) as is the case with vertebrate muscle. However, in vertebrate muscle the graded depolarisation eventually reaches a threshold and a full action potential is generated. Insect muscle fibres, however, are affected directly by synaptic depolarisations generated at the many synaptic sites and it is the summation of these individual depolarisations which

causes contraction.

As stated previously, it is the interaction of the neurotransmitter with the post-synaptic membrane that, by opening specific ion channels, causes the ion permeability change. The point or points of interaction of the neurotransmitter on the post-synaptic membrane are called receptors and are envisaged as specific membrane proteins that combine with the transmitter in a highly specific manner.

The concept of receptors for specific ligands was first postulated by Langley (1905) when studying the paralysing effects of nicotine. He suggested that nicotine must combine with some, then unknown, constituent of the muscle cell, which he termed a "receptive substance". 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.

Today, although the exact mechanisms of receptor-ligand interactions are not known, a model has been suggested by Changeux and his colleagues (Heidmann and Changeux, 1978; Changeux et al., 1976) for the neurotransmitter-receptor interaction based on the nicotinic cholinergic system. The model is based on the theory that the elementary membrane unit is composed of at least two distinct

structural elements; the receptive unit and the biologically active unit or ionophore, which might be part of the same polypeptide chain or equally could be carried by distinct subunits. By analogy with the known behaviour of regulatory enzymes and in agreement with the Katz and Thesleff (1957) model for desensitization, it was further postulated that the regulator may exist in at least three discrete and interconvertible conformational states (Fig. 5). These states, referred to as resting R, active A and desensitized D, would differ primarily in the affinity of the acetylcholine receptor site for cholinergic ligands. The affinity for agonists would increase from R (low affinity) to A (medium affinity) to D (high affinity); on the other hand the affinity for competitive antagonists would be lower in the A than in the R or D states. Postulated conformational states of the acetylcholine regulator may also differ in their affinity for non-competitive blocking agents; in the D and A states the affinity for these compounds would be higher than the R state. Finally, the ion channel would be open only in the A state of the regulator.

Although evidence for this hypothetical model derives from studies on the nicotinic acetylcholine-receptor system of the electroplax and the skeletal neuromuscular junction of vertebrates, it is not unrealistic to suppose that the same or very similar interactions occur in other receptor-neurotransmitter systems in which the time taken to respond to neurotransmitter is very fast (1 - 3 milliseconds). This would indicate that essentially the same events may be taking place; namely interaction of the neurotransmitter with the receptor which results directly in opening of an ion channel or channels, followed by desensitization after

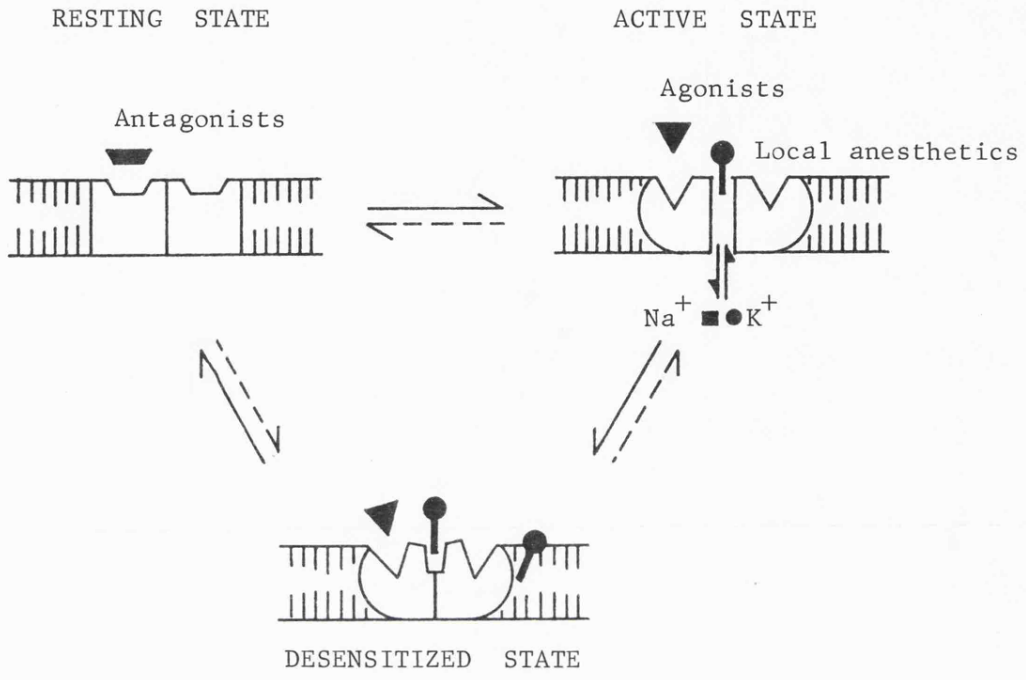


FIG. 5 A Model of the Acetylcholine Regulator

(after Changeux et al., 1976)

prolonged application of the transmitter. Clearly such receptor-ion channel systems differ fundamentally from those in which the response is mediated through second messengers such as cyclic AMP (Fain, 1978).

To date the most advanced work in elucidating the mechanism and consequences of receptor-neurotransmitter interaction has been carried out on the nAChR from the electroplax and vertebrate muscle. This situation has arisen for two reasons: firstly, the discovery of the snake venom α-neurotoxins and their high-affinity, specific binding to the nicotinic receptor (Lee and Chang, 1966); and secondly, the abundant source of nicotinic cholinergic receptor in the electric organ of the electric fishes (see, Changeux, 1975).

The α-toxins, because of their specificity and high affinity for the nicotinic acetylcholine receptor and also because they can be radiolabelled without loss of their biological activity (Miledi and Potter, 1971; Berg et al., 1972; Biesecker, 1973; Dolly and Barnard, 1974; Barnard et al., 1979), have been invaluable as tools in the identification and purification of the acetylcholine receptor protein and also in autoradiographical studies to examine the density and distribution of receptors in intact cell membranes (see Dolly, 1979).

Complementary to this use of the α-toxins was the rich source of nicotinic acetylcholine receptors in the electric organs of electric fishes. The electroplaque is a highly assymmetric cell which receives multiple innervation on only one of its faces. The extent of innervation is such that the subsynaptic areas occupy up to 50% of the total surface of the innervated membrane in Torpedo

marmorata. In Electrophorus electricus the innervated face is much more convoluted and subsynaptic areas represent only 1.4 - 2% of the total surface. Nevertheless both fishes provide a rich source of nicotinic acetylcholine receptor (Changeux, 1975).

The combination of these two factors greatly facilitated the characterisation and purification of the nicotinic acetylcholine receptor from electroplaques. In denervated vertebrate skeletal muscle, acetylcholine receptors have been shown to proliferate over the whole muscle surface (Ginetzinsky and Sharmarina, 1942; Axelsson and Thesleff, 1959; Miledi, 1960). This phenomenon has been exploited in the purification of the muscle receptor using the procedures developed from studies on the electroplaque.

The identification, purification and characterization of putative acetylcholine receptors from other tissue and of other receptors have been greatly hindered by the lack of receptor enriched tissue and/or specific, high affinity ligands analagous to the α-toxins.

Numerous neurotransmitters have been suggested in insects (Table 1). The list ranges from the small peptide proctolin on which only limited electrophysiological work has been done (Miller, 1980), and it is not known whether it has a true neurotransmitter role or is a neuromodulator at the visceral neuromuscular junction, to acetylcholine for which there is considerable biochemical and electrophysiological data to suggest it as an excitatory neurotransmitter in the CNS (see Sattelle, 1980 a). It must be emphasised that the status of the compounds cited in Table 1 as neurotransmitters is, in some cases, still the subject of considerable speculation and controversy. A number of criteria must be fulfilled before a

TABLE 1 PUTATIVE NEUROTRANSMITTERS IN INSECTS

Neurotransmitter	Site of Action
Acetylcholine[1]	Central Nervous System
GABA[2]	Central Nervous System
Glycine[2]	Central Nervous System
L-Glutamate[2]	Neuromuscular Junction
L-Aspartate[3]	Neuromuscular Junction
GABA[2]	Neuromuscular Junction
5-Hydroxytryptamine[4]	Visceral & Cardiac Neuromuscular Junctions
Dopamine[4]	Visceral & Cardiac Neuromuscular Junctions
Octopamine[4]	Visceral & Cardiac Neuromuscular Junctions
Proctolin[4]	Visceral & Cardiac Neuromuscular Junctions

[1] see Sattelle 1980a

[2] see Usherwood 1978

[3] Irving & Miller 1980

[4] Miller 1980

chemical can be termed a neurotransmitter and these are (Werman, 1966):

1. That the transmitter candidate is synthesised and stored in the nerve terminals.

2. That it is released from the nerve terminals by neural stimulation.

3. That a specific mechanism for removing the transmitter candidate from the synaptic cleft exists.

4. That it mimics the action of the natural transmitter at the synapse and interacts with the receptor at the post-synaptic membrane in the same way as the receptor interacts with the natural transmitter.

Having established that a compound is a strong candidate for being a neurotransmitter through fulfillment of the above criteria, the next step is to isolate and characterise its site of interaction on the post-synaptic membrane, the receptor. However, to ensure that a true physiological receptor is being studied rather than some non-specific binding site, again a number of criteria must be satisfied (Birdsall and Hulme, 1976; Creese, 1978):

A. A component of specific binding should saturate with increasing concentrations of the ligand, indicating a finite number of sites.

B. Specific binding should increase linearly with increasing tissue concentrations.

C. Pharmacologically effective concentrations of receptor-active ligands should displace the saturable component of binding, whereas pharmacologically effective concentrations of drugs with different receptor specificity should be ineffective.

D. The saturable component of binding should be localised to specific regions of tissues known from pharmacological and physiological experiments to contain the receptor.

E. The isolated receptor molecule, when incorporated into a model membrane, should endow the membrane with the property of chemical excitability.

Of these five criteria the last one is the most difficult to satisfy and it is only the nicotinic acetylcholine receptor from the electroplaque which has been reconstituted into a lipid vesicle, endowing it with the required chemical excitability (Sobel et al., 1980; Heidmann et al., 1980; Wu and Raftery, 1981; Anholt et al., 1980; Lindstrom et al., 1980 c). Only this receptor satisfies all five of the above criteria and so can be termed, without reservation, a physiological receptor.

In insects putative neurotransmitter receptors are at various stages of identification and characterisation. Evidence is still accumulating for L-glutamate as a neurotransmitter and only limited study of the biochemical properties of the receptor has been carried out. The greatest advances have been in the isolation and characterisation of putative acetylcholine receptors from insect CNS.

ACETYLCHOLINE RECEPTORS IN INSECT CNS

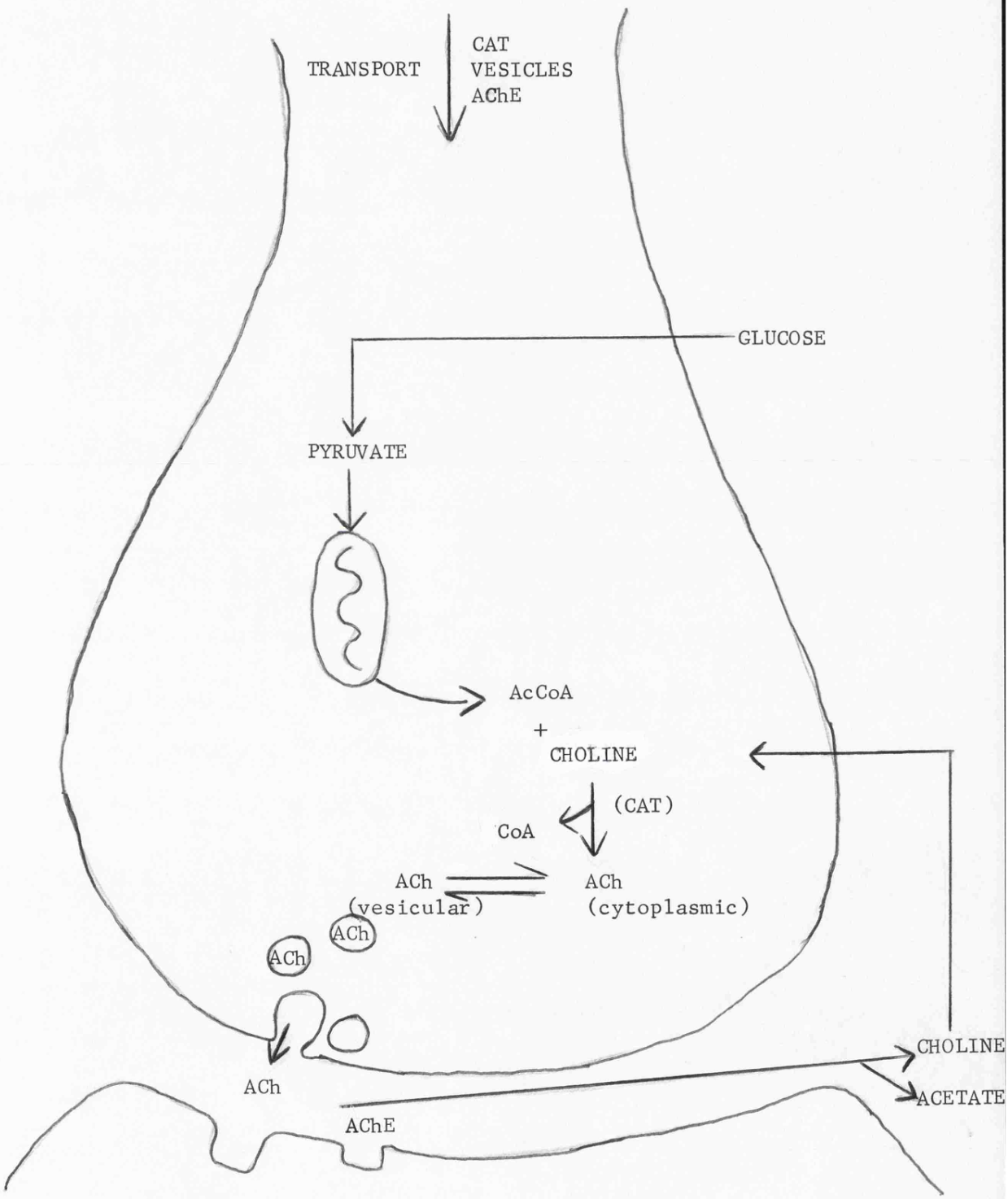
Few species of insects have been studied and in those that have there are rarely both biochemical and electrophysiological data for the same insect. This must be borne in mind when examining the evidence for acetylcholine being a neurotransmitter in insect CNS. Similar reservations must apply to the identification of the acetylcholine receptor because lack of complementary data for all species inevitably results in new information obtained from a particular species being taken as the general rule until proven otherwise.

Acetylcholine as a Neurotransmitter in Insect CNS:

Fig. 6 is a diagramatic representation of the chemical events which take place at the vertebrate neuromuscular junction. The high concentration of many of the components of the cholinergic system in insect CNS strongly suggests the existence of a similar functional system in this tissue.

Acetylcholine, choline acetyltranferase and acetylcholine esterase have been found, at high concentrations, in the CNS of various insects (see, Colhoun 1963; Pichon, 1974). The ability of insect central nervous tissue to synthesise acetylcholine *in vitro* has been demonstrated (Tobias *et al.*, 1946; Lewis, 1953; Smallman, 1956 , 1975; Frontali, 1958; Prescott *et al.*, 1977). Acetylcholinesterase has been localised, cytochemically, in areas along the membranes of axon branches in the neuropile of *Periplaneta americana* (the cockroach) (Smith and Treherne, 1965; Frontali *et al.*, 1971; Hess, 1972) and also in the neuropile of locust metathoracic ganglia (Mandelstom, 1967). These enzymic activities indicate that synthesis and breakdown of acetylcholine occurs in

FIG. 6 Chemical Events at the Vertebrate Nicotinic Acetylcholine Synapse



ACh = Acetylcholine

AcCoA = Acetyl Coenzyme A

CAT = Choline Acetyltransferase

AChE = Acetylcholine Esterase

this tissue and so in part satisfy criteria 1 and 3 (p.15)

The release of acetylcholine from insect ganglia by pre-synaptic nerve stimulation has not yet been demonstrated and early pharmacological work indicated an insensitivity of central neurones to acetylcholine even when desheathed, acetylcholine having no effect at concentrations lower than 1.0×10^{-3} M (Pumphrey and Rawdon-Smith, 1937; Roeder et al., 1947; Roeder, 1948; Twarog and Roeder, 1956, 1957). The same authors, however, noted that upon addition of the acetyl-cholinesterase inhibitor, eserine (1×10^{-6} M) this threshold value was lowered to 1×10^{-4} M. It was not until the work of Shankland et al. (1971) that a threshold value for acetylcholine sensitivity was obtained (1×10^{-6} M) which was more representative of a physiological concen-tration. The lower threshold value was obtained by the authors considering three factors which could affect acetylcholine reaching its site of action and then accounting for them. Firstly, they allowed for diffusion of acetylcholine into the ganglion; secondly, they considered the possibility that endogenous acetylcholine was interfering with the system and so inhibited its synthesis by pre-treatment with hemicholinium-3 (an inhibitor of choline transport) and finally they prevented the rapid hydrolysis of acetylcholine before it reached the synaptic area by pre-treatment of the tissue with either dichlorvos or paraoxon (potent cholinesterase inhibitors). Such preparations were found to be highly sensitive to acetylcholine. This same threshold value of 1×10^{-6} M has been obtained consis-tently using the more sophisticated sucrose-gap recording technique, in the presence of eserine (1×10^{-6} M) (Callec and Sattelle, 1973; Sattelle et al., 1976).

The results, both from studies of enzyme activities and

electrophysiological recordings, lent support to the suggestion that acetylcholine is a neurotransmitter in insect CNS and although all four points on p. 15 have not been satisfied, the implication is strongly positive.

Putative Acetylcholine Receptors in the Insect CNS

Both the electroplaque of electric fish and the post-synaptic membrane of vertebrate skeletal neuromuscular junctions each possess a homologous population of acetylcholine receptors. The receptors have a high affinity for nicotinic agonists and antagonists and little or no affinity for muscarinic ligands. Such a well defined system is not apparent in insect CNS. Three types of putative acetylcholine receptor have been suggested on the basis of their pharmacological specificity. They are:

1. Receptors which have an affinity for both nicotinic and muscarinic ligands - the mixed receptor (nmAChR).
2. Receptors which have an affinity for only muscarinic ligands - the muscarinic receptor (mAChR).
3. Receptors which have an affinity for only nicotinic ligands - the nicotinic receptor (nAChR)

(See Sattelle, 1980 a)

Controversy surrounds the status of these three suggested receptors and it is not known whether they exist in vivo as three distinct receptors or are all part of the same receptor system which has been disrupted during tissue fractionation and subsequent purification procedures. Most of the evidence for the possible existence of these putative acetylcholine receptors has accumulated from radiolabelled ligand binding studies and there are little or no complementary electrophysiological data for the same insect.

1. Acetylcholine Receptors with Mixed Muscarinic-nicotinic Properties:

Putative acetylcholine receptors of mixed muscarinic-nicotinic specificity have been reported in extracts of housefly (Musca domestica) heads (Eldefrawi and O'Brien, 1970; Eldefrawi et al., 1970, 1971; Cattell and Donnellan, 1972; Donnellan et al., 1975; Mansour et al., 1977). The earliest report of cholinergic ligand binding to extracts of insect CNS was that of Eldefrawi and O'Brien (1970) where they showed saturable binding of the cholinergic ligand [^{3}H] muscarone, (Kd 2.4 x 10^{-6} M; Bmax 70 nmol/g head protein) to a 100,000 x g supernatant of an aqueous homogenate of housefly heads. This binding was inhibited by both nicotinic (nicotine, curare and gallamine) and muscarinic (atropine and pilocarpine) ligands (40 - 70% inhibition at 10^{-4} - 10^{-5} M) and it was therefore concluded that [^{3}H] muscarone was binding to an acetylcholine receptor of mixed nicotinic-muscarinic affinity. The same workers went on to complement this study, by demonstrating binding of several additional radiolabelled cholinergic ligands to a similar high speed fraction. Nicotine, decamethonium, dimethyl-d-tubocurarine and atropine all had binding constants in the micro-molar range (Eldefrawi et al., 1971). Furthermore, they concluded that [^{3}H] nicotine and [^{3}H] muscarone were binding to the same macromolecule on the basis of their having the same number of binding sites, the inhibitory effects of six cholinergic ligands were the same in each case and the binding molecules for both radiolabelled ligands were sensitive to trypsin and chymotrypsin but not to phospholipase C (Eldefrawi et al., 1970).

Initial studies of Cattell and Donnellan (1972) were concerned with the use of chloroform-methanol to extract proteolipids from

flyhead homogenates. In later experiments by this same group (Donnellan et al., 1975) an aqueous extract of the same preparation was shown to have the pharmacological characteristics of both their own proteolipid extract and the aqueous extract of the Eldefrawi group. Subsequently, the chloroform-methanol extraction method was abandoned because of the difficulties in the manipulation of proteolipids. Donnellan et al. (1975) also reported two apparent binding constants for [^{3}H] acetylcholine (0.15 and 8.3 μM), and [^{3}H] decamethanium (0.52 and 6.7 μM) but α-bungarotoxin, the potent nicotinic antagonist at the vertebrate skeletal neuromuscular junction, had no effect (Clarke and Donnellan, 1974). A twenty-five fold purification of this putative receptor with respect to [^{3}H] decamethonium binding was achieved by gel-filtration on Sephadex G-200 (Mansour et al., 1977). This partially purified preparation bound only 1 pmol [^{125}I]-α-bungarotoxin/mg. protein compared to 1000 pmol [^{3}H]-decamethonium/mg. protein and acetylcholine displaced [^{3}H] decamethonium binding with an inhibition constant (K_i) of 1 x 10^{-5} M. Further purification of this preparation proved difficult in that affinity chromatography resulted in a 90% loss of total binding activity accompanying any increased purity. Nevertheless, two fast migrating protein bands which apparently accounted for all the binding activity were detected by acrylamide gel electrophoresis (Mansour et al., 1977).

Jewess et al. (1975) achieved an 800-fold purification of a decamethonium-binding component from fly-heads by gel filtration through Sepharose 6B and Sephadex G-200 columns. Although previous work by this group (Donnellan et al., 1975) had found an enrichment of cholinergic ligand binding in an 80,000 x g supernatant fraction

of flyheads homogenised in water, the purification mentioned above was carried out on a solubilised (0.12 M NaCl) 80,000 - 190,000 x g pellet fraction. The flyheads in this instance had been homogenised in 0.32 M sucrose as opposed to water which suggests that the cholinergic binding component is readily soluble in water but not in an isotonic solution.

Polyacrylamide gel electrophoresis in the presence of SDS of this partially purified mixed receptor, revealed two major polypeptide bands of Mr 83,000 and 91,000. By isoelectrofocussing in polyacrylamide gels, two major protein bands were observed with pI values of 4.7 and 4.9 and gel permeation studies attributed an Mr of 360,000 to the native binding protein. The native receptor and subunits all stained as glycoproteins and gas-liquid-chromatography of the alditol acetate derivatives of the native receptor's neutral sugars (3% W/W) has demonstrated that mannose, glucose and galactose are the major constituents (Donnellan and Harris, 1977; Harris et al., (1980).

The same pharmacological specificities were reported for this purified preparation as for both the membrane bound and soluble fractions. High and low affinity binding sites were attributed to radiolabelled acetylcholine, decamethonium and nicotine. α-Bungarotoxin again had no inhibitory effects on binding of these ligands. The muscarinic antagonist quinuclidinyl benzilate (QNB) was also reported to bind to this purified preparation, but the binding did not saturate at concentrations as high as 1 μM. A dissociation constant (K_D) of 0.689 μM was reported for binding of this ligand (Cattell et al., 1980 a).

Tripathi et al. (1979) have also reported purification of a putative mixed receptor. They determined a molecular weight of

360,000 Mr for the native receptor protein which was composed of two subunits Mr 82,000 and 90,000. Only one major protein band of pI 4.8 was obtained after isoelectrofocussing. These results are in general agreement with those of the Donnellan and Cattell group although there is a discrepancy in the size of the subunits and in the number of bands observed upon isoelectrofocussing. Tripathi et al. (1979) also reported that α-bungarotoxin had no effect on [^{3}H] decamethonium binding.

In summary, a cholinergic binding protein of mixed nicotinic-muscarinic pharmacology has been purified with an Mr of 340,000 - 360,000 and two subunits of Mr 83,000 and 91,000 or 82,000 and 90,000. The native protein has an isoelectric point of 4.8, or possibly exists in a monomer and a dimer form pI 4.7 and 4.9, or alternatively as two distinct receptors. α-Bungarotoxin had no effect on the binding of any of the radiolabelled ligands tested to either the soluble, particulate or partially purified preparation. However, rarely, especially in the early reports, was radiolabelled α-bungarotoxin binding measured in the fraction which was enriched in [^{3}H] decamethonium binding, neither were any other fractions assayed for specific α-bungarotoxin binding activity.

2. Acetylcholine Receptors with Muscarinic Properties:

The potent muscarinic antagonist QNB, when radiolabelled, has proved to be a useful receptor probe in studies on vertebrate brain tissue (Yamamura and Snyder, 1974 (a), (b), (c); Yamamura et al., 1974 (a), (b); Snyder et al., 1975) and on isolated brain synaptosomes (Yamamura and Snyder, 1974 (a)).

[^{3}H] Quinuclidinyl benzilate binding has been demonstrated in fractions of fruitfly (Drosophila melanogaster) heads (Dudai and

Ben-Barak, 1977; Dudai, 1977; Haim et al., 1979) housefly heads (Jones and Sumikawa, 1981 b) and locust (Locusta migratoria) central ganglia (Breer, 1981). In all cases binding increased with tissue concentration, and saturated at higher (2 nM) concentrations of [^{3}H] QNB, giving K_D's of 0.7×10^{-9} M, 0.26×10^{-9} M and 0.77×10^{-9} M for fruitfly, housefly and locust respectively. Dissociation constants calculated from on- and off- rate constants, ranged from 0.08×10^{-9} M - 0.7×10^{-9} M. All three preparations also showed a pharmacological specificity for muscarinic ligands, of which atropine, scopalamine, pilocarpine and dexetimide were the most effective inhibitors of [^{3}H] QNB binding. Acetylcholine, in the presence of antiacetylcholinesterases, at a concentration of approximately 10^{-5} M, inhibited binding by 50% in both housefly and fruitfly preparations, and 10^{-4} M acetylcholine displaced more than 80% of [^{3}H] QNB binding in the locust preparation. Jones and Sumikawa (1981) also reported an 80% inhibition of binding by benzoquinonium (10^{-4} M), a potent vertebrate skeletal neuromuscular nicotinic antagonist and lobeline (10^{-4}) a vertebrate ganglionic blocker. No such inhibition by nicotinic ligands was reported by the other two groups but neither group reported testing these two particular ligands and Jones and Sumikawa (1981) found little inhibition with any of the other nicotinic ligands tested. It is possible therefore that inhibition of [^{3}H] QNB binding by benzoquinonium and lobeline is a peculiarity of these two particular ligands. The pharmacological binding specificities, with the exception of benzoquinonium and lobeline, are indicative of a classical muscarinic acetylcholine receptor.

The QNB binding component was shown to sediment between 500 x g and 20,000 x g regardless of whether homogenisation was in isotonic sucrose or in buffer of low ionic strength (Haim et al., 1979). Solubilisation, the first step towards purification, of the QNB binding component proved difficult and to date 100% solubilisation of binding activity has not been reported. The putative receptor is unstable even in isotonic media at $4^{o}C$, losing binding activity with a half-life of 24 hours (Dudai, 1980). Triton X-100, the detergent used to solubilise the nicotinic acetylcholine receptor from both vertebrate muscle (Stephenson, 1980) and invertebrate CNS (Hall, 1980), inactivated this putative muscarinic receptor (Haim et al., 1979). The same authors also reported that 2 M NaCl released less than 20% of the binding activity into the supernatant from a high-speed centrifugation. When this apparently solubilised fraction was passed through a Sepharose 4B column, binding activity was eluted by 2 M NaCl in the void volume, indicating the presence of large aggregates or possibly small membrane fragments.

It would appear therefore that before the putative muscarinic acetylcholine receptor can be purified and fully characterised, further attention must be given to tissue solubilisation procedures that ensure retention of the [^{3}H] QNB binding activity.

3. Acetylcholine Receptors with Nicotinic Properties:

As stated previously, early studies on the binding of radiolabelled nicotinic cholinergic ligands to insect CNS preparations showed no susceptibility to α-bungarotoxin. It was not until Hall and Teng (1975) demonstrated by autoradiography that [^{125}I]-α-bungarotoxin bound in a non-uniform way to frozen sections of

fruitfly CNS, that the early reports that the α-bungarotoxin insensitive cholinergic binding component was the only acetylcholine receptor in insects, were reassessed.

Following the autoradiographical demonstration of [^{125}I]-α-bungarotoxin binding, radiolabelled α-bungarotoxin was shown to bind specifically and saturably to particulate fractions from fruitfly heads (Schmidt-Nielsen et al., 1977; Rudloff, 1978; Dudai, 1977). Binding increased linearly with tissue concentration and saturated with a K_D of 1.1 - 1.8 x 10^{-9} M. Dudai (1980) calculated a K_D of 0.6 x 10^{-9} M from measurements of the on- and off-rate constants. The same author obtained a Hill coefficient of 0.96 for [^{125}I]-α-bungarotoxin binding, indicating no cooperativity and a Hill coefficient of 0.5 for acetylcholine binding which could be an indication of either negative cooperativity or a heterogeneity of agonist binding sites. The concentration of [^{125}I]-α-bungarotoxin binding sites (B_{max}) ranged from 0.0075 to 0.8 pmol/mg. protein. Drug specificity was very similar to that of the classical nicotinic acetylcholine receptor in that [^{125}I]-α-bungarotoxin specific binding was strongly inhibited by nicotinic ligands (nicotine and d-tubocurarine) and muscarinic ligands (atropine and pilocarpine) had little effect. Decamethonium, which has been used in identification of the putative mixed receptor, had little effect.

Similar nicotinic acetylcholine receptor properties have been reported for fractions of housefly heads (Eldefrawi and Eldefrawi, 1980; Harris et al., 1979; Cattell et al., 1980 a, b) and for locust central ganglia (Breer, 1981). However, Cattell et al. (1980 b) reported that when housefly heads were homogenised in 0.32 M sucrose the α-bungarotoxin binding component was enriched

in the pellet fraction of an 80,000 x g centrifugation but the activity was found in the supernatant if the heads were homogenised in water. The same group (March et al., 1980) have subsequently reported an enrichment of binding in the supernatant fraction from centrifugations at 20,000 x g and 150,000 x g, after homogenisation of heads in buffered sucrose. This work will be dealt with in more detail in the discussion (p.156).

In order to purify and further characterise the putative nicotinic receptor it must be obtained in a soluble form. The α-bungarotoxin binding component from housefly heads in the preparation of March et al., (1980) seems to be unusual in this respect because all other workers in the field have had to solubilise the membrane-bound receptor with detergent. Schmidt-Nielsen et al. (1977) reported a preparation of fruitfly heads homogenised in buffer containing the non-ionic detergent Triton X-100 (1% ^{v}/v). Binding to this detergent extract showed similar characteristics and pharmacological specificities to the membrane-bound receptor but no data for total binding activities were reported. On the other hand, Dudai (1978) reported difficulties in solubilising the α-bungarotoxin binding component with Triton X-100 (1% ^{v}/v) and achieved only partial release of binding activity into a 100,000 x g supernatant after treatment with a combination of NaCl (0.12 M) in Triton X-100 (1% ^{v}/v). Furthermore, this apparently solubilised Triton-NaCl extract sedimented after further centrifugation at 200,000 x g for 2 hours and Dudai concluded that most of the material may therefore, represent small membrane fragments or aggregates. Treatment of the particulate α-bungarotoxin binding component of fruitfly heads with the anionic detergent deoxycholate

(1% ^{w}/v) at a pH of 9.0 was reported to give a more efficient extraction than Triton X-100 (1% ^{v}/v) at the same pH (Jimenez and Rudloff, 1980). The deoxycholate extract was included in a Sepharose 6B column whereas the Triton extract was excluded and appeared in the void volume.

Despite the apparent difficulty in solubilisation of the particulate binding component, a scheme has been proposed which gives up to a 1200 fold purification of [^{125}I]-α-bungarotoxin binding activity (Gepner, 1979; Hall, 1980). Triton X-100 (1%) in combination with NaCl (0.1 M) was employed in the solubilisation procedure but again no recovery of total binding activity is given and the criterion for solubility was release into a 30,000 x g supernatant. The "solubilised" fraction was then purified by adsorption onto an affinity column consisting of α-cobratoxin covalently linked to Sepharose 4B, followed by elution with carbamyl-choline (0.2 M). The specific binding activity was 60-1100 pmol/mg protein and a dissociation constant of 1.5 x 10^{-10} M was calculated from the on- and off- rate constants. Pharmacological studies showed a specificity for nicotinic ligands similar to that of the membrane-bound receptor. Molecular weights estimated were 500,000 Mr for the receptor-toxin complex from gel filtration chromatography (without accounting for contribution of bound detergent to the Stokes' radius) and 300,000 from sedimentation in a discontinuous sucrose density gradient ($S_{20,W}$ = 11.5).

Localisation of [^{125}I]-α-Bungarotoxin Binding:

For the three putative receptors, the nicotinic, the muscarinic and the mixed, results of binding studies satisfy the first three

criteria (p. 15) for defining a physiological receptor, i.e. binding is saturable and specific, it increases linearly with tissue concentration and shows pharmacological specificity.

Localisation of binding activity by autoradiographic techniques so far has only been demonstrated for [^{125}I]-α-bungarotoxin binding to frozen sections of the central ganglia from fruitfly, moth (Manduca sexta) and cockroach (Periplaneta americana).

Hall and Teng (1975) first demonstrated a non-uniform distribution of [^{125}I]-α-bungarotoxin binding sites in frozen sections of fruitfly. Binding was confined to neural tissue in the head and thorax. Attempts to introduce [^{125}I]-α-bungarotoxin into whole brain tissue were unsuccessful (Rudloff, 1978), consequently all subsequent work has been carried out on frozen sections.

Three groups of workers independently concluded from autoradiographical studies that toxin binding in fruitflies was confined to synaptic areas of the neuropile and was absent from nerve tracts and cell bodies in the CNS (Schmidt-Nielsen et al., 1977; Dudai and Amsterdam, 1977; Rudloff, 1978). This localisation of toxin binding to neuropile regions was later confirmed in the moth (Hildebrand et al., 1979). However, Sattelle et al., (1981) working on the 6th abdominal ganglion of the cockroach, observed not only dense binding in the neuropile region as expected but also densely concentrated toxin-binding sites in the periphery of the ganglion, a non-synaptic region occupied by glial cells and neuronal cell bodies.

In all cases binding of [^{125}I]-α-bungarotoxin, as shown by autoradiographical studies, has proved to be pharmacologically specific. In the fruitfly, toxin binding was blocked by preincubation

with unlabelled toxin (1×10^{-7} M) or d-tubocurarine (1×10^{-4} M), whereas atropine (1×10^{-4} M) reduced but did not eliminate binding (Schmidt-Nielsen et al., 1977; Rudloff, 1978). Toxin binding was also blocked by nicotine (1×10^{-3} M) (Dudai and Amsterdam, 1977).

In the moth (Hildebrand et al., 1979) binding was blocked by acetylcholine (1×10^{-3} M; in the presence of 10^{-3} neostigmine), d-tubocurarine (1×10^{-3} M) but QNB (1×10^{-3} M) reduced binding only slightly.

All the data so far, with the exception of the report of Sattelle et al. (1981) of binding to non-synaptic neural regions, provide evidence in support of a synaptic localisation of the α-bungarotoxin binding component.

The Physiological Role of the Putative Acetylcholine Receptors:

For a putative receptor to be assigned a physiological role, biochemical binding data need to be supported by electrophysiological studies in the same tissue, of the same species. Unfortunately, in only one preparation have there been reports of both approaches. Most biochemical binding data have accumulated from studies on insect head ganglia, a tissue which has yet to be extensively investigated electrophysiologically. Conversely, detailed electrophysiological data have been presented from work carried out on cercal afferent neurones and giant interneurones in the sixth abdominal ganglion of the cockroach, for which there is only limited complementary ligand binding data. Results of binding experiments on preparations of cockroach abdominal nerve cords are similar to those obtained for preparations of insect head ganglia, in that an [^{125}I]-α-bungarotoxin binding component

has been identified with the specificity expected of a nicotinic acetylcholine receptor. A K_D of 1.1×10^{-9} M was reported for the saturable [^{125}I]-α-bungarotoxin binding, a value which compares favourably with the concentration of α-bungarotoxin (nanomolar range) which completely blocks transmission in this system (Sattelle, 1978, 1980 b; Sattelle et al., 1980). Furthermore, when the concentrations of various cholinergic ligands required to inhibit [^{125}I]-α-bungarotoxin binding by 50% are compared to concentrations estimated to produce half-maximal physiological actions at cercal-afferent, giant interneurone synapses there is a close correspondence in pharmacological specificity, again suggesting the presence of a functional nicotinic acetylcholine receptor (Sattelle, 1978; 1980 a, b).

A physiological role for the other two putative acetylcholine receptors of insects has yet to be demonstrated. It seems unlikely that these putative receptors contribute substantially to acetylcholine-mediated transmission at cercal-afferent, giant interneurone synapses since both the muscarinic receptor and the mixed receptor are unaffected by α-bungarotoxin in the micromolar range. Nevertheless, some support for the existence of α-bungarotoxin-insensitive acetylcholine receptors on certain insect neurones has emerged from recent electrophysiological experiments. For example, dorsal unpaired median (DUM) neurone cell bodies of the grasshopper (Schistocerca nitens) and cockroach metathoracic ganglia are sensitive to acetylcholine but the resulting depolarisation is not blocked by 1×10^{-6} M α-bungarotoxin (Goodman and Spitzer, 1979; Sattelle et al., 1980). However, the pharmacological specificity of these acetylcholine responses has not been

detailed and it should be noted that these experiments were carried out on cell bodies which, in insects, are devoid of synapses and hence the functional role of such receptors is unclear.

L-GLUTAMATE RECEPTORS AT INSECT NEUROMUSCULAR JUNCTIONS

In contrast to the putative cholinergic neurotransmission system in insect CNS, evidence for L-glutamate being a neurotransmitter at insect neuromuscular junctions and the characterisation of the putative receptors is derived almost entirely from electrophysiological studies. The electrophysiological techniques now used are so sophisticated that not only can the change in ion permeability and hence conductance of the post-synaptic membrane be measured, but also the change in conductance and the lifetime of a single open channel can be determined after either neural stimulation or addition of the putative transmitter or its agonists.

Evidence for L-Glutamate as a Neurotransmitter in Insects:

The first invertebrate muscle on which L-glutamate was shown to have an excitatory effect was that of the crayfish (Procambarus clarkii) (Robbins, 1959; Van Harreveld and Mendelson, 1959). Following these reports a similar effect was reported for muscles from the cockroach (Kerkut et al., 1965 a) and locust (Usherwood and Grundfest, 1965). These studies led to the tentative suggestion that L-glutamate was the excitatory neurotransmitter in these tissues. The suggestion was not readily accepted, one of the main objections being the central role of L-glutamate in intermediary metabolism and its apparent high concentration in insect haemolymph, which was believed to be in direct contact with neuromuscular synaptic receptors. A number of explanations have been proposed that argue against the elimination of L-glutamate as a neurotransmitter candidate solely on these grounds.

The concentration of L-glutamate has been reported to be as high as millimolar in the haemolymph of some insects (Chen et al., 1968), a concentration at which an excitatory response and

subsequent desensitisation would be expected if applied to a muscle preparation. A number of workers noticed no such effects following the application of fresh haemolymph to a muscle preparation (Usherwood and Machili, 1968; Miller *et al.*, 1973) but if the haemolymph was stored at room temperature it developed pharmacological activity equivalent to a solution of L-glutamate having a concentration of 1×10^{-4} M (Miller *et al.*, 1973). These authors suggested that L-glutamate was "bound" in some way in fresh haemolymph and on exposure of the haemolymph to air, was slowly released. This was confirmed by Clements and May (1974) when they reported that 25% of blood L-glutamate was bound to divalent cations, but unlike Miller *et al.* (1973), they found an excitatory response in 50% of the cases tested with fresh haemolymph. They suggested the presence of a physical barrier to the entry of haemolymph to the synapse. The barrier, which may be constituted by the perineurium, was probably destroyed on dissection and hence isolated muscle would show a quite different response to exogenous glutamate from that seen with the muscle *in situ*.

However, Irving and Osborne (1976), dismiss the problem of high L-glutamate in the haemolymph by suggesting that in fact all concentrations previously quoted were gross overestimates. Irving and co-workers claim that the techniques they employ represent a major improvement in the analysis of insect blood and that the data of previous investigators were greatly affected by the conversion, *in vivo* and *in vitro*, of L-glutamine to L-glutamate. This would also account for the time-dependent gain in the ability of haemolymph to elicit a pharmacological response, an effect that Irving and Osborne (1976) also noted.

If the criteria for defining a chemical as a neurotransmitter (p. 15) are considered, the evidence for synthesis, storage and release of L-glutamate in and from the nerve terminals is not conclusive. Similarly there is only little evidence for the presence of any removal mechanism in the region of the post-synaptic receptor.

Glutamate has a central role in many aspects of metabolism and is a significant constituent of most tissues, therefore the fact that it is present, along with many of its metabolising enzymes, in insect nervous tissue does not constitute strong evidence for a potential transmitter role. Clarification of this point would be greatly assisted if isolated synaptosomes could be prepared from insect muscle.

Synaptosomes are isolated nerve-ending complexes and are readily prepared from mammalian CNS (Whittaker, 1965; Whittaker and Barker, 1972; De Robertis, 1967). Precise conditions of homogenisation of the nervous tissue appear to tear the pre-synaptic apparatus together with the attached thickened post-synaptic membrane from the post-synaptic cell. The nerve terminal is broken from its axon and the membrane rapidly reseals resulting in a discrete osmotically sensitive entity which is the synaptosome. Electronmicroscopy shows synaptosomes to retain the morphological components of the pre-synaptic terminal (Gray and Whittaker, 1962) and they exhibit the metabolic capabilities of more intact tissue preparations (see Bradford, 1972); synaptosomes respire linearly when incubated with an exogenous substrate, produce ATP, phosphocreatine and lactate and maintain ionic gradients across their membranes. These properties justify Whittaker's (1969) designation of synaptosomes as 'miniature non-nucleated cells' and provide evidence that synaptosomes behave as might be

expected for an intact nerve terminal. Therefore, in mammalian systems, synaptosomes have been invaluable tools in substantiating evidence for a particular ligand being a neurotransmitter; in studies on the uptake and the release of the putative neurotransmitter and in characterising the pharmacology of these systems. The enzymes responsible for synthesis and degradation of putative neurotransmitters have also been shown to be located in synaptosome preparations. Indeed, most of our understanding of the biochemical organisation of the nerve terminal has come from studies on synaptosomes. Moreover, synaptosomes can be osmotically disrupted so that purified fragments of synaptic membranes are obtained, thus providing an ideal preparation for receptor-ligand binding studies without interference from the other components of the nerve terminal.

In reports which have attempted such an isolation of insect neuromuscular synaptosomes (Donnellan et al., 1974; Briley, 1981) problems of fragility and contamination with vesiculated sacroplasmic reticulum and with fragments of disrupted mitochondria have seemingly precluded the isolation of a pure synaptosomal fraction. Thus in these studies it has not been possible to assign a neurotransmitter role to the glutamate measured in any of the fractions prepared. The same problems apply to the enzymes responsible for the synthesis of L-glutamate, in that their presence in any fraction could equally imply synthesis of glutamate as a metabolite or as a neurotransmitter. If isolated insect neuromuscular synaptosomes were available, studies on the uptake, synthesis, storage and release of L-glutamate using such preparations would greatly clarify the neurotransmitter status of this amino acid.

The release of L-glutamate from intact nerve-muscle preparations upon neural stimulation has not been conclusively demonstrated. Early studies (Kerkut et al., 1965 (b); Usherwood et al., 1968) were criticised mainly because of the large quantities of L-glutamate apparently released; values which were too high (>1% of the total weight of the muscle preparation) to be accounted for solely by release of a neurotransmitter. Nevertheless, Usherwood and his co-workers (1968), did demonstrate that this rather high release of L-glutamate from a locust retractor unguis nerve-muscle preparation was proportional to neural stimulation and was Ca^{++} dependent; but in a later attempt to repeat this work Daoud and Miller (1976) could find no correlation between neural stimulation and release of L-glutamate. All of these studies measured release of L-glutamate into perfusates of whole muscle preparations and therefore it is possible that a proportion of the L-glutamate measured was due to leaching from non-synaptic regions which could mask any neurally-promoted release of the amino acid. Recently, Takeuchi et al. (1980) demonstrated the release of picomolar amounts of L-glutamate from a thoroughly washed crayfish muscle preparation and the release was proportional to stimulation. The release was shown to drop significantly upon the reduction of Ca^{++} concentration in the bathing medium. Briley (1981) has demonstrated a K^{+} stimulated release of ^{3}H -L-glutamate from pre-loaded membrane vesicles prepared from locust muscle but the release was not Ca^{++} dependent.

A mechanism for removal of L-glutamate from the synaptic cleft after interaction with the post-synaptic receptor has not yet been delineated. Unlike the enzymic inactivation of acetyl-

choline by acetylcholinesterase in cholinergic systems (Rang, 1975) there is no evidence for enzymic removal of L-glutamate at the insect neuromuscular junction (McDonald, 1972; Dowson and Usherwood, 1973). Two possible alternatives have been suggested. The first, a rapid diffusion of the transmitter from the synaptic cleft, has been shown to be adequate on theoretical grounds (Eccles and Jaeger, 1958) but the suggestion has not been subjected to experimental verification. The second possibility is that there is a high-affinity uptake system for L-glutamate in the synaptic region. An enhanced uptake of labelled L-glutamate by a stimulated intact nerve muscle preparation has been demonstrated autoradiographically (Faeder and Salpeter, 1970; Faeder et al., 1974) at cockroach myo-neural junctions. Uptake was into tracheole cells, sheath cells, (especially in the junctional region) and into the post-synaptic muscle region.

However, autoradiographical studies cannot be taken as conclusive. They need to be complemented by biochemical investigations on sub-cellular preparations to localise the sites of high affinity uptake systems for L-glutamate. Briley (1981) has demonstrated both high and low affinity uptake of L-glutamate into a vesiculated membrane preparation from locust muscle. The high affinity system showed a high Na^+ dependence whereas the low affinity system was relatively independent of Na^+.

The most convincing evidence for L-glutamate being the excitatory neurotransmitter at the insect neuromuscular junction lies in the numerous electrophysiological demonstrations that it interacts with and elicits the same response from the post-synaptic membrane as the natural transmitter (see Gerschenfeld, 1973;

Usherwood and Cull-Candy, 1975; Anwyl, 1975; Usherwood, 1978 b). The most recent and probably the most compelling evidence of this type comes from investigations of glutamate currents and excitatory post-synaptic currents (EPSCs) in voltage-clamped locust muscle fibres (Anwyl and Usherwood, 1974 a; b; Anwyl, 1977 a; b) and from measurements of membrane voltage and current noise generated by application of glutamate to locust muscle fibres (Anderson et al., 1976; 1977).

When a neurotransmitter interacts with its receptor on the post-synaptic membrane the result is the opening of a channel or channels which allows the passage of particular ions down their electrochemical gradient. This conductance of ions gives rise to an electrical current which is dependent on the particular ion channels which have been opened and hence reflects the specificity of the neurotransmitter. That is, one neurotransmitter may open channels for Cl^- ions whereas another neurotransmitter opens channels for Na^+ ions, the post-synaptic current measured in each case will be different and will be characteristic of the ion channels associated with the particular neurotransmitter.

Recent studies (Anwyl and Usherwood, 1974 a, b; 1975 a, b; Anwyl, 1977 a, b) have shown that the EPSCs measured on application of glutamate to voltage-clamped locust muscle, are the same as the EPSCs measured after neural stimulation. This strongly suggests that glutamate and the natural transmitter are opening the same ion channels.

Further encouraging evidence supporting the suggestion that L-glutamate is the natural transmitter, derives from studies of transmitter noise, a phenomenon first described by Katz and Miledi (1972). When an agonist is applied to a synapse the primary event

observed is a change of potential across the post-synaptic membrane. Accompanying this change are minute fluctuations in conductance. These fluctuations form the basis of transmitter noise and arise from random variations in the collision rate between agonist and receptor molecules and hence in the opening and closing of the ion channels. Similarly, when a nerve is at rest spontaneous miniature excitatory post-synaptic potentials (min EPSPs) are still produced by leakage of transmitter. The amplitude of the min EPSPs are seen to be discrete multiples of the smallest min EPSP recorded, which is taken to represent the opening of a single channel. Thus the larger min EPSPs correspond to two or more channels opening simultaneously. Measurement of transmitter noise can therefore quantify the number, lifetime and conductance of channels involved in a min EPSP.

Miniature EPSPs can be produced by iontophoretic application of agonists and the lifetime and conductance of a single channel are specific for the applied drug. Anderson et al. (1976; 1978) working with the extensor tibia muscle of the locust have reported similar results for the lifetime of channels opened by the natural transmitter and iontophoretically applied L-glutamate, a strong indication that the two are identical. Application of quisqualic acid, a glutamate agonist, gave a channel lifetime 2.2 times longer than that associated with L-glutamate (Anderson et al., 1978) thus exemplifying the dependence of the specificity of the open ion channel's characteristics on the ligand applied.

Junctional and Extrajunctional L-Glutamate Receptors:

Extrajunctional receptors for L-glutamate have been found on locust leg muscle fibres (Lea and Usherwood, 1973; Usherwood and

Cull-Candy, 1975; Cull-Candy and Usherwood, 1973; Cull-Candy, 1976 a). Both junctional and extrajunctional receptors display heterogeneity in terms of their pharmacological properties and, to a lesser extent, in terms of the ionophores that they gate.

Recently, reports have suggested the co-existence of three pharmacologically distinct sub-populations of L-glutamate receptors on locust muscle post-synaptic membranes (Clark et al., 1979 a; Gration et al., 1979). This distinction results from their preference for either DL-ibotenate or L-aspartate or neither. The depolarisations arising from application of these two ligands cannot be accounted for by solely a pre-synaptic action because the same depolarisations were recorded on muscle which had been denervated for 18 days, after which time the axon terminal has degenerated (Usherwood, 1963; Rees and Usherwood, 1972; Usherwood, 1973).

The first sub-population of junctional glutamate receptors is activated by DL-ibotenate and therefore is assumed to prefer L-glutamate in its fully extended form (Fig. 7). The second sub-population has a high affinity for L-aspartate and therefore appears to prefer L-glutamate in its fully folded conformation. Finally, the last sub-population and the largest, is activated by neither DL-ibotenate nor L-aspartate and therefore seemingly prefers L-glutamate in its partially folded conformation. Questions yet to be answered are whether or not all three sub-populations co-exist at all insect excitatory neuromuscular junctions, whether they gate the same ionophores and whether all three contribute to the EPSC.

Extrajunctional L-glutamate receptors can be divided into

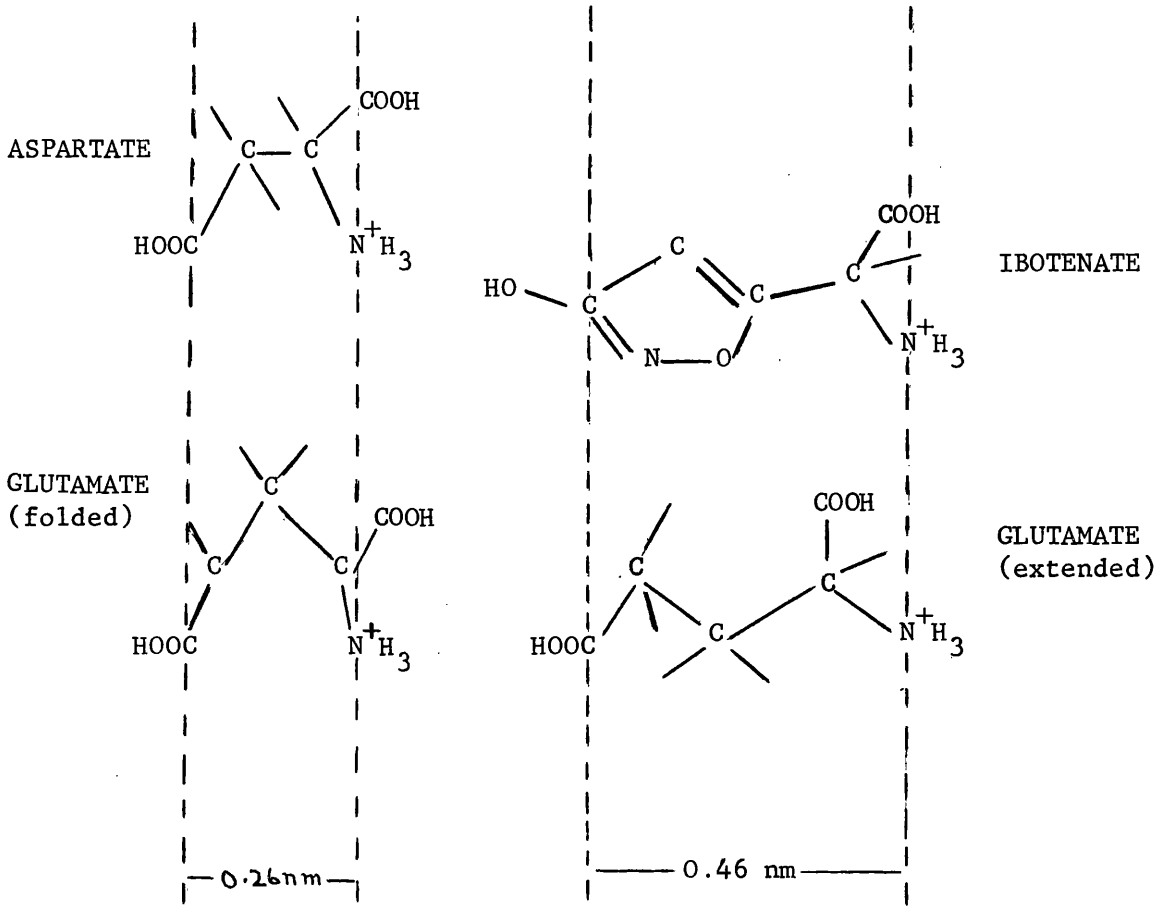


FIG. 7 Diagramatic Representation of the Folded and Extended Conformations of Glutamate

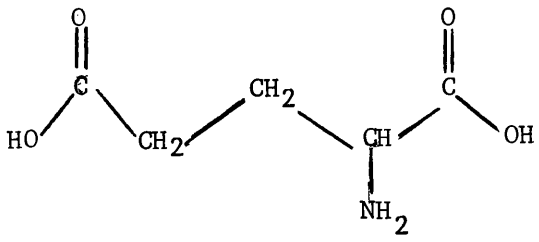
two pharmacologically and physiologically distinct populations. One population, D-receptors, is very similar to the glutamate junctional receptor in that it gates ionophores for Na^+, K^+ and possibly Ca^{++} and the two receptors have identical reversal currents (Clark et al., 1979 b). It has not yet been established whether the D-receptor population is pharmacologically homogeneous, although a sensitivity to quisqualate and not to DL-ibotenate has been reported (Usherwood, 1978). The second population of extrajunctional receptors, H-receptors (Mathers and Usherwood, 1978) gates for Cl^- ions and unlike D-receptors, the H-receptors are insensitive to quisqualate but sensitive to DL-ibotenate (Usherwood, 1978 a). Both receptor population-densities are normally quite low, relative to the junctional receptors and upon denervation or local injury only the D-receptor population has been shown to proliferate (Cull-Candy, 1975; Usherwood, 1969; Mathers and Usherwood, 1978). A functional role has yet to be attributed to these extrajunctional receptors.

The Pharmacology of L-Glutamate Receptors:

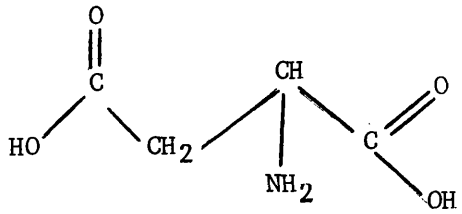
To date no specific highly potent agonist or antagonist for L-glutamate has been reported. Kainate, quisqualate, DL-ibotenate and L-aspartate are only very weak agonists, in comparison to L-glutamate, in both vertebrate CNS and at the invertebrate neuromuscular junction. The search for potent antagonists of L-glutamate has been mainly concerned with the effects of analogues of glutamate and aspartate on mammalian CNS preparations.

From electrophysiological studies putative L-glutamate receptors in mammalian CNS have been divided into three receptor populations on the basis of the effects of selective antagonists

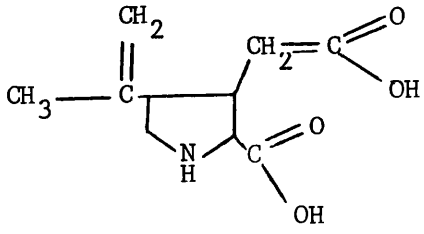
on responses to a range of L-glutamate agonists (Figs. 8 and 9) (see, Davies et al., 1980). These three receptor populations are: firstly, those that are activated by N-methyl-D-aspartate (NMDA) and are selectively blocked by a variety of antagonists, the most potent of which is 2-amino-5-phosphonvalerate (2 APV); secondly, quisqualate-induced responses which are selectively blocked by L-glutamic acid diethyl ester (GDEE) and finally receptors which are activated by kainate. The latter receptor population is not selectively inhibited by any one antagonist but can be distinguished from quisqualate receptors by γ-D-glutamylglycine (γDGG) but γDGG also inhibits NMDA responses. All of these antagonists together with 2-amino-5- phosphonobutyric acid (2 APB) and cis-2,3-piperidine dicarboxcyclic acid (cis-2,3-PDA) (Fig. 9) block L-glutamate induced responses with varying degrees of discrimination. However, there is a poor correspondence between conclusions drawn from electrophysiological studies and conclusions drawn from binding studies and often the pharmacology deduced from binding studies is conflicting. [^{3}H] Kainate has been shown to bind with high affinity to rat brain membrane fractions. Quisqualate and L-glutamate also bind with a high and a moderate affinity respectively but neither ibotenate nor NMDA bind at all (Simon et al., 1976; Johnston et al., 1979). Conversely, Snodgrass (1979) has reported that [^{3}H] NMDA binds with high affinity to cerebellar membranes of rat and mouse brain, with an inhibition pattern of binding consistent with the results of electrophysiological studies. The number of [^{3}H] L-glutamate binding sites exceeds those for kainate or NMDA by ten-fold or more (Simon et al, 1976; Snodgrass, 1979; Foster and Roberts, 1978) and in general these sites appear to



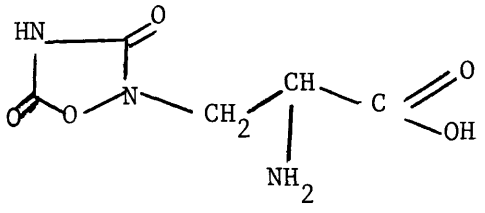
glutamate



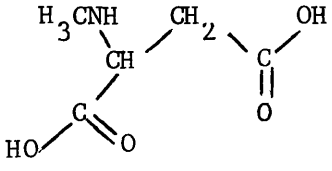
aspartate



kainate

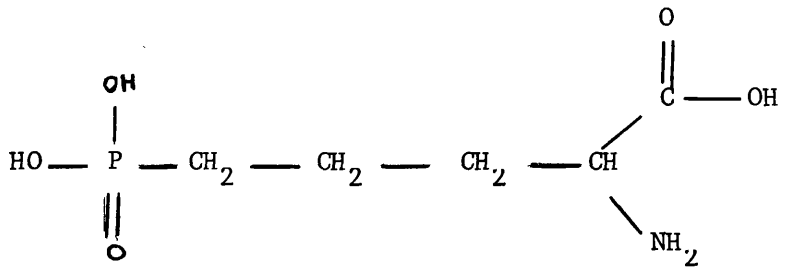


quisqualate

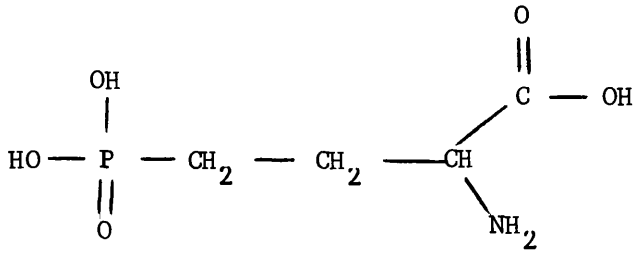


N-methyl-D-aspartate

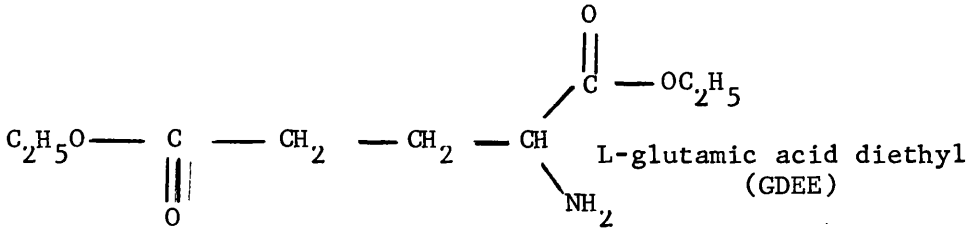
FIG. 8 Structure of Glutamate and Potential Agonists



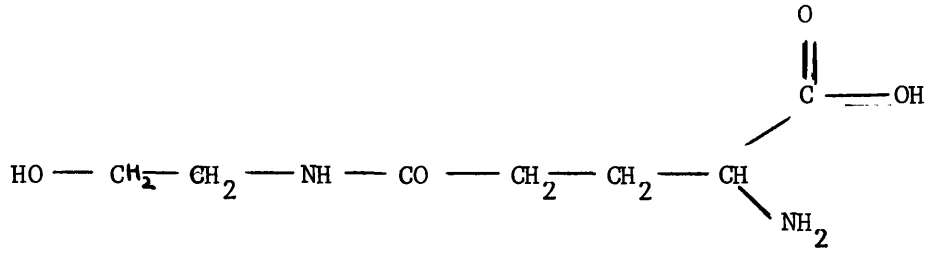
2-amino-5-phosphonovaleric acid (2APV)



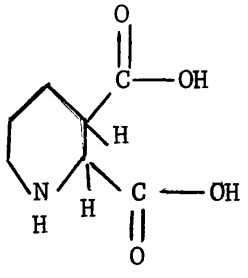
2-amino-4 phosphonobutyric acid (2APB)



L-glutamic acid diethyl ester (GDEE)



γ-D-glutamylglycine (γDGG)



cis-2,3-piperidine dicarboxylic acid (cis-2,3-PDA)

FIG 9 Structures of Excitatory Amino Acid Antagonists

resemble those classified on the basis of electrophysiological experiments as quisqualate receptors, in that neither kainate nor NMDA inhibited [^{3}H] L-glutamate binding to cerebellar membranes, whereas quisqualate was almost as effective as L-glutamate itself. On the other hand, the potent quisqualate antagonist GDEE, as observed from electrophysiological studies, inhibited binding only weakly (Foster and Roberts, 1978).

The results from both electrophysiological studies and binding studies are interesting and encouraging in that further investigation of a wider range of glutamate analogues may bring to light a potent antagonist of this putative neurotransmitter, which may also be effective at the insect neuromuscular junction. Moreover, the interaction of the antagonists with various agonists of L-glutamate may enable distinctions to be made between different populations of L-glutamate receptors and may permit the elucidation of the physiological role of such receptors.

Very few of these putative antagonists have been examined in insect systems. Cull-Candy et al. (1976) reported antagonism by iontophoretically applied DL-2 APB of an L-glutamate-induced response at the locust neuromuscular junction. However, when DL-2 APB was bath-applied it had no effect even at very high concentrations (5×10^{-4}M). The authors proposed that these observed differences in effectiveness may be more apparent than real and may only reflect a difference in the concentration of drug reaching the post-synaptic receptors. In binding studies with proteolipids isolated from locust muscle (Cull-Candy et al., 1976) DL-2 APB was shown to inhibit L-glutamate binding although the inhibition constant (K_i; 65 μM) indicates a relatively low affinity for the receptor.

Unfortunately, no equivalent of the potent antagonist, α-bungarotoxin, at the vertebrate skeletal neuromuscular junction has yet been found for insect neuromuscular junctions. More effort is now being put into studies on the effect of natural toxins and venoms on insect nerve-muscle transmission (see, Leake and Walker, 1980).

The most interesting natural venom under investigation at the moment is that of the digger wasp, Philanthus triangulum. The female wasp lays its eggs on honeybee workers which it has paralysed by stinging. The venom causes paralysis apparently by blocking the neuromuscular system at the periphery (Rathmeyer, 1962) and subsequent experiments on the locust have yielded no evidence for any effects on axonal conduction or muscular contraction (Piek et al., 1971). The venom appears to have a complex mode of action at the locust neuromuscular synapse and has been shown to contain at least three active compounds of low molecular weight (Piek and Spanjer, 1978). At least one pre-synaptic action has been attributed to the venom, as judged by its effect on the frequency of miniature EPSPs, at relatively low concentrations (Piek and Spanjer, 1978). A post-synaptic action of the venom is also suggested by the reduction in glutamate-induced and neurally-evoked contractions when applied to the retractor unguis muscle of the locust (May and Piek, 1979). The authors propose that the venom binds to the glutamate ion-channel in the open conformation, as bath-applied glutamate potentials are blocked non-competitively, indicating a site of action that is different from the receptor site. The complexity of the mode of action of the digger wasp venom may be elucidated by analysis of the specific actions of

the three active components (Piek and Spanjer, 1978) and thus distinction between the relative contributions of pre- and post-synaptic action to the neuromuscular block may ensue.

Biochemistry of L-Glutamate Receptors

Biochemical studies on L-glutamate receptors on insect muscle lie far behind electrophysiological studies. Biochemical identification and characterisation of a putative receptor usually involve radiolabelled-ligand binding studies, using a ligand which has a high affinity and specificity for the receptor. No such ligand is available for the putative L-glutamate receptor and workers have had to resort to using the radiolabelled putative transmitter in such studies. This poses problems in that the off-rate is likely to be quite high and more importantly the assessment of the specificity of binding may be difficult (See discussion p. 104).

First reports of specific L-glutamate binding receptors were made adopting the procedures of De Robertis (1975) for extracting putative receptors as proteolipids. Two of the isolated proteolipids exhibited specific binding of L-glutamate with K_D's of 8 μM and 50 μM (Lunt, 1973; James *et al.*, 1977). Binding was also shown to increase with tissue concentration and the proteolipid with the higher affinity for L-glutamate also showed the pharmacological specificity expected for an L-glutamate receptor (James *et al.*, 1977).

However, as stated previously, working with proteolipids presents many problems, not only in the measurement of binding but also in the further characterisation of the binding macromolecule. In general the techniques that are used for the physiochemical characterisation of proteins are applicable only to those proteins

that are readily soluble in aqueous systems. Therefore, as proteolipids are, by definition, soluble only in chloroform-methanol mixtures the conventional techniques cannot easily be used to determine the physical parameters of the proteolipid receptors. Because of these considerations, attention has turned to measurement of glutamate binding to intact membrane fragments prepared from insect muscle.

Specific binding of L-glutamate to a single set of binding sites, with a K_D of 0.5 μM, was reported for a whole muscle preparation from locust solubilised in deoxycholate (Lunt, 1973). Later studies were carried out on a sub-fraction of locust muscle, using ultrafiltration (Donnellan et al., 1975) to separate bound from free L-glutamate. As with the isolated proteolipids (James et al., 1977) two populations of specific binding sites were reported which differed in their dependence on Na^+ (James et al., 1977). One site was Na^+ independent, had a K_D of 0.53 μM and a B max of 25 pmol/mg of protein. The other site was dependent on Na^+ and had a lower affinity for L-glutamate, with a K_D of 21.6 μM and a B max of 175 pmol/mg of protein. Pharmacological specificity of these two sites, using putative agonists and antagonists suggested a receptor-like function for the binding site of higher affinity whereas the Na^+ independent lower affinity binding site showed some characteristics of an uptake system. However, these early membrane-fragment binding studies are open to criticism in that only D-glutamate was used to account for non-specific binding.

In an impure preparation it is inevitable that a proportion of the L-glutamate binding measured will be to non-neuroreceptor binding sites (see discussion, p.105). D-Glutamate, in excess,

was used on the premise that as it is physiologically inactive but has the same structure as the L-isomer, it will occupy only the non-specific glutamate binding sites. However, as very little is known about the specific interactions of L-glutamate with its receptor at the molecular level, it is questionable whether or not D-glutamate occupies all of the non-specific binding sites or if indeed it is totally inert with respect to L-glutamate specific binding (see Discussion, p. 77). In studies on mammalian brain the method of "unlabelled ligand excess" is now widely used in the determination of the specific L-glutamate binding component. The method is based on the assumption that if the specific radioactivity of the radiolabelled ligand is reduced by addition of a large excess of unlabelled ligand, then specific binding will become insignificant whereas non-specific binding will be essentially unchanged. The assumption is rationalised algebraically by Turner (1980) and specific [^{3}H] L-glutamate binding is defined as the total binding minus the amount bound in an excess of unlabelled L-glutamate (see, Discussion, p.104).

Using this method to account for non-specific binding, Cleworth et al. (1980) reported two specific binding sites for L-glutamate in a subfraction of locust femur muscle. One saturated rapidly in 15 minutes and was independent of Na^+ and the binding to the other was linear over a period of one hour and was dependent on the presence of Na^+. The data presented in this report were insufficient to estimate K_D's or B max's for either binding site.

There has been a considerable amount of work done on the binding of L-glutamate to putative receptors in mammalian brain and rather more progress has been made in this area than in the

insect studies. However, the same problems of lack of a diagnostic glutamatergic ligand and the ubiquitous distribution of the amino acid are pertinent. Furthermore, early binding studies to mammalian brain were limited with respect to the concentration range of ligand which could be investigated because only low specific activity [^{14}C]-L-glutamate was available.

Bearing these considerations in mind, first reported binding sites for L-glutamate in this tissue were of rather low affinities with K_D's in the micromolar range (Roberts, 1974; Michaelis et al., 1974). Roberts also noted a marked difference in glutamate binding in the presence and absence of Na^+. De Robertis et al. (1976) had similar problems of a restricted concentration range but demonstrated binding to three proteolipids isolated from whole rat brain with K_D's of 0.3 μM, 5 μM and 55 μM and corresponding B max's of 0.53, 32 and 166 nmol/mg protein. The Na^+ dependence of binding to these three sites was not investigated so it is not possible to attribute them with either receptor binding or an uptake function. It would seem unlikely that the two binding sites of lower affinity are neuroreceptors because the reported concentration of these sites is so high as to render a synaptic origin improbable.

More recent studies have utilised radiolabelled L-glutamate of a much higher specific activity. Consequently, the ability to accurately measure binding at lower concentrations of L-glutamate has revealed binding sites of much higher affinity than was previously reported. A single population of L-glutamate binding sites has been demonstrated on freshly-prepared cerebellar synaptic membranes with a K_D of 744 nM and a B max of 73 pmol/mg protein (Foster and Roberts, 1978). The pharmacology of this binding site was in

keeping with the characteristics expected for a post-synaptic glutamate receptor. However, a later study by the same workers (Sharif and Roberts, 1980 a) estimated a K_D of 300 nM and a B max of 117 pmol/mg protein. The reasons for the increased affinity and increased concentration of binding sites appear to be changes in the preparation procedure, namely, increased periods of ultrasonication and more extensive washing of the fractions. On investigation of even lower concentrations of [^{3}H]-L-glutamate, binding sites with K_D's in the low nanomolar region were obtained. Biziere et al. (1980) reported two binding sites of K_D's 11 nM and 80 nM for homogenates of whole rat brain and Roberts (1981) detected multiple binding sites to rat striated synaptic membranes when a [^{3}H]-L-glutamate range of 0.05 - 25 nM was examined. One of these binding sites had a K_D of 16.6 nM and a B max of 400 fmol/mg protein and thus corresponds with the high affinity (K_D = 11 nM) binding site reported by Biziere et al (1980). As yet, no pharmacological data are available for these very high affinity binding sites and indeed, as the authors themselves point out, their physiological significance is far from clear.

The majority of binding studies outlined above were carried out in an Na^+-free medium with the intention of minimising possible binding to a high affinity glutamate uptake site. A number of workers have subsequently investigated the dependence of [^{3}H]-L-glutamate binding on Na^+ concentration with a view to revealing such an uptake system. Baudry and Lynch (1981) demonstrated in rat hippocampal membranes that low concentrations of Na^+ (0.5 - 5 mM) inhibited L-glutamate binding whereas higher concentrations (10 - 150 mM) stimulated it. Two binding sites were thus revealed,

one high affinity, Na^+-independent site, K_D 750 nM, and another of lower affinity, K_D 2.4 μM, which was dependent on the presence of sodium. The authors attribute a high affinity glutamate uptake system to the Na^+-dependent binding site, as its regional distribution compares favourably with that of glutamate uptake into brain homogenates. On the other hand, the regional distribution of the high affinity Na^+-independent binding site is markedly different. Vincent and McGeer (1980) also compared Na^+-dependent glutamate binding with high-affinity glutamate uptake in rat striatum but unlike the majority of other workers, they could not detect [^{3}H]-L-glutamate binding in the absence of sodium. Nevertheless, the authors concluded from pharmacological studies and from lesion studies that about half of the Na^+-dependent glutamate binding sites in rat striatum represent high-affinity uptake sites on the corticostriatal terminals. The remainder of the sites are located on striatal neurones and may also be uptake sites. Apart from these two studies (Baudry and Lynch, 1981; Vincent and Mc Geer, (1980)little attention has been given to the putative L-glutamate uptake system. More detailed studies of L-glutamate binding to mammalian brain have been mainly concerned with the Na^+-independent component - the putative neuroreceptor.

Sodium independent L-glutamate binding appears to be very sensitive to low temperatures (Foster and Roberts, 1978). Specific [^{3}H]-L-glutamate binding to cerebellar synaptic membranes was almost abolished by freezing in liquid nitrogen or storage at -30°C and was undetectable after storage at 4°C for 20 hours. Non-specific binding was unaffected by any of these procedures. The addition of cryoprotectants such as glycerol afforded no protection to the

specific binding component. Two storage procedures have been suggested which do not have a detrimental effect on binding. The first method involves lyophilisation of the membranes immediately after preparation and not only confers stability to the preparation but also enhances binding as demonstrated by an increase in B_{max} from 117 pmol/mg protein to 227 pmol/mg protein (Sharif and Roberts, 1980 a). The second storage procedure in which the specific binding capacity of synaptic membranes is preserved was reported by Chang and Michaelis (1980) and requires rapid freezing of the tissue at -80°C followed by thawing in a controlled manner according to the method of Rudnick (1977) for platelet membrane vesicles. Both of the procedures enable storage of synaptic membranes for at least one month without loss of binding activity.

Preliminary studies by Sharif and Roberts (1980 a) and Michaelis et al. (1980) have suggested the presence of an endogenous glutamate inhibitor in synaptic membrane preparations. Either incubation of the tissue at 37°C for 30 minutes followed by extensive washing (Sharif and Roberts, 1980 a) or treatment of brain synaptic membranes with 1% or 0.5% cholate buffer solutions (Michaelis et al., 1980) apparently resulted in the release of the proposed endogenous inhibitor into a 50,000 x g supernatant. Binding was enhanced in both cases and cholate treatment resulted in the appearance of a set of high-affinity (K_D = 3.2 nM) low capacity (B max = 24 pmol/mg protein) binding sites, not seen in untreated membranes. Readdition of the supernatant (found to contain no glutamate or any other detectable amino acids) to the washed, incubated membranes resulted in a potent inhibition of specific L-glutamate binding (Sharif and Roberts, 1980 a). Michaelis et al. (1980) have tentatively suggested

that this endogenous inhibitor is a brain ganglioside for two reasons; firstly, cholate treatment of the membranes extracted N-acetyl neuraminic acid, a marker for gangliosides and secondly, addition of a mixture of bovine brain gangliosides to the assay media produced a dose-dependent inhibition of binding. This effect has been interpreted as a reflection of the possible functional importance of glycosyl residues on the glutamate receptor (Roberts, 1981).

To date little is known of the functional groupings involved in the receptor-lipid interactions occurring in mammalian brain synaptic membranes. A very preliminary report by Sharif and Roberts (1980 b) suggests firstly that a membrane glycoprotein could be involved, as indicated by decreased specific binding in the presence of trypsin and the inhibition of binding by concanavalin A. The authors also suggest that membrane lipids are crucially involved in the maintenance of the glutamate receptor conformation, as judged by the decrease in binding after treatment with phospholipase, polyene antibiotics or extraction of membrane lipid with a 50% acetone :water mixture or treatment with 0.5% Triton X-100. Finally, it has been suggested (Michaelis, 1979; Michaelis et al., 1980) that the glutamate binding site may be a metallo-protein (glycoprotein) with an Fe_2S_2 centre at the glutamate receptor, as azide and strong field iron ligands have been shown to inhibit binding.

Regulation of the glutamate binding site is also unclear, but recently a series of experiments carried out by Baudry and Lynch, (1979; 1980; Baudry et al., 1980) provides evidence for an involvement of cations in the regulatory process. Monovalent cations

such as Li^+, Cs^+, Ru^+, K^+ and Na^+ in the low millimolar range all show significant inhibition of Na^+-independent binding in hippocampal synaptic membrane preparations, whereas low micromolar concentrations of Ca^{++}, Mg^{++} and Mn^{++} effectively enhance the binding (Baudry et al., 1980). In these studies the number of sites rather than their affinity is changed and the authors suggest involvement of a Ca^{++} regulated thiol-protease that regulates the number of glutamate receptor sites in hippocampal synaptic membranes (Baudry and Lynch, 1980).

In several other systems guanine nucleotides have been shown to regulate receptor sensitivity. Such a regulation may also apply to glutamate receptors as Sharif and Roberts (1980 c) have demonstrated substantial inhibition by these nucleotides, especially GTP, at mircomolar concentrations, of [^{3}H]-L-glutamate binding to cerebellar synaptic membranes, an effect not observed with adenine nucleotides.

Solubilisation of rat brain synaptic membranes has been achieved using Triton X-100 (Michaelis, 1975; Sharif and Roberts, 1980 a). Michaelis (1975) then went on to partially purify a glutamate binding glycoprotein by affinity chromatography on concanavalin A. Gel electrophoresis revealed a small acidic protein of 13,800 Mr which was not related to the enzymes involved in glutamate metabolism. Binding was inhibited by concanavalin A and iron ligands thus providing further evidence for the suggested metalloglycoprotein nature of the receptor. Furthermore, gangliosides, the proposed endogenous inhibitor, also inhibited binding.

In fulfilling the criteria for defining a physiological neuroreceptor (p. 15) point E, incorporation of the isolated

receptor molecule into a model membrane and thus endowing the membrane with the property of chemical excitability, is probably the definitive test. A very preliminary report has been made of the incorporation of the metallo-glycoprotein of Michaelis into a variety of membrane preparations and some binding activity was retained (Grubbs and Michaelis, 1979). In similar experiments, Kusnetsov and coworkers solubilised the hippocampal glutamate binding site with Triton X-100 and then achieved partial purification by chromatography on Sepharose 4B. Incorporation of both the detergent extract and the partially purified protein into lipid films prepared from whole brain phospholipids, was then attempted. Glutamate-induced permeability changes were observed but spontaneous permeability changes, of equal magnitude, also occurred in aged preparations (Kuznetsov and Mogilyanskii, 1975). A more recent study by the same workers described the incorporation of "Triton X-100 solubilised membrane fragments" into a planar lipid bilayer (Kolomytkin et al., 1979 a, b). Glutamate-induced permeability changes were again noted but only if incorporation was into both sides of the lipid membrane. This latter observation implies that some sort of interaction between components in the two halves of the bilayer is necessary for the formation of functional channels. The authors conclude that these glutamate induced permeability changes, especially for Na^+, are consistent with those of the synaptic glutamate receptor. However, a great deal more work is necessary before the above system can be termed a reconstituted functional glutamate receptor.

The initial part of the work described in this thesis was an attempt to further characterise this putative L-glutamate receptor in subfractions from the locust, Schistocerca gregaria, interthoracic

muscle, using the method of "unlabelled ligand excess" to account for non-specific binding. As will be seen, many problems were encountered both in obtaining a suitable membranous subfraction with minimal non-specific binding and in the binding assay itself. In contrast, the second part of this thesis is devoted to characterisation of the α-bungarotoxin binding component, a putative nicotinic acetylcholine receptor, from the same insect's supra-oesophageal ganglia. The problems were minimal relative to the glutamate study and characterisation of a membranous, solubilised and consequently partially purified binding component was achieved. Both studies sought to provide basic knowledge about the neurotransmitter receptors of the insect nervous system that would permit comparisons to be made with their better characterised mammalian counterparts. Such receptor sites, once characterised, would constitute attactive targets for novel insecticides.

SECTION 1 : STUDIES ON PUTATIVE L-GLUTAMATE RECEPTORS AT THE LOCUST NEUROMUSCULAR JUNCTION

MATERIALS

Locusts, (Schistocerca gregaria), were supplied by Larujohn, Colwyn Bay, U.K.

Enzymes, substrates and co-factors were obtained from Boehringer-Mannheim, West Germany, or Sigma Chemical Company Ltd., London, U.K.

General reagents were obtained from B.D.H. Chemicals, Poole, Dorset, U.K.

L-[G-^{3}H]-glutamate (35Ci/mmol) was obtained from the Radiochemical Centre, Amersham, Bucks., U.K.

Centrifugation procedures were carried out using an MSE model 18, Beckman L5 50B Ultracentrifuge, Beckman Airfuge and Beckman Microfuge B.

Spectrophotometric assays were performed on an Aminco-Bowman spectrofluorometer or a Cecil CE 212 UV spectrophotometer coupled to a Heathkit model 1R-18M multispeed chart recorder.

Radioactivity was determined by liquid scintillation spectrometry using a Packard Tricarb model 3385.

METHODS

1. Tissue Preparation:

All experiments were carried out using the total intrathoracic musculature from two-week old adult locusts (Schistocerca gregaria). As a routine, the head, abdomen and legs of the locust were cut off; the thorax was then cut ventrally and gently pulled open to reveal the muscles. Most of the fat body was removed by blotting with tissue papers and the entire thoracic muscle was dissected out and stored on ice. The muscle (approx. 3 g from 20 locusts) was homogenised (2% w/v) in a solution of locust saline (Table 2) pH 7.0,in a Potter-Elvejham homogeniser with a motor driven pestle (speed, 500 rpm; radial clearance 0.15 mm). The homogenate was then filtered through nylon bolting cloth (159 µM mesh) and the filtrate subjected to the fractionation scheme shown in Fig.10.

2. Electron Microscopy:

The morphology of the P_2 fraction was examined by electron-microscopy. Table 3 outlines the technique used to prepare tissue.

Fractions were fixed as suspensions by dropwise addition of an equal volume of 6% glutaraldehyde in 0.1M cacodylate buffer, pH 7.1, containing 0.2M sucrose (final concentration of 3% glutaraldehyde). Samples were post-fixed in 1% osmium tetroxide in the same buffer after washing of the fixed suspensions by centrifugation. Pellets were dehydrated through a series of alcohols and embedded in Spurr's resin. Ultrathin sections were cut (LKB microtome) and stained with uranyl acetate and Reynold's lead citrate. Grids were viewed in a Jeol 100 x c electron microscope equipped with a camera.

TABLE 2

Locust Saline (Hoyle, 1953)

0.15	M	Choline chloride (or sodium chloride for Na rich saline)
10	mM	Potassium chloride
2	mM	Calcium chloride
5	mM	Magnesium chloride
4	mM	Potassium hydrogen carbonate
6	mM	Potassium dihydrogen phosphate

pH 7.0

FIGURE 10

Fractionation Scheme for Locust Muscle

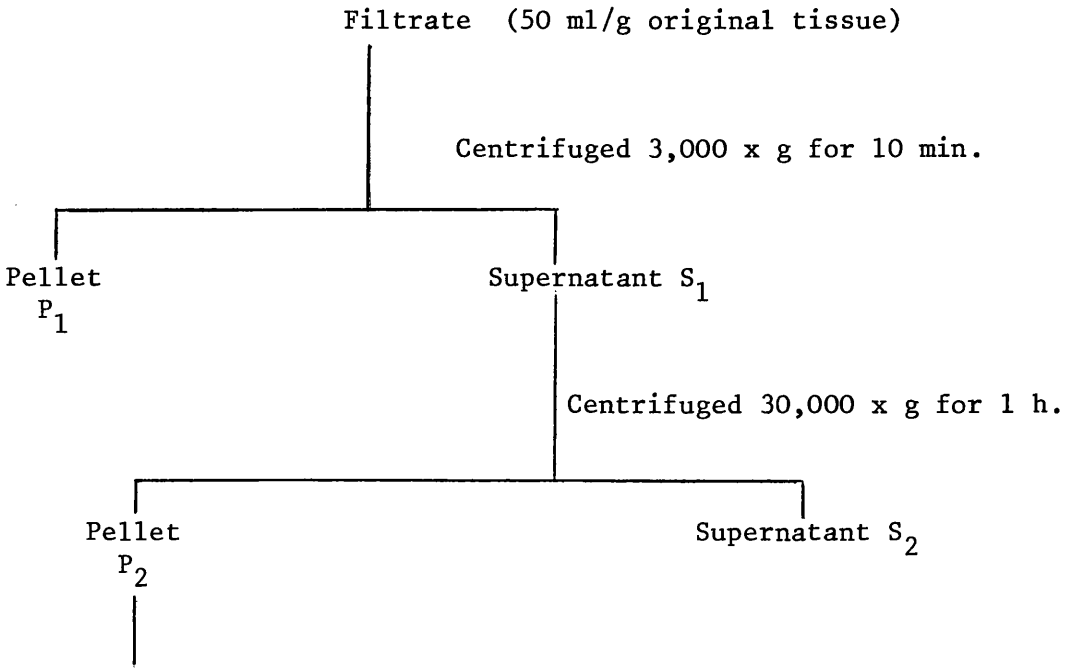


TABLE 3

Preparative Procedure for Electron Microscopy of P_2 Fraction from Locust Muscle

STAGE	CONDITIONS	TIME
Prefix	3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.1, containing 0.25 M sucrose, room temperature	60 min
Wash	Cacodylate/sucrose buffer, by centrifugation and resuspension	45 min
Postfix	1% Osmium tetroxide in cacodylate/sucrose buffer	60 min
Wash	Cacodylate/sucrose buffer	Overnight
Dehydration	70% ethanol	30 min
	95% ethanol	30 min
	100% ethanol	3 x 20 min
Embedding	Spurr's Resin	3 - 4 hr
Polymerisation	60°C oven	2 - 3 days
Sectioning	Ultrathin sections, LKB microtome Om U3	
Staining	Uranyl acetate in 70% ethanol (saturated solution)	10 min
	Reynold's lead citrate	10 min
Observations	Jeal 100 x C microscope	

3. Measurement of Enzyme Activities:

Assay procedures were those of Donnellan et al, (1974) unless otherwise stated. Spectrophotometric assays were performed at 30°C using silica cuvettes of 1 cm light path. Reaction volumes were 3.0 ml.

a) L-Aspartate:2 Oxoglutarate Aminotransferase:

(E.C. 2.6.1.1.; GOT)

Glutamate oxaloacetate transaminase activity was measured by coupling oxaloacetate production to the oxidation of NADH by malate dehydrogenase.

$$\text{L-asp} + \alpha\text{KG} \xrightarrow{\text{GOT}} \text{OAA} + \text{L-Glu}$$

$$\text{OAA} + \text{NADH} + \text{H}^+ \xrightarrow{\text{MDH}} \text{L-malate} + \text{NAD}^+$$

Extracts were added to reaction mixtures containing 50 mM KH_2PO_4, pH 7.9, 0.1% w/v Triton X-100, 40 mM L-aspartate, 0.22 mM NADH and 5 U malate dehydrogenase (MDH, Boehringer pig heart, mitochondrial, 5 mg/ml in 5% glycerol) NADH oxidation was monitored at 340 nm after the addition of 5mM α-ketoglutarate.

b) L-Glutamate:NAD(P) Oxidoreductase: (E.C. 1.4.1.3.; GDH)

Glutamate dehydrogenase activity was assayed by the reductive amination of α-ketoglutarate and followed by monitoring the decrease in absorbance of NADH at 340 nm.

$$\alpha\text{KG} + \text{NADH} + \text{H}^+ + \text{NH}_3 \xrightarrow{\text{GDH}} \text{L-glu} + \text{NAD}^+ + \text{H}_2\text{O}$$

Extracts were added to reaction mixtures containing 50 mM Tris-HCl, pH 7.9, 0.1 % w/v Triton X-100, 100 mM ammonium acetate, 1mM ADP and 0.22 mM NADH. The reaction was started by the addition of 5mM α-ketoglutarate.

c) L-Alanine:2-oxoglutarate Aminotransferase:

(E.C. 2.6.1.2.; GPT)

Glutamate-pyruvate transaminase activity was assayed by coupling pyruvate production to the oxidation of NADH by lactate dehydrogenase.

$$\text{L-ala} + \alpha\text{KG} \xrightarrow{\text{GPT}} \text{pyruvate} + \text{L-glu}$$

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{L-lactate} + \text{NAD}^+$$

Extracts were added to reaction mixtures containing 50 mM KH_2PO_4, pH 6.9, 0.1 % w/v Triton X-100, 40 mM alanine, 0.22 mM NADH and 10 U lactate dehydrogenase (Boehringer, pig heart, 5 mg/ml in 2.2 M $(NH_4)_2SO_4$). NADH oxidation was monitored at 340 nm after addition of 5 mM α-ketoglutarate.

d) L-Glutamate 1-Carboxy-Lyase:

(E.C. 4.1.1.15.; GAD)

Glutamate decarboxylase activity was assayed by measuring the production of $[^{14}C]CO_2$ from L-[^{14}C]glutamate (Langcake and Clements, 1974).

$$\text{U-}[^{14}\text{C}]\text{glutamate} \xrightarrow{\text{GAD}} \text{U-}[^{14}\text{C}]\text{gaba} + [^{14}\text{C}]\text{-}CO_2$$

0.8 ml samples were added to 25 ml conical flasks containing 1.0 ml imidazole acetate buffer (0.2 M imidazole corrected to pH 6.5 with acetic acid) and containing 10 mM mercaptoethanol and 1mM pydridoxal phosphate. To these flasks was added 200µl of 0.54 M L-glutamate which included 1 µCi of L-U-[^{14}C]-glutamate. The flasks were sealed immediately with air-tight, rubber seals and were incubated for 1 h at 37°C. The reaction was terminated by injection of 0.8 ml 2N H_2SO_4 followed by 0.2 ml 0.5 M Na_2CO_3 to flush out the CO_2. Labelled CO_2 was absorbed by 200 µl hyamine hydroxide on filter paper

wicks in plastic centre wells. Flasks were left overnight to ensure complete absorption of $[^{14}C]CO_2$. Centre wells were placed directly into vials containing 10 ml toluene: PPO (100%:0.5%; v/v: w/v). Activity was calculated from the specific activity of the labelled glutamate which gave rise to stoichiometric amounts of labelled CO_2.

e) Sn-Glycerol-3-phosphate:(acceptor) Oxidoreductase:

(E.C. 1.1.9.9.5.; GPox or glycerol 3 P DHase)

Glycerolphosphate oxidase activity was monitored by coupling the oxidation of L-glycerol-3-phosphate to the reduction of ferricyanide to ferrocyanide.

glycerol-3-phosphate + acceptor = dihydroxyacetone phosphate + reduced acceptor

Extracts were added to reaction mixtures containing 130 mM KCl, 10 mM KH_2PO_4 1mM EGTA (pH 7.1), 1 mM KCN and 1mM potassium ferricyanide. The decrease in absorbance at 425 nm due to ferricyanide reduction was followed after the addition of 66.7 mM D, L-glycerol-3-phosphate.

f) Adenosine triphosphatase:

Total ATPase activity was followed at 340 nm using a coupled enzyme assay involving lactate dehydrogenase and pyruvate kinase (PK) (Balerna et al., 1975).

$$ATP + H_2O \xrightarrow{ATPase} ADP + Pi$$

$$ADP + PEP \xrightarrow{PK} ATP + pyruvate$$

$$pyruvate + NADH + H^+ \xrightarrow{LDH} L\text{-actate} + NAD^+$$

The reaction mixture contained 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 10 mM KCl, 2 mM ATP, 0.15 mM NADH, 2 mM phosphoenol

pyruvate (PEP), LDH (Boehringer, pig heart 5 mg/ml in 2.2 M $(NH_4)_2SO_4$, 3 units/assay) and PK (Boehringer, rabbit muscle, 10 mg/ml in 50% glycerol, 9 units/assay). The assay was repeated in the presence of 1 mM ouabain.

4. The Binding Assay:

Membranes (P_2) were suspended (2-4 mg protein/ml) in either sodium rich or sodium free locust saline pH 7.0 (Table 2). Membrane samples (∿ 200 µg protein) were incubated at 23°C for 15 min in the presence of [^{3}H]-L-glutamate (0.002 - 200 µM final conc; 29 Ci/mm). Non-specific binding was accounted for by incubation in the presence of either 1 mM D-glutamate or 0.5-20mM L-glutamate. Bound [^{3}H]-L-glutamate was separated from free [^{3}H]-L-glutamate by one of the following methods:

a) The Filtration Assay:

Samples were incubated in a final volume of 500 µl. Aliquots were removed (150 µl) and filtered through Whatman 2.5 cm GF/C filters. Filters were washed with 2 x 2 ml ice cold locust saline. Total filtration time (i.e. of sample plus washes) was 3 - 30 sec. Filters were dried at room temperature and either placed immediately in 5 ml scintillation fluid (70:30:0.5% toluene: Triton X-100: PPO v/v/w) or trimmed to the exact size of the millipore tower using a 1 cm diameter bore cutter and then placed in the above scintillant. Filters, either cut or uncut were left for 12 hours at room temperature and then counted in Packard Scintillation counter. Filter blanks were in the absence of membranes.

b) The Microfuge Assay:

Samples were incubated in a final volume of 500 µl. Aliquots were removed (150 µl) and centrifuged at 2,000 x g for 10 min in a Beckman microfuge (Model B). The supernatant was removed using a pasteur pipette and the pellet superfically washed with 2 x 0.5 ml ice cold locust saline. 50 µl of soluene - 350 were added to the pellet and left to solubilise for 6 hours. The solubilised pellet was then transferred using a pasteur/pipette to a scintillation vial containing 5 ml of the above scintillant. The centrifuge tube was rinsed with 100 µl scintillant and the wash also was placed in the vial. Samples were left in scintillant for 12 hours before counting to allow chemiluminescence to subside.

c) The Microairfuge Centrifugation Assay:

Samples were incubated in a final volume of either 500 µl or 150 µl directly in the centrifuge tube. If incubated in a final volume of 500 µl, aliquots (150 µl) were taken and centrifuged for 1 min at 100,000 x g in a Beckman airfuge. Samples incubated directly in the centrifuge tubes were also centrifuged for 1 min at 100,000 x g. The supernatant was removed and the pellet washed with 2 x 200 µl of ice cold locust saline, after which 50 µl of Soluene 350 were added and the pellet was left to solubilise for at least 6 hours. The end of the centrifuge tube containing the solubilised pellet was then cut off into a scintillation vial containing 5 ml of scintillant (70: 30: 0.5% toluene: Triton X-100: PPO (v/v/w)) plus 0.5 ml distilled water. The scintillation vial was then mixed for 30s on a whirlimixer and left to stand for 12 hours to allow chemiluminescence

to subside. Samples were then counted for radioactivity in a Packard scintillation counter, of counting efficiency 30%. Quenching was accounted for by an external standard. Results were expressed as pmol [^{3}H]-L-glutamate bound/mg protein.

5. Thin Layer Chromatography to Check for Metabolism of [^{3}H]-L-Glutamate:

10 x 10 cm plates of Baker-Flex F cellulose (prewashed in 2 M NaCl) were used to check for metabolism of [^{3}H]-L-glutamate during the binding assay.

Solvent system: propan-2-ol/formic acid/water 40/2/10v/v/v.

Plates were run in two dimensions at right angles in the same solvent, and the amino acid spots visualised with ninhydrin, marked. Spots were bleached by spraying the plates with 1% H_2O_2. Plates were cut into 1 cm squares and the squares counted in 5 ml Triton/toluene/PPO 70/30/0.5 v/v/w to check for other radioactive spots not ninhydrin positive. P_2 was incubated at 25^{o}C with labelled L-glutamate and samples taken at 0, 15, 30 and 60 mins. Protein was precipitated with an equal volume of ice cold ethanol and the supernatants dried under a stream of nitrogen. Aliquots were applied to TLC plates, together with standards.

α-ketoglutarate was visualised under U.V. by spraying the plates with 0.1% semicarbazide in 0.15% sodium acetate, prior to ninhydrin.

6. Protein Determination:

Proteins were determined by the method of Lowry et al (1951)

RESULTS AND DISCUSSION

1. The Choice of the Subcellular Fraction for Further Study

The fractionation scheme outlined in Fig.10 , is a modification of that reported by James (1977) (Fig. 11). It was my experience that the yield of P_2 from the latter method was very low. In an attempt to improve this position, the modified fractionation scheme outlined in Fig.10 was adopted; this eliminates the centrifugation steps A and B (Fig.10) and substitutes one centrifugation at 3,000 x g for 10 minutes thereby increasing the yield in the subsequent 30,000 x g pellet but probably at the expense of purity.

The P_2 pellet fraction was initially examined by electron-microscopy (Plates 1 and 2) and as was expected is contaminated by large mitochondria. However, numerous membrane fragments, in some cases vesiculated, are also visible.

To further characterise the P_2 membrane fraction the distribution of a number of enzymes characteristically found in insect muscle was investigated. Enzyme activities were assayed as described in the Methods (p. 65). Results are given as the means of either 2 or 3 experiments (No. of expts) and are expressed as RSA (relative specific activities) where:

$$\text{RSA} = \frac{\%\text{ total enzyme activity recovered in the fraction}}{\%\text{ total protein recovered in the fraction}}$$

An RSA value of greater than unity indicates an enrichment of the enzyme activity in that fraction.(Table 4) GPT, GDH, GOT, GPox and GAD are all glutamate metabolising enzymes and are considered to be associated with mitochondria (Donnellan et al., 1974), thus an enrichment of these in any fraction is indicative of mitochondrial enrichment in that fraction. All of the above mentioned enzymes are

Fig. 11

Fractionation Scheme of James (1977)

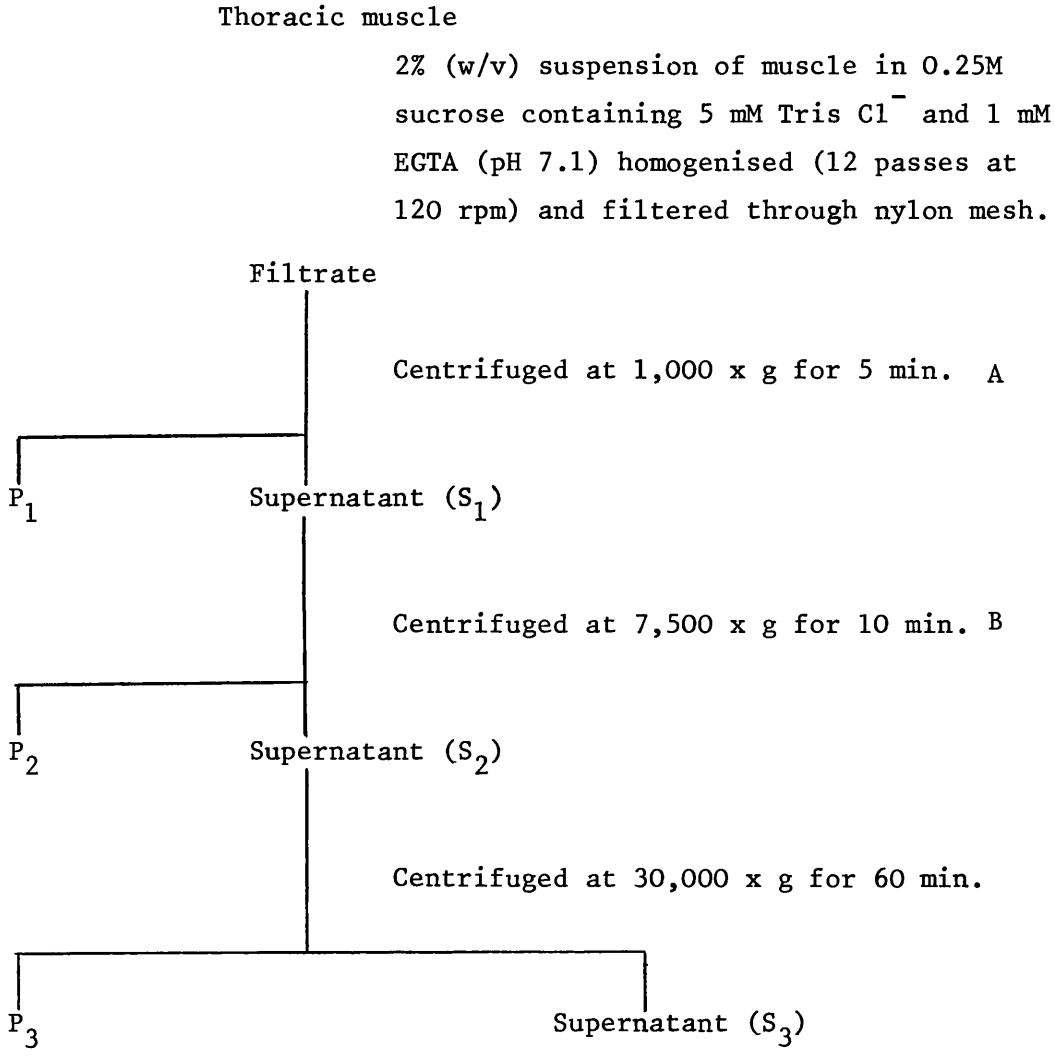
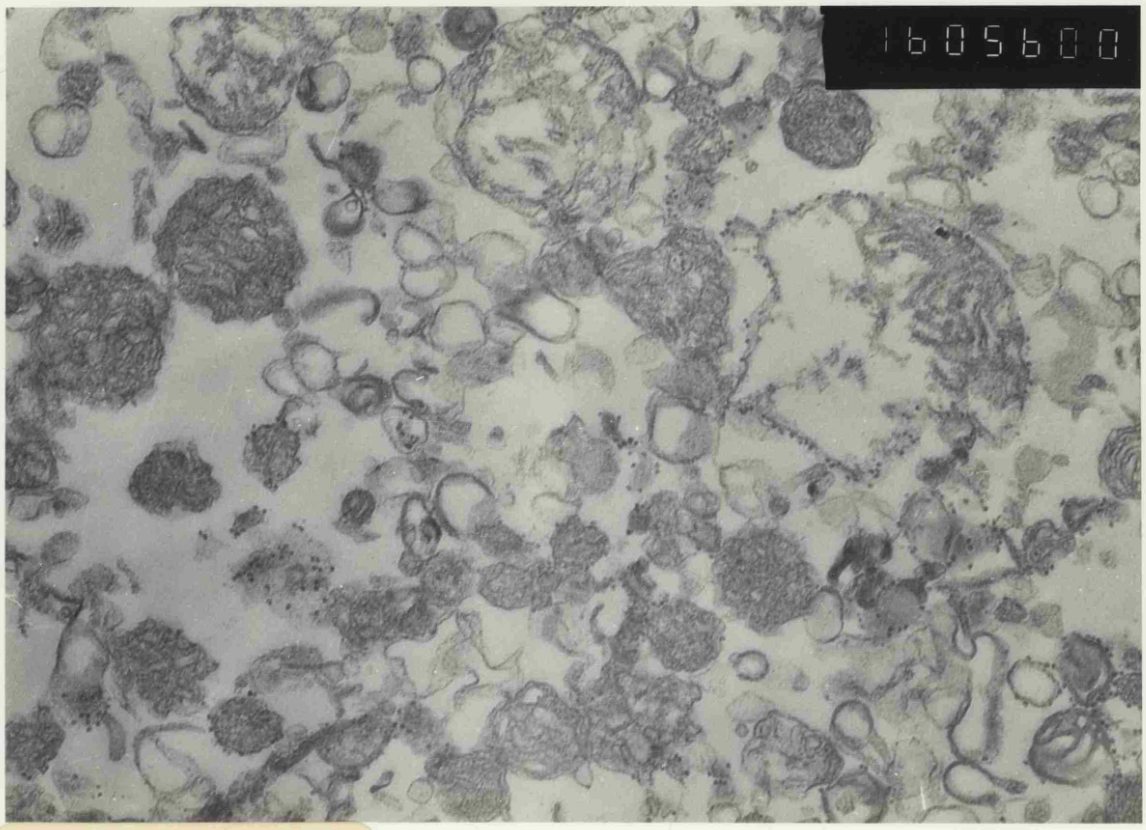
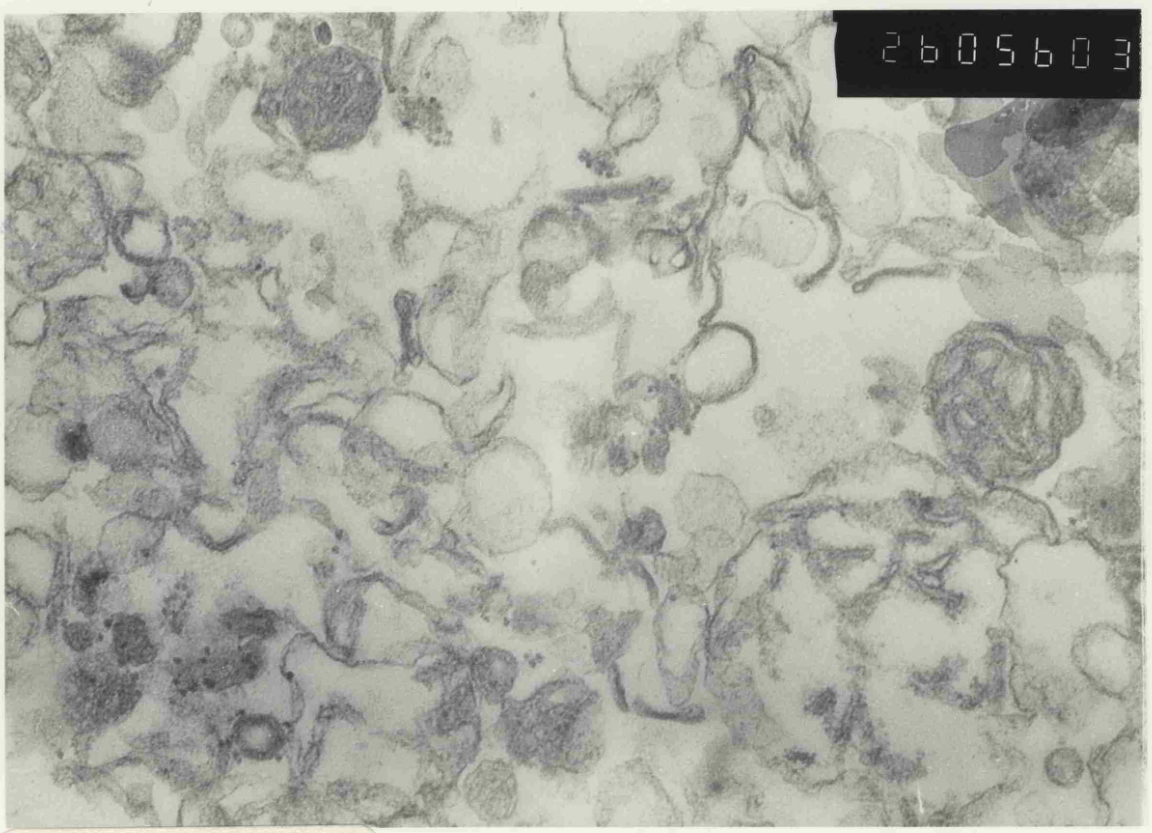


PLATE 1 Electronmicrograph of the P_2 Membrane Fraction from Locust Muscle



0.1 µm

PLATE 2 Electronmicrograph of the P_2 Membrane Fraction from Locust Muscle



0.1 µm

TABLE 4

Enzyme Localisation in Fraction from Locust Thoracic Muscle

	ENZYME																	
	GPT (3)			GDH (2)			GOT (3)			GPox (2)			GAD (2)			ATPase (3)		
Fraction	% P.	% E	RSA	% P.	% E	RSA	% P	% E	RSA	% P	% E	RSA	% P	% E	RSA	% P	% E	RSA
TH	100	100	1	100	100	1	100	100	1	100	100	1	100	100	1	100	100	1
P_1	31.3	41	1.31	31.3	42.5	1.214	31.3	32.3	1.03	34.1	49.4	1.46	24	61	2,54	31	13.3	0.43
S_1	67.3	27.3	0.41	67.3	8.68	0.129	67.3	43	0.64	54.4	9.74	0.17	97	75.4	0.78	33	4.62	0.14
P_2	7.3	5.3	0.73	7.33	3.52	0.48	7.3	4.5	0.62	2.4	3.3	1.37	13	34.6	2.66	9.4	47	5.01
S_2	22.5	9.2	0.41	22.5	4.56	0.203	22.5	38	1.69	41	5.33	0.13	75	48.1	0.64	17	13.4	0.79

Figures in brackets are the number of times the experiment was carried out.

Results are expressed as % P (% protein), % E (% enzyme) activity and RSA values where

Relative Specific Activity = (Percentage total enzyme activity recovered in the fraction)/ (Percentage total protein recovered in the fraction).

enriched in the P_1 fraction, suggesting that this fraction is composed mainly of mitochondria. The greatest RSA value for GOT is in the S_2 fraction with enrichment also in the P_1 fraction,which is consistent with the bimodal distribution of this enzyme reported by Donnellan et al (1974), in subcellular fractions of fleshfly (Sarcophaga barbata) flight muscle. Of all of these enzymes only GPox and GAD had RSA values greater than one in both the P_1 and P_2 fractions. The enzyme GPox has been shown to be firmly bound to the inner membrane of flight muscle mitochondria (Chance and Sacktor, 1958; Donnellan et al., 1970; 1974). Therefore, an enrichment in the P_2 fraction suggests the presence of these membranes and/or whole mitochondria in this fraction.

The GAD activity was predominantly in the P_2 fraction, again suggesting the presence of mitochondria in the fraction because the intramitochondrial location of this enzyme has been established in preparations of fleshfly flight muscle (Langcake and Clements, 1974). However, GAD may possibly have a role in the synthesis of GABA as an inhibitory neurotransmitter in nerve endings.

The distribution of total ATPase activity indicates considerable enrichment in P_2 but the activity was not sensitive to ouabain Briley (1981) found that in similar preparations from locust muscle this activity was stimulated by Ca^{++} and was inhibited by azide, features which are characteristic of myofibrillar and/or sarcoplasmic reticulum ATPase activity. Thus the enrichment in P_2 may indicate a considerable content of vesiculated saroplasmic reticulum in this fraction. Briley (1981) has also found an enrichment of K^+ stimulated ouabain-sensitive p-nitrophenylphosphate phosphatase (pNPP) activity

in the P_2 fraction. This activity is thought to represent Na^+K^+ ATPase and is usually associated with plasma membranes in vertebrate tissues. An enrichment of pNPP in the P_2 fraction could therefore suggest an enrichment of plasma membrane in this fraction.

The RSA values reported here, in conjunction with the work of Briley (1981) suggest that:-

1. The P_1 fraction is enriched in mitochondria. Some mitochondrial activity (GPox and GAD) is also seen in P_2.
2. Although there is a lot of total ATPase activity in P_2 this activity is not ouabain sensitive and probably corresponds to myofibrillar and/or sarcoplasmic reticulum ATPase activity.
3. pNPP activity in P_2 may indicate an enrichment of plasma membranes.
4. An enrichment of GAD activity in P_2 may also indicate an enrichment in nerve endings if GABA is synthesised within the nerve terminals.

In conclusion the P_2 fraction, as prepared in Fig.10, contains predominantly membrane fragments, the origins of which are uncertain. Mitochondria are also present in this fraction but to a lesser extent than in the P_1 fraction.

2. <u>The Solubility of L-Glutamate in the Scintillation Fluid</u>:

It was noted that the recorded cpm decreased with time when 20µl of [^{3}H]glutamate, isotopically diluted to a concentration of 10^{-5}M, was placed in scintillation fluid and counted. Closer examination revealed that the L-glutamate appeared to be precipitating in a time dependent manner. To overcome this effect 0.5 ml of H_2O were

TABLE 5

The Solubility of L-Glutamate in Scintillation Fluid:

Time	$[^3H]$-L-glutamate	$[^3H]$-L-glutamate + 0.5 ml H_2O	$[^3H]$-L-glutamate + unlabelled L-glutamate	$[^3H]$-L-glutamate + unlabelled L-glutamate + 0.5 ml H_2O
0	1,390,163 cpm	1,338,824 cpm	1,137,999 cpm	1,403,216 cpm
1 hour	96%	100%	70%	100%
22 hours	84%	100%	23%	100%
6 days	73%	99%	20%	98%

5 ml Scintillation Fluid

\+ 20 µl 10^{-5}M $[^3H]$-L-glutamate

\+ 15 µl 5 mM L-glutamate

added to the 5 ml of scintillation fluid in which samples were routinely counted. Table 5 shows the cpm at the intervals indicated for samples in scintillation fluid (5 ml) with and without water and with and without addition of excess unlabelled L-glutamate.

Firstly, it should be noted how the cpm decreased with time in the absence of H_2O (100% to 73% in 6 days). This reduction in cpm is greatly accentuated by the addition of an excess of unlabelled L-glutamate (5 mM; 100% to 70% in 1 hour) where the cpm at zero time is only 80% of the expected value. It appears, therefore, that the detectable cpm are markedly reduced even before counting commences. However, in both cases i.e. with and without an excess of unlabelled L-glutamate, this decrease in cpm with time is to some extent prevented by the addition of 0.5 ml H_2O/5 ml scintillation fluid. Henceforth, 0.5 ml H_2O was routinely added to 5 ml scintillation fluid before counting.

3. Experiments using D-Glutamate to Account for Non-Specific Binding:

In early experiments, incubation of [^{3}H]-L-glutamate (1×10^{-5}M) with the membrane fragments was carried out in the presence of an excess of unlabelled D-glutamate (1 mM) to account for non-specific binding. D-Glutamate was chosen because it has no physiological activity in the insect nervous system and is structually very similar to L-glutamate and therefore any site capable of differentiating between the D and L isomers would be considered to be a specific binding site for L-glutamate.

Binding was carried out as described in the Methods (p.68) and bound [^{3}H]-L-glutamate was separated from free by filtration through GF/C filters. The experiments were carried out in the presence

TABLE 6

Effect of Na^+ on [^{3}H]-L-Glutamte Binding Using D-Glutamate to Account for Non-Specific Binding:

Prep. No.	Binding (pmol/mg prot.)		
	+ Na^+	- Na^+	$\frac{+ Na^+}{- Na^+}$
1	24.6	22.9	93%
2	14.9	12.5	84%
3	39.2	17.2	44%
4	8.7	4.3	11%
5	9.9	13.7	140%
6	10.6	12.6	119%

Results are the means of triplicates S.E. = 20 - 30%

[^{3}H]-L-glutamate $\simeq 10^{-5}$ M

D-glutamate = 5 mM

Choline chloride 0.14 M used in Na^+ free buffer

Binding was as described in the Methods (p. 68).

(0.15M) and absence of Na^+, on the assumption that high affinity uptake of [^{3}H]-L-glutamate would be sodium dependent and therefore addition of sodium to the incubation media may result in an apparent increase in binding.

Table 6 shows the results of 6 experiments, each carried out in triplicate. As can be seen the binding varies considerably from preparation to preparation. Four out of the six experiments showed an increase in [^{3}H]-L-glutamate binding on the addition of Na^+ but for the other two experiments the converse is true. In all experiments the standard error was high.

Increased [^{3}H]-L-glutamate binding to a similar locust muscle membrane preparation, in the presence of Na^+ has been reported by other workers (James, 1977; Cleworth et al., 1980). For this reason and because the majority of experiments reported here in the presence and absence of Na^+ gave similar results, further [^{3}H]-L-glutamate binding experiments reported in this thesis were carried out in a Na^+ free buffer as a means of minimising any high affinity uptake of [^{3}H]-L-glutamate.

The use of D-glutamate to account for non-specific [^{3}H]-L-glutamate binding has not been widely used (James, 1977; Cleworth, personal communication). The majority of workers use the method of "unlabelled ligand excess" where the unlabelled ligand must compete with the labelled ligand for the specific binding sites. This method is considered to be more reliable when the labelled and unlabelled ligands are chemically distinct (see AChR Discussion p.155). However, in the case of L-glutamate such studies are hampered by the lack of any well characterised receptor agonists or antagonists and

therefore the only ligand which can be used with confidence, in such an assay, is unlabelled L-glutamate. A comparison of the two methods, that is, excess D-glutamate or excess L-glutamate for the determination of non-specific and hence specific [^{3}H]-L-glutamate binding, was made.

4. A Comparison of Binding Using Excess D-glutamate and Excess Unlabelled L-Glutamate to Account for Non-Specific Binding:

Binding was carried out as described in the Methods (p.68). Bound [^{3}H]-L-glutamate (10^{-5}M) was separated from free by filtration through GF/C filters. When excess D-glutamate (1 mM) was used to account for non-specific binding;

specific binding = the amount of [^{3}H]-L-glutamate bound in the presence of an excess of unlabelled D-glutamate.

∴ Non-specific binding

= (Amount bound in the absence of unlabelled L- or D-glutamate) - (Amount bound in the presence of D-glutamate).

When an excess of unlabelled L-glutamate (1mM or 5mM) was used to account for non-specific binding;

Non-specific binding

= the amount bound in the presence of an excess of unlabelled L-glutamate.

∴ Specific binding

= (Amount bound in the absence of unlabelled L- or D-glutamate) - (Amount bound in the presence of unlabelled L-glutamate).

TABLE 7

Comparision of Binding in the Presence of an Excess of Unlabelled D-Glutamate and in the Presence of an Excess of Unlabelled L-Glutamate:

Preparation No.	Binding (pmol/mg prot.)				
	A Total	B Non-specific (excess L-glut.)	C Specific A - B	D Specific (excess D-glut.)	E Non-Specific A - D
1	0.523	1.161	-	0.638	0.071
2	1.197	1.512	-	1.049	0.148
3*	1.482	0.831	0.651	1.241	0.241

Results are the means of triplicates S.E. = 20 - 30%.

* Unlabelled L-glutamate at a concentration of 5 mM, 1 mM for preparations 1 and 2.

D-glutamate was at a concentration of 1 mM

[^{3}H]-L-glutamate was at a concentration of 10^{-5}M

A = Total binding i.e. binding in the absence of either excess L - or D-glutamate.

B = Non-specific binding measured in the presence of excess unlabelled L-glutamate

C = Specific binding i.e. (Total binding) - (Binding in the presence of excess unlabelled L-glutamate).

D = Specific binding i.e. binding in the presence of excess D-glutamate.

E = Non-specific binding i.e. (Total Binding) - (Binding in an excess of D-glutamate).

Table 7 shows the results of three experiments, each in triplicate, in which a comparison was made of specific binding measured by incubation in an excess of unlabelled L-glutamate (1mM and 5mM) and by incubation in an excess of unlabelled D-glutamate.

Firstly, it should be noted that it was only when unlabelled L-glutamate concentration was increased from 1mM - 5mM that specific [^{3}H]-L-glutamate binding was displaced. This could be indicative of binding of very high affinity, a large number of specific binding sites, or different affinities for the ligand in the labelled and unlabelled state. The last point is unlikely because tritiation of a ligand has little effect on structural conformation.

If excess unlabelled L-glutamate and excess D-glutamate are equally effective in accounting for non-specific binding, then the figures in columns C and D (specific binding) should compare favourably. This was not the case, as there was apparently more non-specific binding measured in the presence of an excess of unlabelled L-glutamate than could be accounted for by D-glutamate. In such a crude preparation as the P_2 membrane fraction and in consideration of the widespread occurrence of L-glutamate, non-specific (non-neuroreceptor) L-glutamate binding sites would be expected to exceed the specific binding sites. This is borne out by the results of experiments in which unlabelled L-glutamate is used as the displacing ligand and therefore this method was routinely adopted in all further experiments to account for non-specific binding.

5. <u>The Binding Assay</u>:

In all of the binding experiments reported here a number of problems have persisted and have so far precluded any assessment of the significance of the results obtained. The problems are outlined

below:-

1. Filter blanks were inconsistent and sometimes unusually high; in some cases values higher than that of the sample were obtained.

2. Poor reproducibility between triplicates within a preparation (standard error as high as 35% in some cases).

3. Often no displaceable binding,i.e. no specific binding, was obtained.

4. When specific binding was obvious it was not reproducible from preparation to preparation.

In an effort to irradicate or at least to minimise these problems a number of procedures were employed to try to lower the filter blanks and to reduce the non-specific binding.

a) Experiments to Reduce the Binding of [^{3}H]-L-Glutamate to the Filters:

In some of the previous experiments the [^{3}H]-L-glutamate bound to the filters alone (i.e. without P_2 membrane present) was found to be very high, in some cases higher than when the sample was filtered, thus rendering results from that particular experiment invalid. Three possible reasons for high filter blanks were investigated. Firstly, it was thought that the time of filtration (i.e. time for sample plus wash to pass through the filter) may have affected the blanks. Experiments were carried out in which the filtration time was varied from 3S - 10S, a filtration time which is probably still sufficiently rapid to ensure minimal dissociation of [^{3}H]-L-glutamate -receptor complex assuming L-glutamate binds with a similar affinity to its receptor as do other neurotransmitters (AChR Discussion p.154).

TABLE 8

The Effect of Filtration Time on the Non-Specific Binding of [^{3}H]-L-Glutamate to the Filters:

Buffer	Filtration Time (sec.)		
	3 (cpm bound)	5 cpm bound)	10 (cpm bound)
L.S.	551	346	48
L.S. + 1% BSA	299	366	-
L.S. + 5mM L-glutamate	366	385	36
L.S. + 1% BSA + 5mM L-glutamate	345	355	-

L.S. = Locust saline

Results are means of quadruplicates S.E. < 5%

[^{3}H]-L-glutamate was at a concentration of 10^{-5}M

Secondly, the effect of presoaking the filters in 1% (w/v) BSA and/or 5mM L-glutamate was investigated. The rationale for this procedure was to try to occupy all potential non-specific [^{3}H]-L-glutamate binding sites on the filter. Finally, after filtration and washing was complete, the filters were trimmed to the exact diameter of the millipore filtration tower, thus cutting away any [^{3}H]-L-glutamate which had become trapped in the seal between the millipore tower and the scinter support and was therefore unlikely to have been efficiently washed.

Table 8 shows that presoaking in BSA and/or L-glutamate had no effect on the levels of the filter blanks whereas increasing the filtration time from 3S to 10S lowered the radioactivity associated with the filter by a factor of ten. Trimming the filters caused a drop in cpm, from about 1000 cpm to about 100 cpm and also decreased the standard error from 30% to 5% (mean of quadruplicates).

In all subsequent experiments the filters were trimmed after filtration.

b) <u>The Effect of Filtration Time on the Binding of [^{3}H]-L-Glutamate to the Membrane Fragments</u>:

Binding was carried out as described in the Methods (p.68). [^{3}H]-L-glutamate was at a final concentration of 10^{-5}M. Two filtration times were used; 3s and 10s. The filters were trimmed before counting.

Table 9 shows total, non-specific and specific binding of [^{3}H]-L-glutamate for the two filtration times. No improvement in the reproducibility of the overall assay (mean of quadruplicates ± S.E.)

TABLE 9

Effect of Filtration Time on Binding of ^{3}H -L-Glutamate to the P_2 Fraction:

Filtration Time	Binding (pmol/mg prot.)		
	Total	Non-Specific	Specific
3 sec	2.23 ± 0.50	1.23 ± 0.22	1.0 ± 0.46
10 sec	3.39 ± 0.43	2.20 ± 0.60	1.73 ± 0.74

Results are the means of quadruplicates ± S.E.

[^{3}H]-L-glutamate = $10^{-5}M$

Unlabelled L-glutamate = 5 mM

Binding was carried out as described in the Methods (p. 68).

was observed when the filtration time was increased from 3s to 10s.

Although filtration time and trimming the filters had no effect on improving the reproducibility of results from preparation to preparation, in all subsequent experiments the two practices were carried out to try to ensure low blanks.

c) Comparison of [^{3}H]-L-Glutamate Binding Assayed by the Filtration and by the Microfuge Centrifugation Methods:

Binding was carried out at 10^{-5}M [^{3}H]-L-glutamate. Bound [^{3}H]-L-glutamate was separated from free ligand by either filtration through Whatman GF/C filters or by centrifugation at 2,000 x g in a Beckman Microfuge for 5 minutes (Methods p. 69).

The microfuge centrifugation method of separating bound from free [^{3}H]-L]glutamate was even less reliable that the filtration method giving greater standard error (Table 10). It was noticed that the resultant pellet after centrifugation at 2,000 x g was very small and loosely packed, suggesting that the membranes may not all have sedimented. Extended centrifugation times failed to produce tightly packed pellets such that it was difficult to remove the supernatant without possible loss of pelleted material. Thus the microfuge method of separating bound from free [^{3}H]-L-glutamate was not considered further.

d) The Effect of Freeze-Thawing the P_2 Membrane Fraction on [^{3}H]-L-Glutamate Binding:

Freeze-thawing of the P_2 membrane fragments was carried out because of two possible factors which could be affecting [^{3}H]-L-glutamate binding to its putative receptor. Firstly, it was highly probable that the membrane fragments of the P_2 suspension were reforming

TABLE 10

Comparision of Binding Assayed by the Microfuge Centrifugation and by the Filtration Methods:

Preparation No.	Assay	Binding (pmol/mg prot.)		
		Non-Specific	Total	Specific
1	Centrifugation	1.35 ± 0.53	2.49 ± 0.8	1.14 ± 0.48
1	Filtration	0.704 ± 0.55	2.064 ± 0.33	1.36 ± 0.32
2	Centrifugation	5.4 ± 1.02	5.3 ± 2.23	-
2	Filtration	2.20 ± 1.21	3.93 ± 0.86	1.73 ± 0.74

Results are the means of quadruplicates ± S.E.

$[^3H]$-L-glutamate = 10^{-5}M

Unlabelled L-glutamate = 5 mM

Binding was carried out as described in the Methods (p. 68).

as vesicles, as suggested by the electronmicrographs (Plates 1 and2) and so perhaps rendering a number of specific L-glutamate binding sites inaccessable. Secondly, Olsen et al., (1980),when investigating binding to putative GABA receptors in preparations of mammalian CNS, reported improvement of binding after freeze-thawing which he attributed to release and removal by washing of a possible endogenous inhibitor of [^{3}H]GABA binding, which may have been endogenous GABA, and would have occupied specific binding sites and so caused an underestimation of binding sites. A similar situation has been suggested for glutamate binding to preparations of mammalian brain (Roberts,1981) and the same could be possible when examining [^{3}H]-L-glutamate binding to locust muscle.

The P_2 membrane suspension was twice subjected to freeze-thawing in liquid nitrogen. At the end of each cycle the membranes were recentrifuged at 30,000 x g for 20 min and the pellets resuspended in fresh locust saline. The binding was carried out as described in Methods (p. 68). [^{3}H]-L-glutamate was at a concentration of 10^{-5}M and bound [^{3}H]-L-glutamate was separated from free [^{3}H]-L-glutamate by the filtration method.

Table 11 compares binding to a sample which has been freeze-thawed x 2 and one that has not. Freeze-thawing appeared to have a detrimental effect on total and hence specific binding as non-specific binding was relatively unchanged. It should also be noted that this is a particularly good preparation in that there is apparently a high level of specific binding and the standard error is low (mean of quadruplicates, S.E. < 10%). From these results it is impossible to determine whether the purpose of freeze-thawing has been achieved

TABLE 11

Effect of Freeze-Thawing on [^{3}H]-L-Glutamate Binding:

Freeze-Thawed	Binding (pmol/mg prot.)		
	Non-Specific	Total	Specific
0	2.03 ± 0.23	14.77 ± 1.4	12.73 ± 1.4
x2	1.61 ± 0.025	8.67 ± 0.185	7.06 ± 0.189

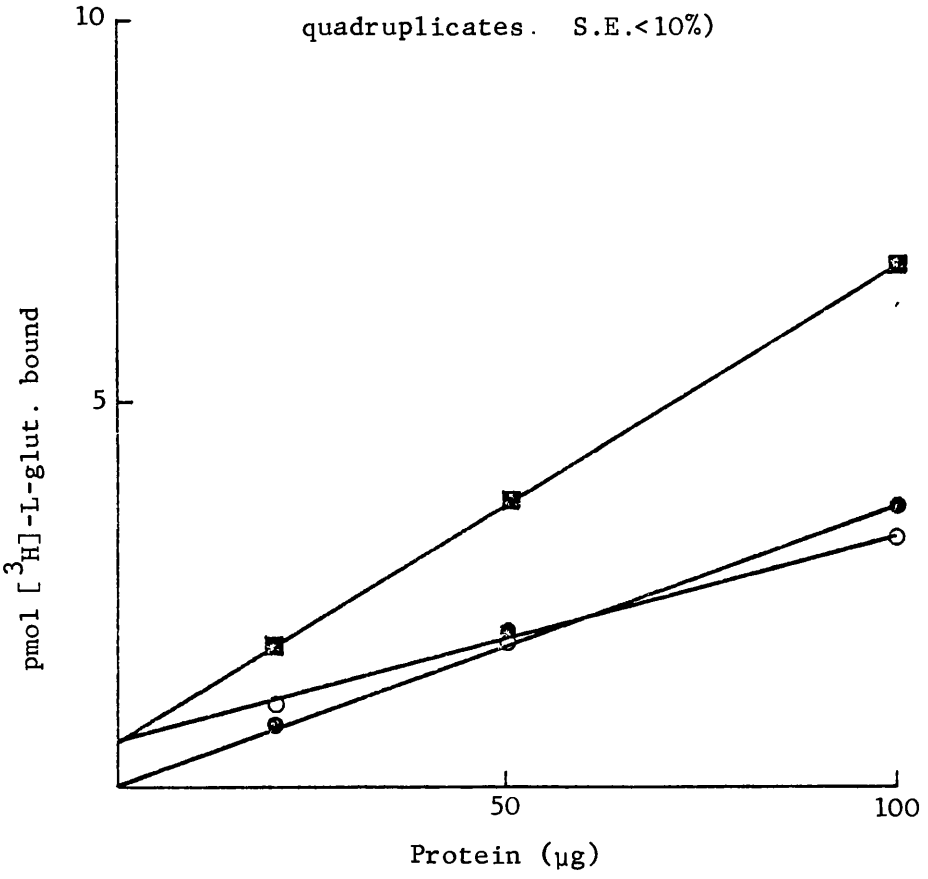
Results are the mean of quadruplicates ± S.E.

[^{3}H]-L-glutamate = 10^{-5}M

Unlabelled L-glutamate = 5 mM

Binding was carried out as described in the Methods (p. 68).

Fig. 12 The Effect of Varying the Protein Concentration on [^{3}H]-L-Glutamate Binding to the P_2 Muscle Fraction. [^{3}H]-L-glut., 10^{-5}M; unlabelled L-glut. 5mM; bound [^{3}H]-L-glut. was separated from free by filtration. ■ total Binding; ○ non-specific binding; ● specific binding. (Results are means of quadruplicates. S.E.<10%)



but it appears that the putative L-glutamate receptor from locust muscle is not as stable to freeze-thawing as the putative GABA receptor from mammalian CNS.

6. The Effect of Protein Concentration on [^{3}H]-L-Glutamate Binding (Filtration Assay):

If the specific binding of [^{3}H]-L-glutamate that is observed in these experiments represents binding to membrane receptor sites in the P_2 fraction, then the binding should increase linearly with protein concentration (Introduction p. 15).

Protein was varied from 20 - 100 µg at a [^{3}H]-L-glutamate concentration of 10^{-5}M and specific binding was found to increase proportionally (Fig. 12) (Mean of quadruplicates, S.E. < 10%). It should also be noted that both total and non-specific [^{3}H]-L-glutamate binding intersect the y-axis at the same point and this corresponds to the filter blank. Therefore, for this preparation of membrane fragments the assay appears to measure a true specific binding. On frequent repetition of this experiment the majority of the results that were obtained were similar but on occasion, although total binding still increased with increased protein,non-specific binding varied considerably, suggesting that for many preparations an excess of unlabelled L-glutamate was not effective in quantitatively displacing specific binding at each membrane concentration.

7. The Effect of Storage at 4°C on Specific [^{3}H]-L-Glutamate Binding:

In view of the great variability encountered between different membrane preparations it was decided to investigate the feasibility of preparing large quantities of membranes, sufficient to provide

TABLE 12

The Effect of Storage on Specific [^{3}H] -L-Glutamate Binding:

Time (days)	Specific Binding
0	100%
1	56%
2	23%

Results are the means of quadruplicates S.E. < 10%

[^{3}H] -L-glutamate = 10^{-5}M

Unlabelled L-glutamate = 5 mM

Binding was carried out as described in the Methods (p. 68).

material for a complete set of binding data. Membranes (P_2) were prepared and stored at 4^oC for up to 2 days. At various times the P_2 membrane fraction was assayed for [^{3}H]-L-glutamate (10^{-5}M) binding by the filtration assay (Methods p. 68). Table 12 shows the specific binding of [^{3}H]-L-glutamate at the times stated, expressed as a percentage of the specific binding to the fresh preparation. It was found that after 2 days specific binding drops to only 23% of the activity of the fresh membranes. Consequently, all subsequent assays of binding activity were carried out on freshly prepared P_2 membrane fragments.

8. The Effect of Varying the Unlabelled L-Glutamate Concentration on [^{3}H]-L-Glutamate Binding (Filter Assay):

Binding was carried out at a [^{3}H]-L-glutamate concentration of 10^{-5}M and at 3 concentrations of unlabelled L-glutamate;- 5mM, 10mM and 15mM (Methods p. 68).

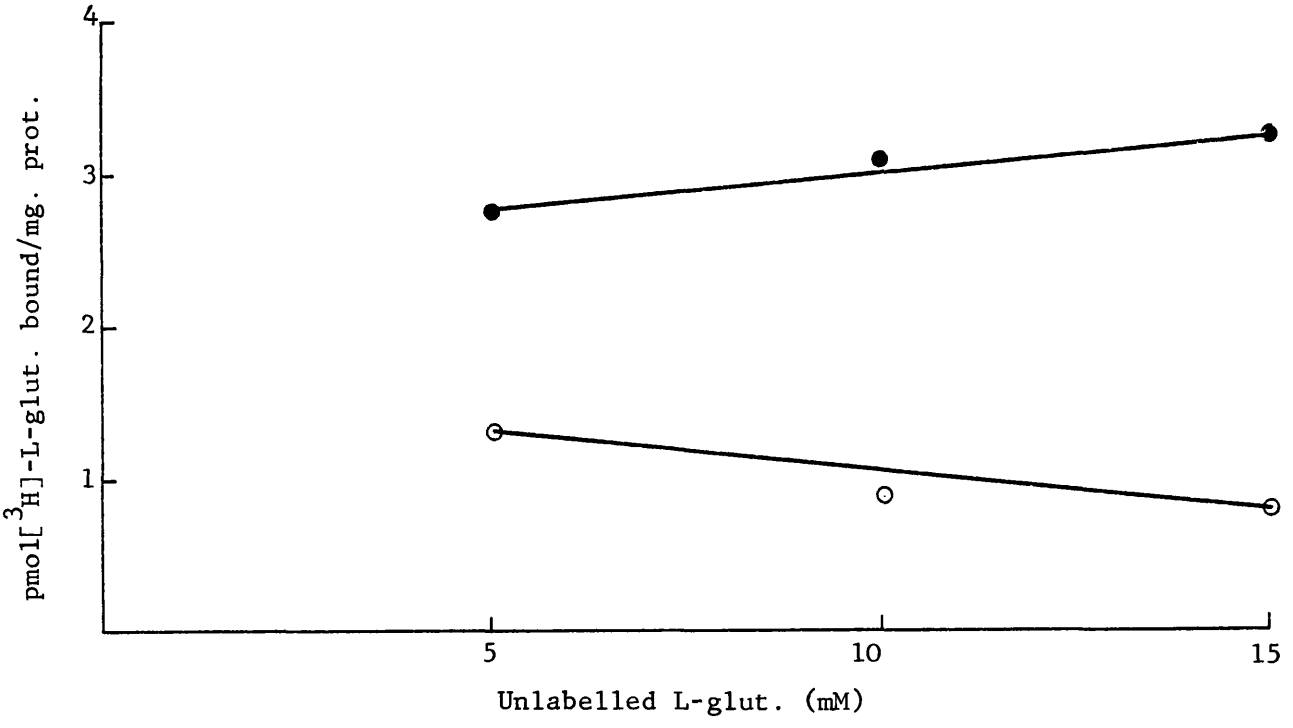
No significant change in specific [^{3}H]-L-glutamate binding was observed for the three concentrations of unlabelled ligand.(Fig. 13). A concentration of 5 mM unlabelled L-glutamate was used in all subsequent experiments because higher concentrations of unlabelled L-glutamate raised the possibility of displacement of labelled ligand from some non-specific as well as from specific binding sites.

9. Saturability of [^{3}H]-L-Glutamate Binding (Filter Assay):

Demonstration of saturable specific [^{3}H]-L-glutamate binding to the P_2 membrane fragments would provide evidence for a specific L-glutamate receptor in this locust muscle preparation, (Introduction p. 15). Such data would also give an indication of the affinity of

Fig. 13 The Effect of Varying the Concentration of Unlabelled L-Glutamate on [^{3}H]-L-Glut. Binding to the P_2 Muscle Fraction.

[^{3}H]-L-glut., 10^{-5} M; bound [^{3}H]-L-glut. was separated from free by filtration. ● specific binding; ○ non-specific binding. (Results are the means of quadruplicates S.E.< 12%)



the ligand for its putative receptor.

The [^{3}H]-L-glutamate concentration was varied from 1μM - 150μM and binding was carried out as described in Methods (p.68). As may be seen (Fig 14a) specific binding does not saturate but tails off at the higher concentrations. Scatchard analysis of the data (Fig. 14(b)) suggests the presence of two binding sites, one of high affinity, K_D 62.75μM and B_{max} 36.51 pmol/mg protein and one of lower affinity K_D 380μM and B_{max} 193 pmol binding sites/mg protein. The two lines have correlation coefficients of 0.65 and 0.77 respectively. These results are not definitive, firstly because of the poor correlation coefficients and secondly,when the experiment was repeated the specific [^{3}H]-L-glutamate binding profile was inconsistent, on some occasions the points being so scattered that no clear binding profile could be discerned. In addition,the standard error of the mean of triplicates within any one preparation for either the total or non-specific binding was often as high as 30%. The latter observation could infer an inability of the filtration method to separate bound [^{3}H]-L-glutamate from free [^{3}H]-L-glutamate efficiently and/or with constancy . An alternative method of separating membrane bound ligand from free ligand is centrifugation of the sample after incubation at a sufficiently high g force to rapidly pellet the membrane fragments and the bound [^{3}H]-L-glutamate, leaving the free [^{3}H]-L-glutamate in the supernatant. The centrifugation procedure also offers the advantage that until the membrane pellet becomes firmly packed, the equilibrium of ligand binding is not disturbed.

Fig. 14(a) The Effect of Concentration on [^{3}H]-L-Glutamate Binding to the P_2 Muscle Fraction.

Unlabelled L-glut. 5mM; bound [^{3}H]-L-glut. was separated from free by filtration; ■ total binding; ○ non-specific binding; ● specific binding. (Results are the means of two experiments each in triplicate. S.E. < 15%).

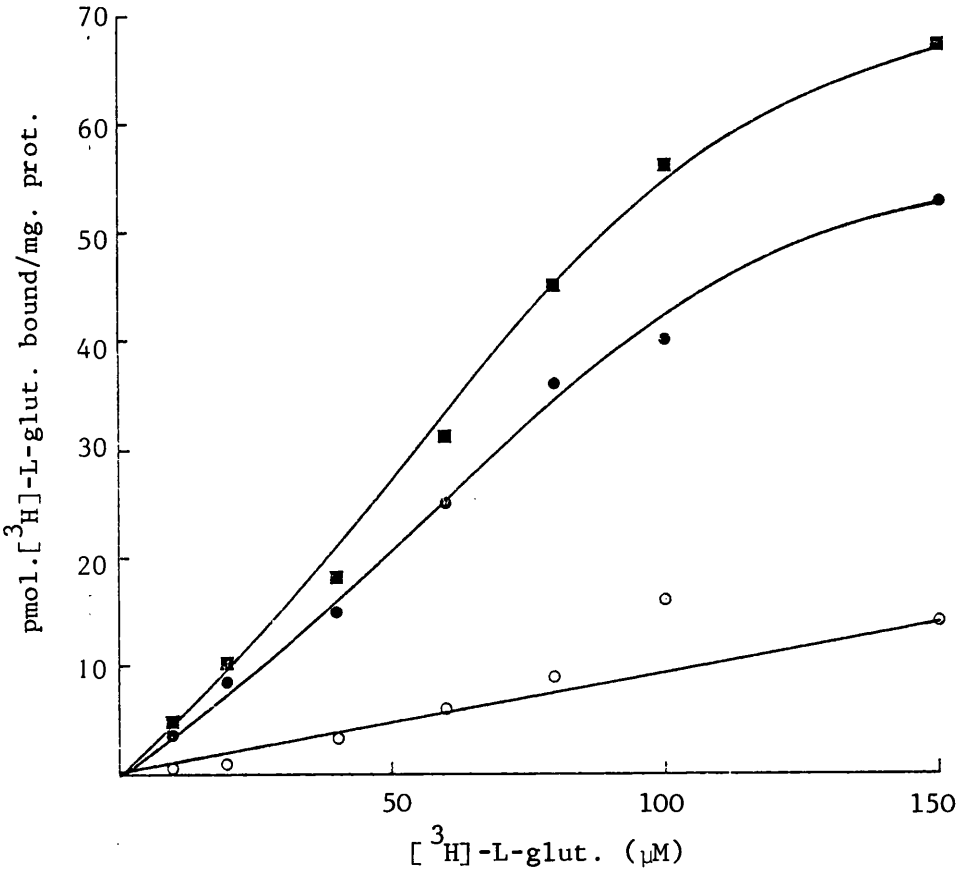
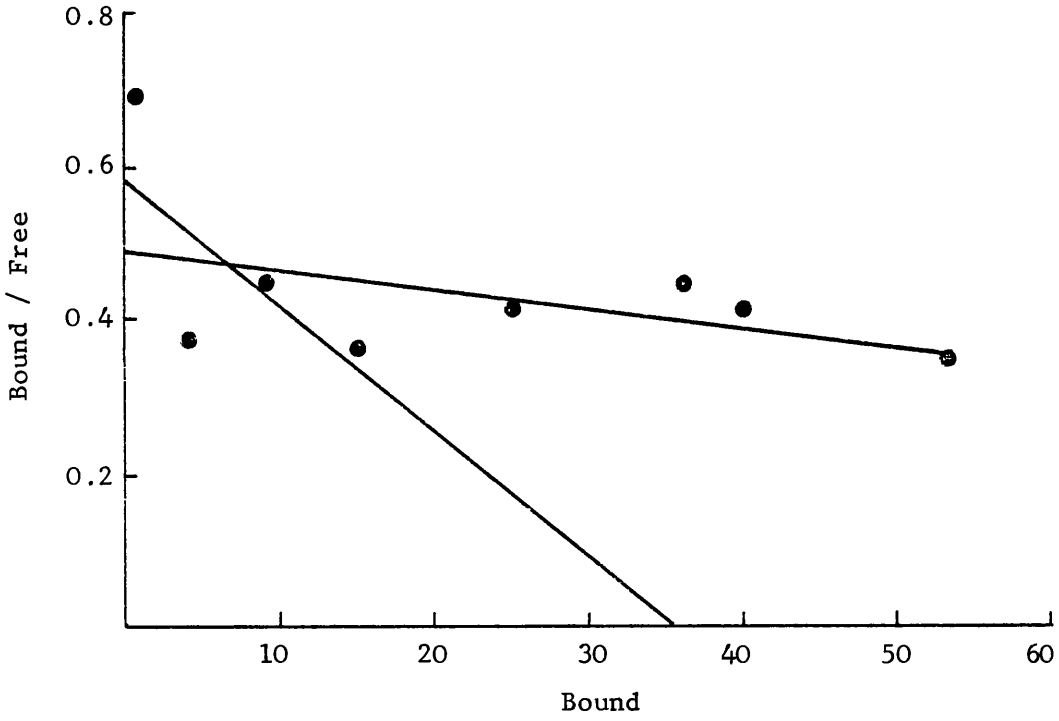


Fig. 14 (b) It should be noted that the experimental values presented in Fig. 14 (b) are composites of the two proposed binding sites and this analysis may not give true values of K_D and B_{max}. A more rigorous treatment of the data is that of Rosenthal, H.E. (1967), Anal. Biochem., 20, p.525. This and other interpretations are discussed in Nørby, J.G., Ottolengli, P. and Jensen, J. (1980), Anal. Biochem., 102, p.318. Such a rigorous analysis was not applied to the data in Fig. 14 (b) because only an approximation of the K_D's was required from these preliminary results.

Fig. 14 (b) Scatchard analysis of the data represented in Fig. 14 (a)



10. A Comparison of the [^{3}H]-L-Glutamate Binding Assayed by the Filtration and Airfuge Centrifugation Methods:

Earlier attempts of separating bound [^{3}H]-L-glutamate from free by centrifugation used a Beckman microfuge, at its maximum force of 2,000 x g. This method proved unsuccessful even though the samples were centrifuged for 5 min (Results Table 10), hence the method was abandoned on the grounds that the Beckman microfuge could not produce a sufficiently high g force to quickly form a firm pellet of the membranes. However, the Beckman airfuge reaches a force of 100,000 x g in less than 10s and it was found that the P_2 membrane fragments had formed a tight pellet after a centrifugation time of 1 min at this g force.

Binding was carried out as described in the Methods (p. 68) [^{3}H]-L-glutamate was separated from free by either filtration through Whatman GF/C filters or by centrifugation in a Beckman airfuge at 100,000 x g for 1 min.

Table 13 shows total, non-specific and specific binding of [^{3}H]-L-glutamate, measured by both methods for 4 separate membrane preparations. Specific [^{3}H]-L-glutamate binding was consistently higher and the standard error lower ($<$ 8%) as assayed by the airfuge centrifugation method than [^{3}H]-L-glutamate specific binding as assayed by the filtration method. Therefore, the airfuge centrifugation method of separating bound [^{3}H]-L-glutamate from free [^{3}H]-L-glutamate is apparently more efficient and more reliable for any one preparation (S.E. $<$ 8%) than the filtration method (S.E. $<$ 60%). However, it should be noted that there is no improvement in the inconsistency of the amount of [^{3}H]-L-glutamate bound from preparation to preparation.

TABLE 13

Comparision of Binding Assayed by the Airfuge Centrifugation Procedure and by the Filtration Assay:

Prep. No.	Assay	Binding (pmole/mg protein)		
		Non-Specific Binding	Total Binding	Specific Binding
1	Centrifugation	24.23 ± 1.56	56.68 ± 2.26	32.46 ± 2.7
1	Filtration	17.83 ± 2.97	21.94 ± 1.30	4.11 ± 3.2
2	Centrifugation	59.45 ± 1.77	67.28 ± 2.41	7.83 ± 2.99
2	Filtration	19.35 ± 0.57	24.42 ± 1.59	5.07 ± 1.69
3	Centrifugation	26.06 ± 3.74	319.09 ± 11.23	293.03 ± 11.8
3	Filtration	32.0 ± 1.31	109.7 ± 4.54	77.7 ± 4.73
4	Centrifugation	44.8 ± 6.68	172.34 ± 4.01	127.54 ± 7.79
4	Filtration	17.01 ± 0.69	76.66 ± 3.21	59.65 ± 3.28

Results are the means of quadruplicates ± S.E.

$[^3H]$-L-glutamate = 10^{-5} M

Unlabelled L-glutamate = 5 mM

This would suggest a variable as yet undetected, and independent of the method of assaying specific binding of [^{3}H]-L-glutamate. Nevertheless, because of the improved S.E. the airfuge centrifugation assay was used in all subsequent experiments, unless stated otherwise.

11. Comparison of the Specific Binding of [^{3}H]-L-Glutamate in the Various Fractions:

Previous experiments had been carried out on the P_2 membrane fraction, on the assumption based on the work of James (1977) that this fraction was enriched in the putative L-glutamate receptor. To confirm this assumption, binding was assayed in all fractions.

Binding was carried out as described in the Methods (p. 68). [^{3}H]-L-glutamate was at a concentration of 10^{-5}M. Bound [^{3}H]-L-glutamate was separated from free [^{3}H]-L-glutamate by the filtration assay for the S_2 fraction and by the airfuge centrifugation for the total homogenate, P_1, S_1 and P_2 fractions.

Table 14 , shows the specific binding, purification (taking specific binding in the total homogenate as unity), total protein recovered and hence total binding activity recovered. The greatest specific binding activity was in the P_2 fraction, representing a purification of 4 with respect to the total homogenate. However only 20% of total [^{3}H]-L-glutamate binding activity was recovered in this fraction. A lot of specific binding activity appeared in the P_1 fraction (69%) but non-specific binding to this fraction was also very high. When choosing a suitable fraction for further study of [^{3}H]-L-glutamate binding, minimal non-specific [^{3}H]-L-glutamate binding to that fraction is preferred because of the unsatisfactory methods

TABLE 14

Comparison of Specific Binding in Different Fractions:

Fraction	Specific Binding (pmol/mg prot.)	Purification	Tot. Prot. (mg)	Tot. Binding (pmol)	% Binding Recovered
Tot. Hom.	17.1 ± 3.6	1	226.5	3873	100%
P_1	33.4 ± 3.0	2	80.0	2672	69%
S_1	6.69 ± 0.86	0.39	124.5	832.9	22%
S_2	-		58.25	-	-
P_2	70.8 ± 1.5	4	10.9	771.72	20%

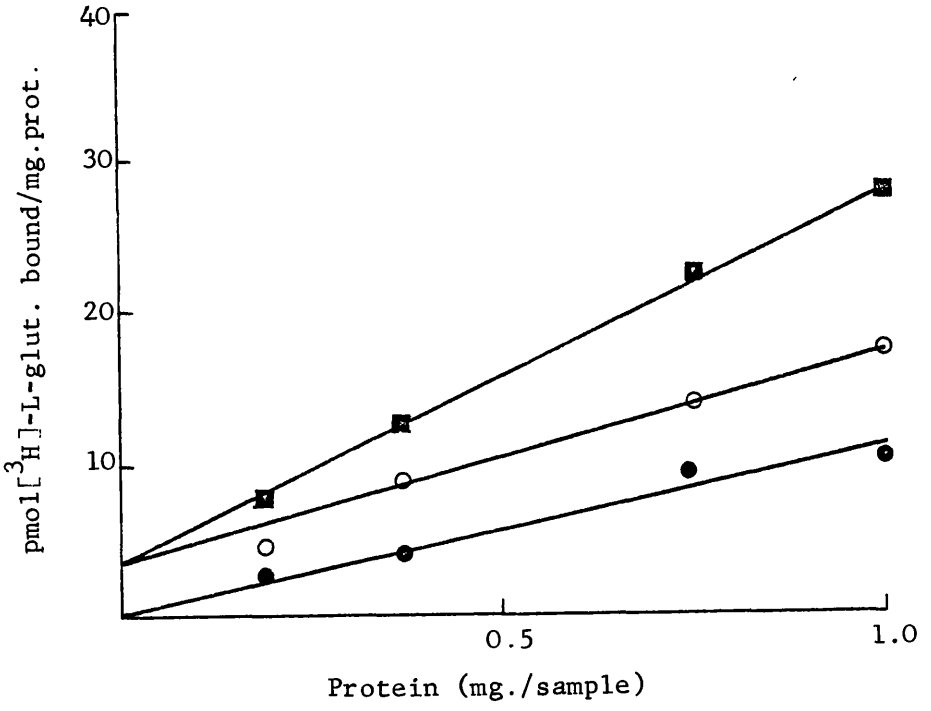
Results are the means of triplicates ± S.E.

[^{3}H]-L-glutamate = 10^{-5} M

Unlabelled L-glutamate = 5 mM

Binding was carried out as described in the Methods (p. 68).

Fig. 15 The Effect of Varying the Protein Concentration on [^{3}H]-L-Glut. Binding to the P_2 Muscle Fraction. [^{3}H]-L-glut. 10^{-5} M; unlabelled L-glut. 5mM; bound [^{3}H]-L-glut. was separated from free by airfuge centrifugation. ■ total binding; ○ non-specific binding; ● specific binding. (Results are the means of triplicates. S.E.<10%)



available to account for non-specific binding. Thus the P_2 fraction was chosen in preference to the P_1 fraction for further study.

12. The Effect of Varying Protein Concentration on the Binding of [^{3}H]-L-Glutamate (Airfuge Centrifugation Assay):

It was necessary to show that specific binding of [^{3}H]-L-glutamate to the P_2 membrane fraction was linear with respect to protein concentration for the airfuge centrifugation method of assay.

Binding was carried out as described in the Methods (p. 69); [^{3}H]-L-glutamate was at a concentration of 10^{-5}M. Protein was varied from 0.19 mg to 1.0 mg.

Specific [^{3}H]-L-glutamate binding increased proportionally with protein (Fig. 15 Mean of triplicates S.E. < 10%). Both the lines representing total and non-specific [^{3}H]-L-glutamate binding failed to go through the origin but intersected the y-axis at the same point; this corresponds to non-specific binding to the separating material (see AChR Discussion p. 155) and to [^{3}H]-L-glutamate trapped in the pellet.

13. The Effect of Varying the Concentration of Unlabelled L-Glutamate on the Specific Binding of [^{3}H]-L-Glutamate (Airfuge Centrifugation Assay):

Binding was carried out as described in the Methods (p. 69). [^{3}H]-L-glutamate was at a concentration of 10^{-5}M and unlabelled L-glutamate was varied from 1 mM - 20 mM. The results are shown in Table 15. Increasing the unlabelled L-glutamate in this concentration range caused no further displacement of specific binding. It can

TABLE 15

The Effect of Varying Cold L-Glutamate on Non-Specific and Specific Binding as Assayed by the Airfuge Centrifugation Assay:

Total Binding = 64.73 pmol/mg protein
±1.92

Unlabelled L-Glutamate (mM)	Binding (pmol/mg prot.)	
	Non-Specific	Specific
1	27.45 ± 2.23	37.28 ± 2.94
5	28.54 ± 2.12	36.19 ± 2.86
10	27.96 ± 1.27	36.77 ± 2.30
20	28.19 ± 2.3	36.54 ± 2.99

Results are the means of quadruplicates ± S.E.

$[^3H]$-L-glutamate = 10^{-5}M

Binding was carried out as described in the Methods (p. 69).

therefore be assumed from this experiment that all the specific L-glutamate specific binding sites are occupied by unlabelled L-glutamate when the unlabelled ligand is at a concentration of 5 mM. This concentration of unlabelled L-glutamate was used to account for non-specific binding in all subsequent experiments.

14. The Dependence of [^{3}H]-L-Glutamate Binding on Concentration (Airfuge Centrifugation Assay):

Binding was carried out as described in the Methods (p. 69). Unlabelled L-glutamate was at a concentration of 5 mM and [^{3}H]-L-glutamate was varied initially from 1.5 - 110 nM and then from 1μM - 200μM. Bound [^{3}H]-L-glutamate was separated from free [^{3}H]-L-glutamate by the airfuge centrifugation assay.

The lower concentration range was investigated because of reports by other workers (Biziere et al., 1980; Roberts, 1981) of K_D's in the nanomolar range, for membrane preparations from rat brain. Using locust muscle P_2 membrane fragments, the lower concentration range of [^{3}H]-L-glutamate was investigated numerous times. The same problems persisted as has been obvious throughout this work, namely poor reproducibility of specific binding of [^{3}H]-L-glutamate.

Fig.16 (a)(b) and (c) show examples of specific binding of [^{3}H]-L-glutamate obtained in different experiments, apparently under the same experimental conditions. As can be seen, not only the amount of [^{3}H]-L-glutamate specifically bound is inconsistent from preparation to preparation but also the binding profile varies considerably. Fig.16(a) suggests saturation, Fig.16 (b) shows no indication of approaching saturation,and Fig.16 (c) suggests either two binding

Fig. 16 The Effect of Concentration on Specific [^{3}H]-L-Glutamate Binding to the P_2 Muscle Fraction. Unlabelled L-glut. 5mM; Bound [^{3}H]-L-glut. was separated from free by airfuge centrifugation. (a), (b) and (c) are the results of 3 separate expt. (Results are the means of triplicates, S.E.<9%)

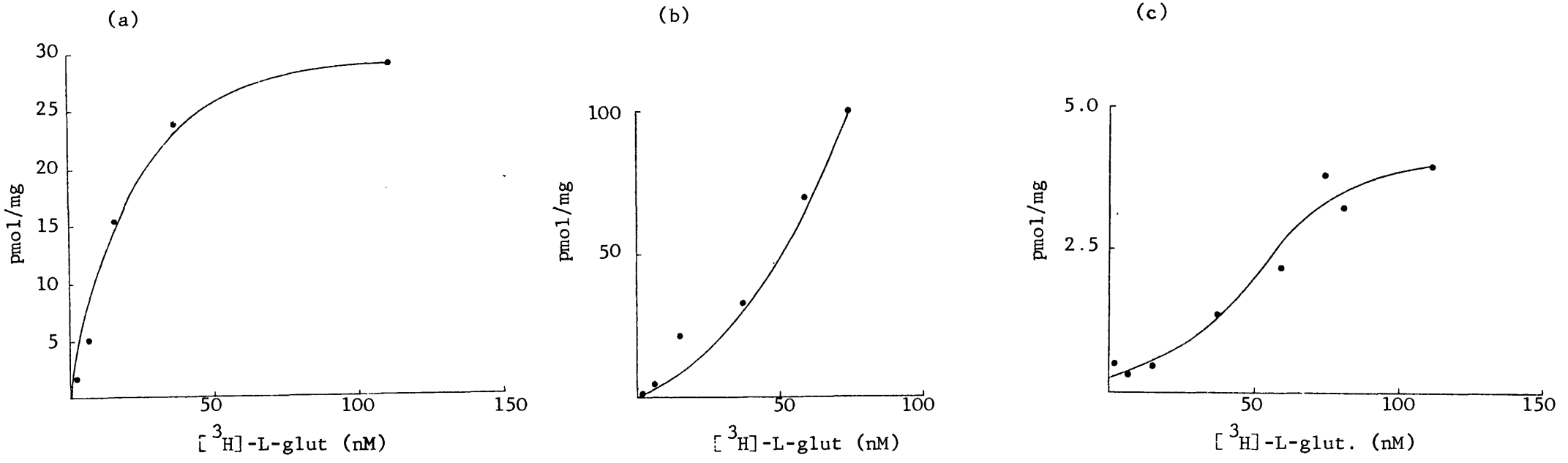


TABLE 16 A Summary of the K_D's Obtained for L-Glutamate Binding to the P_2 Muscle Fraction

Preparation Number	K_D		
1		63.0 μM	380 μM
2	2.0 nM	63.0 μM	
3	12.5 nM	1.3 μM	
4	47.9 nM		

sites or cooperativity. These results are just examples of the diversity of results obtained and on some occasions no displacement and hence no specific [^{3}H]-L-glutamate was observed. However, on some occasions, Scatchard analysis of the binding data yielded a value for K_D. A summary of the different K_D's calculated for different preparations is given in Table 16 . Included in this table are the results of experiments carried out over a wider concentration range, 0.1 μM - 200 μM.

Although these K_D values are very scattered, the presence of three binding sites for [^{3}H]-L-glutamate can be tentatively suggested; a high affinity binding site in the nM range, a medium affinity binding site in the lower μM range and a low affinity binding site in the high μM region.

15. Experiments to Investigate the Variation in [^{3}H]-L-Glutamate Binding from Preparation to Preparation

The previous set of experiments which investigate the dependence of [^{3}H]-L-glutamate binding on concentration exemplifies the variation in specific [^{3}H]-L-glutamate binding to this P_2 membrane fraction, under apparently the same conditions of preparation and assay. The standard error of the mean of triplicates was low, < 8% for all these preparations, suggesting a constancy in the binding assay for any one preparation. Also it is unlikely that any change in the physical parameters of the binding assay conditions (incubation, centrifugation conditions, washing and counting of sample) of sufficient magnitude to affect the amount of specific binding would go undetected. On the other hand, the preparation of the tissue, although carried out under

TABLE 17

The Effect of Longer Centrifugation Time on RSA of GPox in the S_1 Supernatant:

Fraction	RSA	Specific Binding (pmol/mg prot.)
Tot. Homogenate	1	22.8 ± 3.2
S_1 10 min *	0.174	3.65 ± 0.78
S_1 20 min	0.106	3.44 ± 0.53
S_1 30 min	0.122	3.96 ± 0.91
S_1 45 min	0.132	3.01 ± 0.32
P_2	1.371	94.4 ± 3.35

Results are the means of quadruplicates ± S.E.

[^{3}H]-L-glutamate concentration = 10^{-5}M

Unlabelled L-glutamate = 5 mM

Enzyme activity was assayed as described in the Methods (p. 65).

Binding was carried out as described in the Methods (p. 69)..

* Centrifugation time

apparently the same conditions for every preparation (homogenisation, centrifugation and age of preparation), is more likely to include as yet undetected and therefore unaccounted for, variants which could affect the specific [^{3}H]-L-glutamate binding and therefore a number of possible factors which could affect [^{3}H]-L-glutamate binding to the P_2 membrane fragments were considered.

a) The Effect of Longer Centrifugation Time at 3000 x g on the RSA of GPox in the S_1 Supernatant:

Electronmicrographs (Plates 1 and 2) of the P_2 membrane fraction showed that although this fraction is largely composed of membrane fragments there is still a lot of contamination by mitochondria. Mitochondrial contamination of this fraction was also suggested by the enrichment of enzymes (Table 4) that have a mitochondrial localisation in other insect systems (Donnellan, 1974). It was thought that perhaps mitochondria were interfering with the neuroreceptor specific binding of [^{3}H]-L-glutamate, either by high affinity uptake or metabolism of the labelled L-glutamate. Therefore, in an attempt to reduce the mitochondrial contamination of the P_2 membrane fraction, longer times of centrifugation at 3,000 x g were investigated in relation to the RSA of one typical mitochondrial marker enzyme (GPox), in the supernatant, (S_1). It was hoped that more mitochondria would pellet with the longer centrifugation times and hence a reduction of GPox RSA values would appear in the S_1 supernatant,possibly accompanied by increased specific [^{3}H]-L-glutamate binding.

Table 17 shows the RSA values in the S_1 supernatant after 10, 20, 30 and 45 min. centrifugation at 3,000 x g. Specific [^{3}H]-L-glutamate binding to the S_1 fraction is also shown.

The increased times of centrifugation at 3,000 x g investigated had no effect on the RSA of GPox in the S_1 supernatant, hence there was still an enrichment of this enzyme and also presumably of mitochondria in the P_2 pellet fraction, indicated by a RSA of greater than 1. Also no increase in specific [^{3}H]-L-glutamate binding was apparent in the S_1 supernatant with increased centrifugation time, and therefore in subsequent experiments the tissue was prepared as outlined in Fig. 10.

b) The Effect of Ethanol on [^{3}H]-L-Glutamate Binding:

Ethanol causes an increase in the force of the neurally evoked contractions of the innervated retractor unguis muscle of the locust (McDonald et al., 1974). The authors suggest that alcohol, either directly or indirectly,increases the intracellular concentration of calcium by interacting with the cellular membranes to promote calcium ion influx to the contractile mechanism of the muscle fibres. If this is the case then it is possible that the interaction of the alcohol with the cellular membranes could be at the L-glutamate receptor and so could disturb the normal ion-gating mechanisms controlled by this receptor. Michaelis et al (1978) have also shown that ethanol administered to rats in vivo causes changes in the [^{3}H]-L-glutamate binding capacity of brain membrane fractions,subsequently assayed in vitro.

The [^{3}H]-L-glutamate from The Radiochemical Centre, Amersham, used in the experiments in this thesis was in solution in 2% ethanol. When isotopic dilution of [^{3}H]-L-glutamate is carried out the final concentration of ethanol in a 10^{-5}M [^{3}H]-L-glutamate solution is 4mM.

TABLE 18

The Effect of Ethanol on the Binding of [^{3}H]-L-glutamate:

Final Ethanol Concentration	Binding (pmol/mg protein)		
	Non-Specific	Total	Specific
0	36.44 ± 6.32	65.33 ± 7.55	28.89 ± 9.85
4mM	49.51 ± 3.72	60.68 ± 7.36	11.16 ± 8.25

[^{3}H]-L-Glutamate was at a concentration of 10^{-5}M.

Unlabelled L-glutamate was at a concentration of 5 mM.

Binding was carried out as described in the Methods (p. 69).

Results are the means of quadruplicates ± S.E.

It was thought that in view of both electrophysiological studies on locust muscle of McDonald et al (1974) and the studies on rat brain in vivo carried out by Michaelis (1978), these concentrations of ethanol could be affecting the binding of [^{3}H]-L-glutamate to the P_2 membrane fraction. To see if this was the case, [^{3}H]-L-glutamate binding to the same P_2 membrane fragment preparation was carried out using two samples of [^{3}H]-L-glutamate from the same batch; one blown down to dryness under nitrogen, taken up in locust saline and isotopically diluted to give a final concentration in the incubation tube of 10^{-5}M [^{3}H]-L-glutamate, the other sample was isotopically diluted as usual to a final concentration of 10^{-5}M [^{3}H]-L-glutamate.

As can be seen from Table 18, specific binding of [^{3}H]-L-glutamate to the membrane fragments was higher in the ethanol free sample. Total binding was unchanged by removing the alcohol. Therefore it would appear from this experiment that ethanol at a concentration of 4 mM causes a decrease in specific [^{3}H]-L-glutamate binding.

All samples of [^{3}H]-L-glutamate were therefore taken to dryness before use, in all subsequent experiments. This procedure, however, had little effect in improving the reproducibility of the amount of [^{3}H]-L-glutamate bound from preparation to preparation.

c) The Effect of Incubation of the P_2 Membrane Fragments and [^{3}H]-L-Glutamate in the Centrifuge Tubes:

In previous experiments incubation was carried out in a total volume of 500 µl, of which 150 µl was removed and centrifuged. This 500 µl contained, on average, 200 µg - 300 µg of protein and a final concentration of 10^{-5}M [^{3}H]-L-glutamate and when accounting for

non-specific binding 5 mM unlabelled L-glutamate. When the incubation was carried out in centrifuge tubes, in a total volume of 150 µl, the final concentrations of [^{3}H]-L-glutamate and unlabelled L-glutamate were maintained at 10^{-5}M and 5 mM respectively but the protein was increased from 200 - 300 µg per 150 µl as opposed to 200 - 300 µg per 500 µl previously used. Incubation in the centrifuge tubes would,firstly,eliminate any possible inaccuracies in transferring samples from the incubation tube to the centrifugation tube. In addition,the increased protein concentration should result in an increase in the amount of specifically bound [^{3}H]-L-glutamate per 150 µl sample and the standard error of the mean between triplicates would be expected to be smaller because of the increase in recorded counts associated with one sample.

Incubation in centrifuge tubes initially appeared to improve both the reproducibility within and between preparations. However, after an initial series of experiments, it became obvious that in a majority of experiments more [^{3}H]-L-glutamate was apparently being bound in the presence of an excess of unlabelled L-glutamate than in its absence. In previous experiments the inconsistency of [^{3}H]-L-glutamate binding, and the occasional absence of specific [^{3}H]-L-glutamate binding has been attributed to an,as yet,undetected artifact in the preparation and/or the binding assay. In these experiments it was thought unlikely that the increased protein concentration in the incubation mixture was the cause of the erroneous but consistent results. In a number of the previous experiments using the lower concentration of protein, there was no apparent displacement of [^{3}H]-L-glutamate binding by an excess of unlabelled

L-glutamate, but the amount of labelled glutamate that was bound was so low that it was not possible to determine whether the unlabelled glutamate had in fact caused an increase in binding. A number of possibilities for these as yet inexplicable results were subsequently investigated.

16. Experiments to Investigate the Apparently Increased Binding of [^{3}H]-L-Glutamate in the Presence of an Excess of Unlabelled L-Glutamate:

a) The Metabolism of [^{3}H]-L-Glutamate:

It was thought that perhaps [^{3}H]-L-glutamate was being metabolised by enzymes associated with the P_2 membrane fraction and that increased tissue concentrations, hence also in the associated enzymes, would cause an increase in the amount metabolised. Furthermore, if the enzymes involved were sensitive to positive cooperativity, then an increased concentration of L-glutamate would result in an increased metabolism of both labelled and unlabelled L-glutamate. Alternatively, the phenomenon of increased apparent [^{3}H]-L-glutamate binding in the presence of an excess of unlabelled L-glutamate could be the result of simple mass action. In incubations in which there is a low concentration of substrate, i.e. in the absence of unlabelled L-glutamate, metabolism may be at a low level. However, when the concentration of substrate is increased by the addition of unlabelled ligand, there may be an increase in metabolism and more substrate could be pushed through the system, resulting in an apparent increase in [^{3}H]-L-glutamate binding.

In an attempt to inhibit any possible metabolism of L-glutamate, the metabolic inhibitor monofluroacetate was included in the incubation media and/or the incubation was carried out at 4°C instead of the more usual temperature of 23°C. In the two experiments carried out under the above conditions, there was still an increase in [^{3}H]-L-glutamate binding in the presence of excess unlabelled L-glutamate, indicating that metabolism of the [^{3}H]-L-glutamate was unlikely to be

contributing to the apparent binding.

The presence of possible metabolites of [^{3}H]-L-glutamate in the incubation media was investigated by thin layer chromatography. Protein-free samples were examined after incubation for 0, 15, 30 and 60 min with 10^{-5}M [^{3}H]-L-glutamate with and without 5 mM unlabelled L-glutamate. The plates were run in two dimensions and the standards were run in one dimension along both sides (Methods p. 70). The pattern of stained spots was the same for all the incubation times investigated and only the sample spot corresponding to L-glutamate had any radioactivity associated with it. So it would appear that no metabolism of [^{3}H]-L-glutamate occurs during incubation times up to 60 min.

b) The Effect of Preincubation with Unlabelled L-Glutamate on [^{3}H]-L-Glutamate Binding:

P_2 membrane fragments were incubated with 5 mM unlabelled L-glutamate for 5, 10 and 15 min at 23^{o}C, prior to the addition of [^{3}H]-L-glutamate (final concentration 10^{-5}M) and further incubation for 15 min. Control membrane samples were incubated for the same times and at the same temperature, in the absence of unlabelled L-glutamate, prior to incubation with [^{3}H]-L-glutamate.

Table 19 shows the results from two experiments, each carried out in quadruplicate and both apparently under the same conditions. Firstly, in comparing the results from the two preparations it appears that in preparation (a) there is more [^{3}H]-L-glutamate bound in the presence of an excess of unlabelled L-glutamate whereas in preparation (b) the converse is true and there is apparent specific [^{3}H]L-glutamate

binding as defined by the "excess unlabelled ligand" method of assessment. This exemplifies the variation in [^{3}H]-L-glutamate binding from preparation to preparation where the only apparent difference is that the tissues were prepared on different days and suggests that the probable artifact which causes an increase in [^{3}H]-L-glutamate binding on addition of an excess of unlabelled L-glutamate is not present in every preparation or alternatively is suppressed in some preparations. Secondly, in both preparations the amount of [^{3}H]-L-glutamate associated with the tissue appears to increase with longer pre-incubation times and this increase is greater when excess unlabelled L-glutamate is present.

One supposition, on the basis of the latter observation is that the [^{3}H]-L-glutamate binding capacity of the membranes changes with time. If only preparation (b) is considered, this change is associated with a non-specific [^{3}H]-L-glutamate binding component, as both total and non-specific [^{3}H]-L-glutamate binding increase similarly, leaving specific [^{3}H]-L-glutamate binding unchange. However, the same cannot be said for preparation (a), as there is apparently no specific [^{3}H]-L-glutamate binding to this preparation, as defined by the"excess unlabelled ligand"method of assay. One could speculate that there are two factors affecting the binding of [^{3}H]-L-glutamate to the P_2 membrane fraction, one of which is affected by preincubation of the tissue at room temperature and is obvious in preparation (b) and a second which is manifested by the presence of an excess of unlabelled L-glutamate. Both artifacts could be affecting the [^{3}H]-L-glutamate binding to preparation (a).

TABLE 19

The Effect of Preincubation with Unlabelled L-Glutamate on [^{3}H]-L-Glutamate Binding:

(a) Preparation No. 1

Preincubation Time (min)	+ Excess L-Glutamate (pmol/mg prot.)	- L-Glutamate (pmol/mg prot.)
5	74.9	54.9
10	86.9	55.9
15	90.0	59.9

Results are the means of quadruplicates. S.E. < 8%.

(b) Preparation No. 2

Preincubation Time (min)	+ Excess Unlabelled L-Glutamate	- Unlabelled L-Glutamate
5	33.2	52.3
10	39.4	54.5
15	45.3	60.6

Results are the means of quadruplicates. S.E. < 5%.

[^{3}H]-L-glutamate = 10^{-5}M

Unlabelled L-glutamate = 5 mM

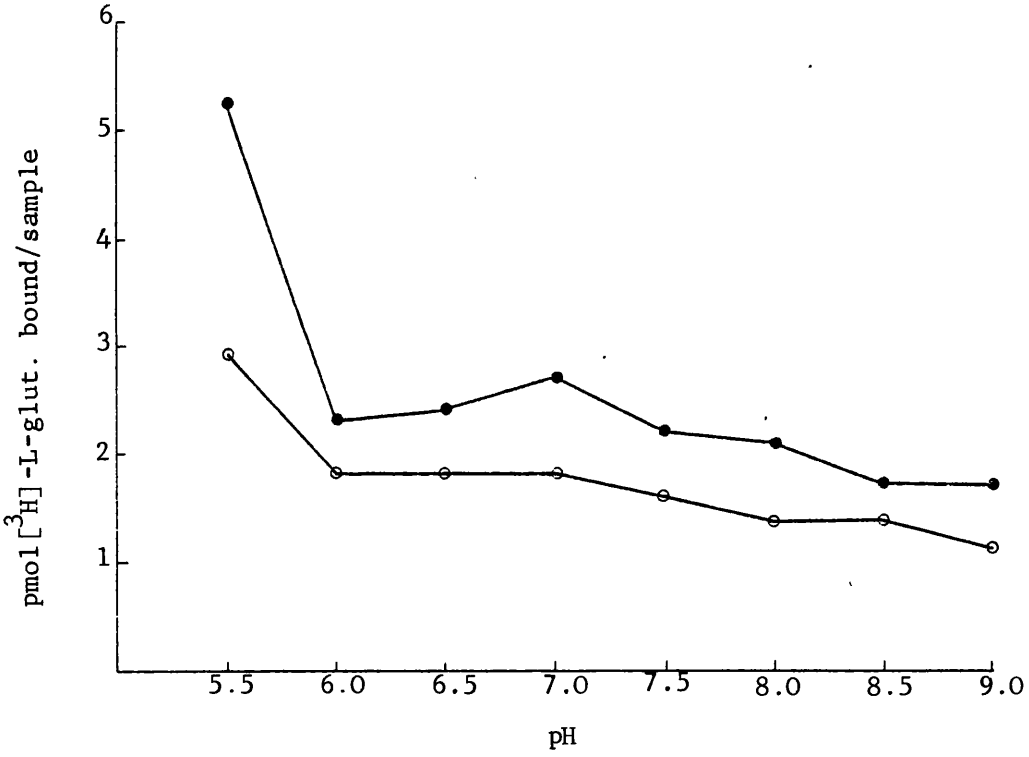
c) Effect of pH on [^{3}H]-L-Glutamate Binding:

Binding was carried out over a pH range of 5.5 - 9. All solutions were adjusted to the required pH prior to incubation. Fig. 17 shows the total binding and the binding in the presence of an excess of unlabelled L-glutamate (5 mM). Fig. 17 shows that most binding occurs at the lower pH range and is unchanged between pH 6 - 7. However,in all cases there was more apparent binding in the presence of an excess of unlabelled L-glutamate indicating that the pH of the incubation media is not responsible for this phenomenon.

Fig. 17 The Effect of pH on [^{3}H]-L-Glutamate Binding to the P_2 Muscle Fraction in the Presence and Absence of Unlabelled L-Glutamate.

Unlabelled L-glut. 5 mM; bound [^{3}H]-L-glut. was separated from free by airfuge centrifugation;

● + unlabelled L-glut. ○ - unlabelled L-glut. (Results are the means of quadruplicates. S.E.<8%)



17. The Effect of Various Marine Toxins on Specific [^{3}H]-L-Glutamate Binding:

Two toxins α and β cephalotoxin (M_r 19,200 and 33,900 respectively) have been isolated from the octopus, Octopus vulgaris (Cariello and Zanetti, 1977). Both these toxins along with a third toxin, suberitine (M_r 28,000), isolated from the marine sponge, Suberitus domuncula (Cariello and Zanetti, 1979) have a paralysing effect, possibly by neuro-muscular blockade, on the crab, Pachigrapsus marmoratus. Furthermore, it was also suggested from preliminary investigations on a preparation of locust muscle that the cephalotoxins act at the synaptic level inhibiting glutamate mediated transmission (Cariello and Zanetti, 1977). For these reasons the cephalotoxins and suberitine were investigated as potential antagonists of L-glutamate binding to the P_2 membrane fragments prepared from locust muscle. All three toxins were in limited supply as they were a gift from Professor Lucio Cariello and were not commercially available. Therefore, only a preliminary study was possible.

Initially the effects of the lower concentrations of toxins on the specific binding of [^{3}H]-L-glutamate (10^{-5}M) were investigated (Table 20a). Non-specific binding was accounted for by incubation in the presence of 5 mM unlabelled L-glutamate. Bound [^{3}H]-L-glutamate was separated from free [^{3}H]-L-glutamate by the airfuge centrifugation assay. The results were expressed as the percentage of specific [^{3}H]-L-glutamate binding displaced by the toxins at the concentrations used. Using the lower concentrations all three toxins displaced specifically bound [^{3}H]-L-glutamate, suberitine being the most potent, followed by β-cephalotoxin and α-cephalotoxin in that

order (Table 20a). In a subsequent preparation the concentration of all three toxins was doubled but only with β-cephalotoxin was there a corresponding increase in displacement of specific [^{3}H]-L-glutamate binding. With suberitine the displacement was decreased by a factor of 2.5, whereas binding was greater than total [^{3}H]-L-glutamate binding when the concentration of α-cephalotoxin was doubled.

After carrying out these experiments a small quantity of only suberitine remained, which was used in later experiments,that showed an increase in [^{3}H]-L-glutamate binding in the presence of excess unlabelled L-glutamate. The effect of suberitine on [^{3}H]-L-glutamate binding was again investigated to see if it too caused an increase in [^{3}H]-L-glutamate binding.

[^{3}H]-L-glutamate (10^{-5}M) binding to the P_2 membrane fragments was assayed in the presence and absence of 5 mM L-glutamate and/or 31 μM suberitine, as described in the Methods (p. 69). Table 20 (b) shows the results of two experiments,each carried out in triplicate. In both cases, unlabelled L-glutamate caused an apparent increase in [^{3}H]-L-glutamate binding. This increase was lower when incubations were carried out in the presence of both unlabelled L-glutamate and suberitine, suggesting that perhaps suberitine reduced the increase in apparent binding of [^{3}H]-L-glutamate seemingly caused by unlabelled L-glutamate. However, suberitine alone did not displace [^{3}H]-L-glutamate binding as in the previous experiments (Table 20a) and in preparation 2 (Table 20b) apparently caused a slight increase in [^{3}H]-L-glutamate binding, although not to the same extent as did unlabelled L-glutamate.

The results are interesting in that suberitine seemed to reduce the increase in [^{3}H]-L-glutamate binding apparently caused by unlabelled L-glutamate. Further study may assist in providing an explanation for the erroneous results obtained in the presence of an excess of unlabelled L-glutamate. Furthermore, these preliminary experiments suggest that α and β cephalotoxin and suberitine may be capable of displacing specifically bound [^{3}H]-L-glutamate and as such may be useful probes for the binding site of the putative glutamate receptor. However, it is clear that considerably more work must be done before their usefulness can be properly assessed.

TABLE 20 (a)

The Effect of Cephalotoxin and Suberitine on Specific [^{3}H]-L-Glutamate Binding:

Toxin	Concentration (μM)	% Displacement of Specific [^{3}H]-L-Glutamate Binding
α-Cephalotoxin	18	24%
α-Cephalotoxin	36	increased binding
β-Cephalotoxin	9	58%
β-Cephalotoxin	18	79%
Suberitine	31	80%
Suberitine	62	33%

[^{3}H]-L-glutamate was at a concentration of 10^{-5}M.

Unlabelled L-glutamate was at a concentration of 5 mM.

Binding was carried out as described in the Methods (p. 68).

Standard error of the mean of quadruplicates < 10%.

TABLE 20 (b)

The Effect of Suberitine on [^{3}H]-L-Glutamate Binding:

Preparation Number	L-Glutamate Present (5 mM)	Suberitine Present (31 μM)	[^{3}H]-L-glutamate Bound (pmol/mg prot.)
1	-	-	229.87 ± 10.6
1	+	-	365.88 ± 5.22
1	-	+	221.11 ± 10.2
1	+	+	301.63 ± 12.16
2	-	-	324.86 ± 3.25
2	+	-	421.24 ± 9.73
2	-	+	344.49 ± 3.46
2	+	+	363.40 ± 6.32

Results are the means of triplicates ± S.E.

Binding was carried out as described in the Methods (p. 68).
[^{3}H]-L-glutamate was at a concentration of 10^{-5}M.

GENERAL DISCUSSION

The major constraint on progress in the biochemical characterisation of glutamate neuroreceptors in both vertebrates and invertebrates is the lack of a diagnostic glutamatergic ligand. The problem is compounded in insects by the inability to obtain a preparation enriched specifically in synaptic membranes. The lack of a ligand that binds to glutamate receptors with a specificity and a high affinity analagous to α-BgTx binding to the vertebrate nAChR has resulted in the use of the natural ligand, L-glutamate, as the receptor probe. In turn this presents serious problems in receptor-ligand binding studies:

1. As the natural neurotransmitter is the only ligand available as a specific probe for the receptor, it follows that it is the only ligand that can be confidently employed in the "excess unlabelled ligand" method (p. 76) of accounting for non-specific binding. Theoretically this should present no problems (Turner, 1980) but in practice a chemically distinct ligand is preferred (see AChR Discussion p. 155).
2. For a compound to be physiologically neuroactive binding to its receptor must be readily reversible to avoid densensitisation and thus allow recovery of the membrane to its resting state. Therefore, if L-glutamate is the neurotransmitter at the insect neuromuscular junction, as postulated, a relatively short half-time of dissociation of receptor-ligand complex in vitro would be anticipated. Consequently, the techniques used in this thesis to separate membrane bound [^{3}H]-L-glutamate from free [^{3}H]-L-glutamate may not be rapid enough to prevent significant dissociation (see AChR Discussion p. 154).

3. L-Glutamate, unlike acetylcholine, plays a central role in intermediary metabolism, and consequently is a substrate for numerous enzymes which represent non-neuroreceptor, but specific, binding sites for this amino acid. Furthermore, if present and under the right conditions these enzymes could metabolise the L-glutamate added to a preparation and, if this is not accounted for, an overestimation of the amount of L-glutamate available for neuroreceptor binding would ensue.

Preparation of synaptosomes from insect muscle has proved difficult because of the fragility of several of the constituents of the muscle cell, in particular the mitochondria, and also because of the cells' high content of sarcoplasmic reticulum. (Donnellan, et al., 1974; Briley, 1981; see Introduction p. 36). Consequently, suitable marker enzymes associated with specific membranes have not yet been delineated. As a result of these insurmountable problems the membrane preparations currently available are ill-defined and heterogeneous.

Clearly, rectification of either of the two major problems - an ill-defined preparation and lack of a diagnostic ligand - would minimise the adverse characteristics of the other. Unfortunately, a solution to either is not apparent in the immediate future because firstly, the discovery of a high affinity specific ligand for L-glutamate would require an intensive study of analogues of the amino acid, which may prove to be pharmacologically active and/or a systematic physiological and biochemical investigation of the numerous natural toxins available. The former line of investigation

has commenced on preparations of mammalian brain (see, Davies et al, 1980) but it is more likely that a study of natural toxins will reveal a potent L-glutamate antagonist, as was the case in the discovery of α-bungarotoxin. However, because the range of natural toxins is so wide and because rarely are both biochemical and physiological studies carried out simultaneously, it would be fortuitous if such a toxin/antagonist was to become immediately apparent. Secondly, the starting material for binding studies is frequently a crude membrane preparation and in such preparations specific binding to non-receptor sites may well obscure the specific binding to the receptor under study.

The fractionation scheme in Fig. 10 was employed with a view to removing the bulk of the mitochondria, a paramount but readily identified contaminant, from the preparation with minimal reduction in the yield of membrane fragments. Both electronmicrographs (Plates 1 and 2) and enzyme studies (Table 4) on the P_2 fraction indicate that this has been achieved. Enzyme studies localised mitochondrial enrichment to the P_1 pellet fraction whereas electron-micrographs showed P_2 to be largely composed of membrane fragments with limited mitochondrial contamination. On the basis of these results the P_2 membrane fraction presented a likely point at which to commence investigations of putative L-glutamate receptors in insect muscle. The conclusions drawn from electronmicrographs and enzyme studies were later confirmed in that the differential centrifugation of Fig. 10 yielded a P_2 membrane fraction which was enriched in specific [^{3}H]-L-glutamate binding activity (Table 14).

From the outset the most persistent problem in the binding

studies lay in obtaining a consistent amount of specific [^{3}H]-L-glutamate binding from preparation to preparation; a problem which was compounded in initial binding experiments using GF/C vacuum filtration to separate bound [^{3}H]-L-glutamate from free [^{3}H]-L-glutamate by the frequently high standard error, often greater than 30% of the sample value, between triplicates and quadruplicates. Poor reproducibility between samples suggests an inconsistency in the method of assaying specific binding activity, possibly attributable to an inefficiency in the separation technique such that not all of the free [^{3}H]-L-glutamate passes through the filter on every occasion. This seems to be a more probable explanation than the likelihood that the binding capacity of the sample changes between samples.

High standard errors within a preparation were curbed to a certain extent by trimming the filter discs after filtration and by rigorously controlling the time taken for the sample and the wash to filter. Thus, in preparations in which reproducible displaceable [^{3}H]-L-glutamate binding was observed, binding was apparently specific L-glutamate receptor bindingas shown by the linearity of specific [^{3}H]-L-glutamate binding with increasing protein concentration and thus is in agreement with point B of the criteria (p.15) for defining a physiological receptor. Specific [^{3}H]-L-glutamate binding was also dependent on the concentration of [^{3}H]-L-glutamate (Fig.14 (a)) but the classic hyperbolic binding curve, indicative of a single class of saturable binding sites, was not obtained. The observed binding profile is suggestive of either two classes of binding sites or positive cooperativity of L-glutamate binding. Only after

extensive study at numerous concentrations can Scatchard analysis of the data distinguish between the two. Unfortunately, the limited data in these studies made it impossible to conclude irrefutably which was the case. If the results are interpreted as two populations of specific L-glutamate receptors, a high affinity (low K_D) population, corresponding to neuroreceptor binding sites and a lower affinity (higher K_D) population possibly corresponding to a specific Na^+ independent (as all experiments were carried out in Na^+ free buffer) uptake system, two K_D's of 62.75 µM and 380µM respectively are obtained. Such an interpretation is corroborated firstly, by evidence to suggest that high affinity uptake is the most probable mode of removal of the natural transmitter from the synaptic cleft (see Introduction p. 36) and secondly by the work of Briley (1981) which demonstrates specific uptake of L-glutamate into a vesiculated membraneous fraction of locust flight muscle, of which only a proportion is sodium dependent. It is quite conceivable that in this ill-defined P_2 membrane fraction specific uptake sites for L-glutamate are contributing to the specific binding component and are possibly interfering with the higher affinity neuroreceptor binding site.

Freeze-thawing of the sample caused a decrease in only the specific [^{3}H]-L-glutamate binding component (Table 11) a reduction perhaps associated with impairment of specific uptake sites for L-glutamate. The effect of freeze-thawing was not investigated any further because the objective of freeze-thawing at this stage in the study was to improve the specific binding capacity of the membranes by possible exposure of previously inaccessable specific binding sites

and/or to remove possible sequestered endogenous L-glutamate (p. 81). A decrease in specific [^{3}H]-L-glutamate binding to a freeze-thawed preparation indicated that this objective had not been attained.

An alternative method of separating bound [^{3}H]-L-glutamate from free [^{3}H]-L-glutamate is by centrifugation (see AChR Discussion p. 153). Initially, separation by centrifugation was attempted using a Beckman microfuge centrifuge, the maximum force of which is 2000 x g. Probably because of the relatively low g force the membranes were not sedimented to a tight pellet and consequently the amount of [^{3}H]-L-glutamate associated with the membranes was inconsistent between triplicates,moreso than when assayed by the GF/C filtration method. On the other hand, the Beckman airfuge produces its maximum force of about 100,000 x g within 15 seconds and after one minute at this g force the membranes had sedimented to a tightly packed pellet. After comparison (Table 13)the airfuge centrifugation method was chosen in preference to the GF/C vacuum filtration method for the following reasons:

1. The airfuge centrifuge appeared to separate more thoroughly bound from free [^{3}H]-L-glutamate as indicated by the increase in the specific [^{3}H]-L-glutamate binding.
2. The standard error of the mean of triplicates was greatly reduced, a reduction that persisted in subsequent preparations.
3. A constant time to separate bound [^{3}H]-L-glutamate from free [^{3}H]-L-glutamate was more readily maintained because with the airfuge centrifugation method this factor is dependent on the speed and the centrifugation time, both of which are readily controlled, whereas the rate of filtration is dependent on

parameters such as water pressure and porosity of the glass Sinter support, which are not so easily controlled.

Despite these improvements using the airfuge centrifugation separation technique, the major problem of poor reproducibility of [^{3}H]-L-glutamate binding from preparation to preparation persisted and is therefore more likely to be a result of some, as yet undetected, artifact(s) in the membrane preparation rather than in the method of assay. All experiments were carried out under the same apparent conditions, with respect to tissue preparation and the binding assay. A prerequisite to improving the specific binding of [^{3}H]-L-glutamate, by changes either in the preparation procedure or in the binding assay conditions, is a standard by which any such improvements can be assessed. The inconsistency in [^{3}H]-L-glutamate binding from day to day does not readily permit this and therefore the effect of alterations could only be compared within any one preparation. Consequently, when a change which apparently improved specific [^{3}H]-L-glutamate binding in one preparation was employed in subsequent experiments and no displacement of [^{3}H]-L-glutamate observed, the validity of such a change was questioned and reversion to the original procedure demanded.

Preliminary attempts at improving the reproducibility from preparation to preparation were unsuccessful and are outlined below:

1. It was thought that the quantity of mitochondria contaminating the P_2 membrane fraction may have been a variable factor. Therefore, longer centrifugations at 3,000 x g were implemented and both the specific [^{3}H]-L-glutamate binding and the appearance of a typical mitochondrial enzyme, GPox, were

monitored in the supernatant. A four-fold increase in centrifugation time had no effect on either the GPox in the supernatant or the specific [^{3}H]-L-glutamate binding to this fraction.

2. Removal of ethanol from the radiolabelled L-glutamate solution (p. 92) appeared initially to improve specific [^{3}H]-L-glutamate binding. However, when routinely removed it did not prevent inconsistent binding from preparation to preparation.

3. Increasing the amount of tissue per incubation sample , on the premise that this would enhance detection of specifically bound [^{3}H]-L-glutamate, had the desired effect in a number of preparations and initially also appeared to promote constant binding from preparation to preparation. However, in a number of subsequent experiments erroneous results were consistently obtained in that more [^{3}H]-L-glutamate was apparently associated with the membranes in the presence of an excess of unlabelled L-glutamate and is therefore contrary to the presumed basis for the "excess ligand" method of defining specific binding. This point will be discussed in greater detail later.

A further anomaly in these binding studies was that for a number of consecutive experiments reproducible, displacable [^{3}H]-L-glutamate binding was obtained, whereas in another batch of consecutive experiments this binding disappeared for no apparent reason. Nevertheless, in the preparations in which displaceable binding was detected, the indications are of a specific L-glutamate binding

component, a conclusion that is tentatively reached for the following reasons:

1. Displacement of [^{3}H]-L-glutamate was obtained in the presence of an excess of unlabelled L-glutamate. The displaced binding can be attributed to specific binding sites as defined by the "excess ligand" method of assay (p. 155).
2. Increasing the unlabelled L-glutamate from 5mM to 20 mM did not result in displacement of more [^{3}H]-L-glutamate, indirectly indicating a finite number of specific binding sites all of which are occupied by 5 mM unlabelled ligand.
3. Displaceable [^{3}H]-L-glutamate binding increased linearly with protein concentration (Fig. 15),strongly suggesting that true specific binding to the tissue was being monitored and not artifactual "specific" binding to the separating materials.
4. Specific [^{3}H]-L-glutamate binding was dependent on the concentration of [^{3}H]-L-glutamate and in some cases (Fig.16a) specific binding appeared to saturate, providing evidence for a finite number of specific binding sites.

The latter observation was unfortunately not the case in every experiment in which the concentration of [^{3}H]-L-glutamate was varied. Nevertheless, bearing these problems in mind,three populations of specific L-glutamate binding sites in this P_2 membrane fraction can be tentatively identified (Table 16). The population of highest affinity has a K_D in the low to mid nanomolar range, the second population has a K_D in the high nanomolar/low micromolar range and the lowest affinity site has a K_D in the middle to high micromolar

range. Distinction between these sites is difficult, firstly because all three are not apparent in every preparation and secondly all three appear to be sodium dependent, a property that has been used to differentiate between high and low affinity binding sites in other preparations (James, 1977; Baudry & Lynch, 1980). A systematic study of the effects of a range of monovalent and divalent cations may aid in the elucidation of L-glutamate binding sites of differing affinities, as work carried out by Baudry and Lynch (1979; 1980) on preparations of mammalian brain has indicated a possible receptor regulatory role for these ions.

Alternatively, the use of potential L-glutamate neuroreceptor agonists and antagonists may distinguish differences between the three populations of putative receptors. A number of analogues of both L-glutamate and L-asparate have been shown to inhibit the action of L-glutamate in the vertebrate CNS (Introduction p. 45 ; Davies et al., 1980) and at the insect neuromuscular junction (see Introduction p. 45 ; Usherwood, 1978). Although all these antagonists inhibit the action of L-glutamate, in a number of cases no cross-reactivity of ligands has been noted, indicating that they could be acting at different sub-populations of L-glutamate receptors.

Because the majority of these studies with potential agonists and antagonists have been with electrophysiological techniques, a major reservation must be borne in mind when drawing analogies with biochemical studies. Electrophysiological studies tend to use the muscle intact, whereas the biochemical approach is to subfractionate the muscle to isolate the neuroreceptor. It is quite conceivable that the receptor would exhibit differentpharmacological properties under these conditions. For example,

the morphology of the intact muscle may present permeability problems to ligands aimed at the receptor molecule. The problem would be less apparent with subcellular preparations. Conversely, the topography of the receptor molecule within the membrane may be important to its function. Isolation may modify its capacity to bind ligands with which it appears to interact *in vivo* (see Davies *et al.*, 1980; Krogesgaard-Larsen, 1980; Introduction, p. 45).

Preliminary studies with the marine toxins, α and β cephalotoxin and suberitine, were encouraging in that they showed an apparent ability to displace specific [^{3}H]-L-glutamate binding. However, when the experiment was repeated with higher concentration of toxins, increased displacement of [^{3}H]-L-glutamate binding was not observed with all three toxins. This may have been due to problems inherent in the preparation. Unfortunately, complete characterisation (IC_{50}, K_D) of the marine toxins, binding was impossible because of the limited supply. They do however, represent an attractive area for further investigation in that their paralysing effects in studies on crusteans *in vivo* are indicative of a possible neuro-muscular blockade (Cariello & Zanetti, 1977; 1979).

The majority of reports of biochemical characterisation of putative L-glutamate neuroreceptors have been concerned with preparations of mammalian brain (see Briley *et al.*, 1981). In comparison to studies on putative insect glutamate receptors, workers investigating mammalian glutamate receptors have an advantage in that well characterised preparations of synaptic membranes are readily available. However, in both instances, binding studies are hampered by the same lack of high affinity specific L-glutamate receptor

probes. Although at a more advanced stage, the position of L-glutamate receptors in mammalian brain is still unclear, the reasons for which are summarised below:

1. The K_D's reported cover a very wide range 11 nM - 55 μM (Table 21). This variation may be partly due to the choice of the brain fraction under study, in that different sections of the brain will not possess identical populations of receptor. Thus a number of receptor populations with different K_D's would be expected in whole brain preparations. As yet, only two populations of binding sites have been reported for such preparations (Michaelis et al., 1974; Biziere et al., 1980). Furthermore, the physiological significance of the highest affinity binding sites (K_D 10 - 20 nM) is doubtful because the rate of dissociation of L-glutamate from such sites would be relatively slow, thus enhancing desensitisation and preventing recovery of the membrane to an excitable state.

2. Minor changes in the preparation procedure, such as increased periods of ultrasonication and more extensive washing of the fractions, appear to result in an increased affinity of the receptors for L-glutamate (Sharif and Roberts, 1980a). In addition, lyophilisation apparently increases the binding capacity of the membranes without affecting the K_D.

3. In a number of reports the profile of [^{3}H]-L-glutamate specific binding does not saturate in the concentration range investigated (Foster and Roberts, 1978; Vincent and McGeer, 1980). Therefore, in these studies a finite number of specific

L-glutamate binding sites cannot be identified.

4. The distinction between sodium dependent and sodium independent binding sites is unclear. Vincent and McGeer (1980) were unable to detect a sodium independent population of L-glutamate binding sites in membrane preparations of rat striatum, whereas Foster and Roberts, (1978) reported an inhibition of specific [^{3}H]-L-glutamate binding in rat cerebellar membranes upon the addition of Na^+ ions (100 mM). On the other hand, high concentrations of sodium (10 mM - 100 mM) have been reported to increase specific [^{3}H]-L-glutamate binding to rat hippocampal membranes, an increase the authors attribute to a high affinity uptake binding site (Baudry and Lynch, 1981).

5. To date, there has been only one report of an attempted purification of the L-glutamate binding component from mammalian brain (Michaelis, 1975). A 200 fold purification was claimed after affinity chromatography on Con A -Sepharose of a Triton X-100 extract of a rat brain preparation. In consideration of the numerous workers in the field it is curious that no reports of purification have appeared in the intervening years.

The above discrepancies in studies on putative L-glutamate receptors from mammalian CNS illustrate that the problems experienced in studies carried out in this thesis are not confined to insects. None of the proposed L-glutamate binding sites are sufficiently well-characterised to permit a unequivocal designation as a synaptic glutamate receptor.

TABLE 21

A Summary of the K_D's and B_{max}'s Obtained for L-Glutamate Binding To Rat Brain:

Preparation	K_D	B_{max} (pmol/mg prot.)	Reference
Membrane fragments from cerebral cortex	8μM	30	Roberts (1974)
Membrane fragments from whole brain	0.2μM 4.4μM	180 880	Michaelis et al, (1974)
Synaptic membranes from cerebellum	774nM	73	Foster & Roberts (1978)
Synaptic membranes from cerebellum	300nM	117	Sharif & Roberts (1980)
Membrane fragments from hippocampus	770nM 2400nM	6.5 75.0	Baudry & Lynch (1981)
Membrane fragments from whole brain	11nM 80nM		Biziere et al, (1980)
Synaptic membranes from striatum	16.6nM	0.4	Roberts (1981)
Proteolipid from cerebral cortex	300nM 5μM 55μM	530 16,600 320,000	De Robertis & Fiszer de Plazas (1976)
Partially purified glycoprotein	0.85μM	65,500	Michaelis (1975)

The most serious problem encountered in [^{3}H]-L-glutamate binding studies to locust muscle membranes was that of increased [^{3}H]-L-glutamate binding in an excess of unlabelled L-glutamate. A similar incongruity has not been reported for binding studies to preparations of mammalian brain. Results indicate (p. 96) that the most obvious explanation for this phenomenon, metabolism of [^{3}H]-L-glutamate, was not taking place over a 60 minute incubation period as neither incubation at 4^{o}C or in the presence of mono-fluoroacetate suppressed the increased binding. Monofluoroacetate is ultimately synthesised to fluorocitrate which is an inhibitor of aconitase, hence the TCA cycle is blocked, resulting in a general block of the cell's metabolism. Therefore major metabolism of [^{3}H]-L-glutamate would be inhibited in the presence of this compound. Furthermore, thin layer chromatography of protein-free samples revealed only L-glutamate to be radiolabelled. Similarly, pH did not appear to be the cause of the increased binding (Fig. 17). Results of experiments in which samples were pre-incubated with and without unlabelled L-glutamate suggest two things: firstly, that the non-specific binding capacity of the membranes increases with time and secondly, that unlabelled L-glutamate, in some cases, further stimulates this increased binding capacity (Table 19). Therefore, in such cases the "unlabelled ligand excess" method of determining non-specific [^{3}H]-L-glutamate binding is not independent of unlabelled ligand. Reasons as to why this should be are not forthcoming. Perhaps low-affinity L-glutamate uptake system(s), which could constitute a non-specific binding component, are activated by incubation of the tissue at room temperature. If this is the case then such a system is apparently stimulated further by an excess of

unlabelled L-glutamate. Moreover, the ratio of radiolabelled L-glutamate to unlabelled L-glutamate at concentrations of 10^{-5}M and 5 mM respectively, is 1:500; Therefore as it is unlikely that any system can discriminate between tritiated and unlabelled L-glutamate, for every pmol of [^{3}H]-L-glutamate associated with the membranes, 500 pmol of unlabelled L-glutamate must also be associated. This would mean that the total amount of L-glutamate associated with one milligram of tissue would be of the order of tens of nanomoles. High concentrations such as this would mask any specific neuroreceptor L-glutamate binding but are within the concentrations determined for uptake of L-glutamate into a similar membranous preparation (Briley, 1981).

If such an uptake system is operating then it must be assumed that positive cooperativity is an inherent part of it, in that unlabelled L-glutamate enhances the uptake of [^{3}H]-L-glutamate. This hypothesis,however, does not explain why this system is only apparent in a few preparations and therefore suggests that the preparations are not identical in every case.

Obviously further investigations into putative L-glutamate neuroreceptor binding to locust muscle demands elucidation and elimination of this problem. Reappraisal of the preparation procedure presents the most logical point at which to start. The tedious operation of monitoring glutamate metabolising enzymes, measuring [^{3}H]-L-glutamate binding and uptake, and making electron-micrographs at every stage in a number of preparations might enable detection of a relationship between variable [^{3}H]-L-glutamate binding to the P_2 membrane fraction and some other variant. Such studies

might also establish the stage in the preparation when the variables are first manifested and thus give an indication of where changes should be made in the fractionation procedure i.e.homogenisation or centrifugation. Until reproducible, specific [^{3}H]-L-glutamate binding is obtained further biochemical characterisation of putative L-glutamate synaptic receptors cannot proceed using this method of receptor identification.

An alternative approach might come from the discovery of a specific, high-affinity ligand which binds to the ionophore controlled by the L-glutamate receptor. If both the receptor and the ionophore reside on the same macromolecule then purification of a radiolabelled ligand-ionophore complex would result in a simultaneous purification of the L-glutamate receptor.

SECTION 2 : STUDIES ON THE PUTATIVE NICOTINIC ACETYLCHOLINE RECEPTOR IN THE LOCUST CNS

MATERIALS

Materials were obtained as stated on p.62. In addition: α-bungarotoxin was obtained from the Boehringer Corporation (Lewes, Sussex, U.K.). ^{125}I ( ) was obtained from the Radiochemical Centre (Amersham, Bucks., U.K.).

^{125}I activity was determined by counting in an LKB 1280 Ultrogamma counter.

Gel filtration reagents were supplied by Pharmacia Ltd. (Hounslow, U.K.).

METHODS

1. Tissue Preparation:-

Two week old adult locusts, in the 5th instar were used for all experiments. The animals were decapitated and the heads were either used immediately or stored frozen at -20°C for periods of up to one week. The supra-oesophageal ganglion was exposed by shaving off the cuticle from the mouth parts to the top of the head and fatty tissue was wiped away. The supra-oesophageal ganglion was then distinguishable, having a deeper orange colour than the surrounding tissue. Using fine forceps the ganglion was removed, taking as little as possible of the optic lobe, and placed on ice. One hundred heads provided, on average, 400 mg of starting material.

2. Tissue Fractionation:

The tissue was fractionated as outlined in the flow diagram in Fig. 18, the details of which are given below.

a) Preparation of the Membrane Fragments:-

The tissue was suspended in buffer A (Table 22) (~5% w/v; i.e. material from 100 heads/8 ml). The suspension was homogenised in a Potter-Elvejham homogeniser with a motor-driven pestle, radical clearance 0.15 mm, 20 passes at 500 rpm. The homogenate was then filtered through nylon bolting cloth (159 μm mesh). The filtrate (taken as the total homogenate) was then centrifuged at 500 x g for 10 min. to remove heavily pigmented eye material. The supernatant (S_1) was carefully removed using a pasteur pipette and centrifuged at 100,000 x g for 30 min. in a Beckman L5-50B ultracentrifuge, using the SW 50.1 rotor (av. radius = 8.35 cm). The pellet (P_2) was resuspended in buffer A (~400 mg starting material (100 heads)/8 ml). All fractions were assayed for [^{125}I]-α-bungarotoxin binding activity and for protein content on the day of

Figure 18

TISSUE FRACTIONATION SCHEME FOR LOCUST

SUPRA - OESOPHAGEAL GANGLIA

100 heads 400 mg ganglia

Homogenised in 8 ml buffer A (5% w/v)

Filtered through nylon mesh (159 μm)

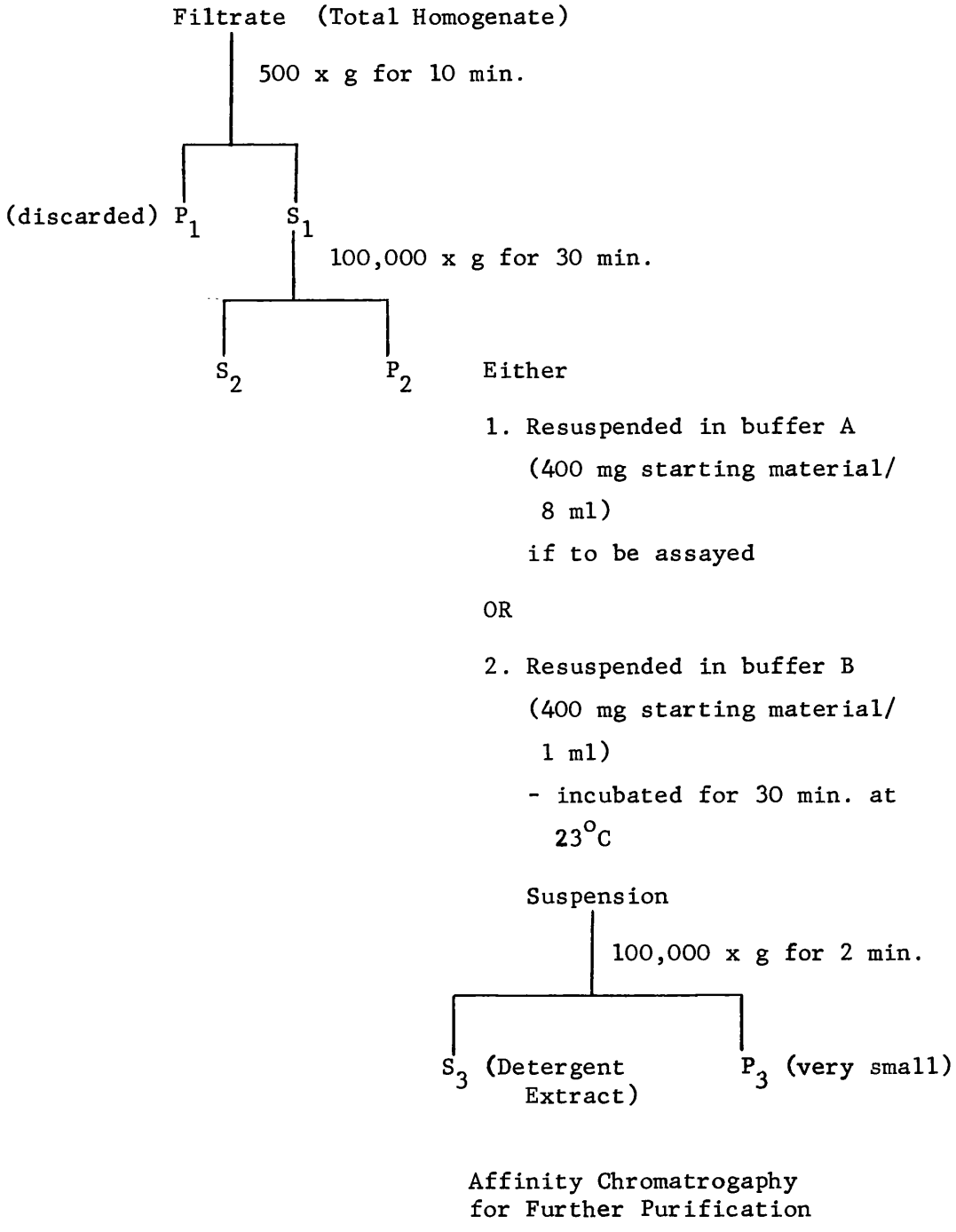


Table 22 Composition of the Buffers used in the Fractionation of Locust Supra-oesophageal Ganglia

Buffer A:

10 mM	Potassium phosphate	
	pH 7.4, containing	
0.25 M	Sucrose	
1 mM	EDTA	
2 mM	Benzamidine hydrochloride	Anti-Proteases
0.1 mM	Benzethonium chloride	
0.1 mM	Phenylmethane sulphonyl fluoride (PMSF)	

Buffer B:

10 mM	Potassium phosphate
	pH 7.4, containing
0.14 M	NaCl
1% (v/v)	Triton X-100 (unless stated otherwise)

The above anti-proteases

preparation.

b) Detergent Solubilisation of the [^{125}I]-α-Bungarotoxin Binding Component from the P_2 Pellet Fraction:

The P_2 pellet fraction was resuspended in buffer B (Table 22) (~400 mg starting material/ml), incubated for 30 min. at 23°C and then centrifuged at 100,000 x g for 2 min. in a Beckman Airfuge. The supernatant (S_3) was retained for assay or used in the further purification of the α-bungaortoxin binding component.

c) Partial Purification of the α-Bungarotoxin Binding Component:

(i) Preparation of the Affinity Beads:

α-Bungarotoxin was coupled to Sepharose 4B following the method of March et al.(1974).

Sepharose 4B (20 ml packed beads) was washed with 0.1 M NaCl (500 ml) and distilled water (250 ml). The beads were resuspended in cold water (20 ml total volume) and 2 M sodium carbonate (40 ml added). The solution was stirred at 4°C. α-Bungarotoxin (2 mg) was dissolved in 0.2 M sodium hydrogen carbonate (40 µl) pH 9.4. Cyanogen bromide (3 g) dissolved in acetonitrile (1 ml) was added to the Sepharose 4B suspension and stirred for 2 min. at 4°C. The mixture was rapidly filtered, washed with ice cold water (200 ml) and the Sepharose 4B beads added to the α-bungarotoxin solution and stirred for 21 h. at 4°C. The beads were washed with water (160 ml) and resuspended in 1 M glycine, pH 9.0 (80 ml). The affinity beads were collected by filtration and washed alternately with 0.1 M acetate buffer, pH 4.0, containing 1 M NaCl (100 ml) and 0.1 M borate buffer, pH 8.0, containing 1 M NaCl (100 ml). This process was repeated three times and the affinity beads were

equilibrated with 0.01 M potassium phosphate buffer, pH 7.4, containing 0.1% (v/v) Triton X-100. The beads were stored at 4°C in the presence of 0.02% (w/v) NaN_3.

(ii) The Purification Procedure:

The detergent extract (S_3) (400 mg starting material/ml) (1 ml) was mixed with 1 ml of toxin-Sepharose beads (α-bungarotoxin coupled to Sepharose 4B cyanogen bromide-activated affinity beads, Methods 2 (c) (i) p.125) and incubated for 90 min. at 23°C with occasional shaking. The affinity beads were then packed into a small column (2 x 1 cm) and non-bound material removed as follows: the column was washed with 4 ml buffer A (Table 22) containing 0.1% (v/v) Triton X-100; this was followed by the same buffer containing 1 M NaCl (4 ml); and finally by 4 ml of the former buffer. The α-bungarotoxin binding component was eluted with buffer A (Table 22) containing 0.1% Triton X-100 (v/v) and 1 M acetylcholine and the elutant was dialysed for 16 h. against 4 x 2 l of 10 mM-phosphate buffer, pH 7.4, containing 0.1% (v/v) Triton X-100 and potassium azide (0.02% w/v). The non-diffusible material and the wash fractions were assayed for α-bungarotoxin binding activity (Methods 4, p.127). The toxin beads were regenerated for re-use by washing with 10 mM-phosphate buffer, pH 7.4, containing 1 M NaCl followed by 10 mM-phosphate buffer, pH 7.4 containing 1% (v/v) Triton X-100 (5 vols. each).

3. The Preparation of [^{125}I]-α-Bungarotoxin

α-Bungarotoxin was labelled with ^{125}I by the method of Urbaniak et al. (1973).

Carrier-free Na ^{125}I (100 m Ci/ml) in dilute NaOH solution (10 µl) was added to α-bungarotoxin (10 µg) dissolved in 0.05 M

potassium phosphate, pH 7.5 (20 µl). Then, 0.5% (w/v) chloramine T in 0.05 M potassium phosphate buffer, pH 7.5, (10 µl) and 0.05 M postassium phosphate buffer, pH 7.5 (10 µl) were added. The mixture was stirred for 1 min. at 23°C after which 0.016% (w/v) sodium metabisulphite in 0.05 M potassium phosphate buffer, pH 7.5, (750 µl) and 1% (w/v) potassium iodide in 0.05 M potassium phosphate buffer, pH 7.5,(200 µl) were added. [^{125}I]-α-Bungarotoxin was separrated from free ^{125}I by passage down a Sephadex G25 column (25 cm x 1 cm) equilibrated with 0.01 M potassium phosphate buffer, pH 7.4, containing 1% (w/v) bovine serum albumin and eluted with the same buffer. Fractions (1 ml) were collected and samples (10 µl) counted in an LKB 1280 Ultrogamma counter. Peak tubes were pooled and the specific activity of the [^{125}I]-α- bungarotoxin was calculated, assuming 100% recovery of protein. [^{125}I]-toxin was stored at 4°C in elution buffer for up to 4 weeks. The biological activity of the labelled α-bungarotoxin was determined by measuring the amount of [^{125}I]-α-bungarotoxin bound to an excess of purified acetylcholine receptor from Torpedo marmorata, prepared by Dr. S. Wonnacott. The method of assay was as described in the methods (p.127). Biological activity is defined as:-

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

The biological activity of the [^{125}I]-α-bungarotoxin prepared and used in all experiments ranged from 50 - 60% with no loss of activity on storage for periods of up to 4 weeks at 4°C.

4. Assay to Determine the Specific Binding of [^{125}I]-α-Bungarotoxin

Samples (85 µl; 30 - 200 µg protein for the TH, P_2 and S_3 fractions; 60 - 100 ng for the partially purified binding component)

were incubated for 90 min. at 23°C in the presence of [^{125}I]-α-bungarotoxin (0.2 - 600 nM) in buffer A for the TH and P_2 fractions and buffer B (Table 22) for the S_3 and partially purified binding component (total volume 150 µl). Non-specific binding was taken as the amount of [^{125}I]-α-bungarotoxin bound in the presence of an excess of d-tubocurarine (10^{-3} M). Results were expressed as pmol [^{125}I]-α-bungarotoxin bound/mg protein.

Bound [^{125}I]-α-bungarotoxin was separated from free toxin by one of the following four methods:

a) The Centrifugation Assay:

For particulate fractions separation was achieved by centrifugation at 100,000 x g for 1 min. in a Beckman Airfuge. The pellets were superficially washed with 2 x 200 µl of buffer A and counted. Blanks contained buffer only.

b) The Ammonium Sulphate Precipitation Assay:

For detergent extracts and for soluble fractions, an ammonium-sulphate precipitation assay, adapted from the method of Meunier et al. (1972) was used. Saturated ammonium sulphate was added after incubation to give a final concentration of 30% (v/v) (75 µl saturated solution/150 µl incubation mixture). The samples were left for 16 h. at 4°C and then filtered on Whatmann GF/C filter discs, presoaked for 15 min. in 1% (w/v) bovine serum albumin. The filter discs were washed with 30% (v/v) ammonium sulphate solution (3 ml) and counted. Blanks contained buffer only.

c) The DEAE Cellulose Filtration Assay:

For the partially purified α-bungarotoxin binding component filtration on DEAE cellulose discs adapted from the method of Schmidt and Raftery (1973) was used. The reaction was terminated

after incubation by vacuum filtration through two DE 81 cellulose filter discs (2 cm diameter). The discs were washed with 10 mM potassium phosphate buffer, pH 7.4, containing 1% (v/v) Triton X-100 and 0.1% (w/v) bovine serum albumin (5 ml). Filtration of the sample and the wash was complete in less than 5 sec. Blanks contained buffer only. The washed discs were then counted.

d) The GF/C Filtration Assay:

In some instances, for the P_2 fraction, filtration on Whatmann GF/C filters discs was used. After incubations the samples were vacuum filtered through GF/C filter discs (2 cm diameter), presoaked for 15 min. in 1% (w/v) bovine serum albumin. The filter discs were washed with buffer A (3 ml) and counted. Blanks contained buffer only.

5. Determination of the Dissociation Rate Constant:

Samples, either the resuspended P_2 particulate fraction (~500 µg/ml) or the partially purified α-bungarotoxin binding component (~400 ng/ml), were incubated in the presence of 3 nM [^{125}I]-α-bungarotoxin for 90 min. at 23°C in buffer A for the P_2 fraction and buffer B without the 0.14 M NaCl, for the partially purified binding component. After incubation a 1000 fold excess unlabelled α-bungarotoxin was added. Samples (150 µl) were taken at intervals up to 180 min. and the amount of [^{125}I]-α-bungarotoxin remaining specificially bound was measured. Bound [^{125}I]-α-bungarotoxin was separated from free by the centrifugation assay (Methods, 4 (a) p.128) for the P_2 fraction and by the DEAE cellulose filtration assay (Methods 4 (c) p.128) for the partially purified binding component. Non-specific [^{125}I]-α-bungarotoxin binding (i.e. in the presence of 10^{-3} M d-tubocurarine) and blanks (buffer only) were

measured for every time point. Results were expressed as percentage [^{125}I]-α-bungarotoxin specifically bound after the addition of the excess unlabelled α-bungarotoxin. 100% binding was taken as that specifically bound before the addition of unlabelled toxin, taking into account the volume dilution by the unlabelled toxin.

6. Determination of IC_{50} Values for the Binding of Various Ligands to the P_2 Pellet Fraction:

Samples (~50 μg protein) were incubated with [^{125}I]-α-bungarotoxin (2.0 nM) (final volume 150 μl) for 90 min. at 23°C in the presence of a range of concentrations (10^{-3} M - 10^{-9} M) of various ligands. Bound [^{125}I]-α-bungarotoxin was separated from free [^{125}I]-α-bungarotoxin by the centrifugation assay (Methods 4 (a) p.127). Non-specific binding was taken as the amount bound in the presence of 10^{-3} M d-tubocurarine. Results were expressed as the percentage of α-bungarotoxin remaining specifically bound at that particular ligand concentration. 100% binding was taken as the amount specifically bound in the absence of ligand. Measurements using acetylcholine were carried out in the presence of neostigmine (10^{-4} M).

7. Sedimentation in a Sucrose Density Gradient:

Continuous sucrose density gradients were prepared by making five step discontinuous gradients with 4%, 8%, 12%, 16% and 20% (w/v) sucrose in buffer B without the 0.14 M NaCl (Table 22) and leaving them to stand overnight (~12 h.) at 23°C.

a) The Detergent Extract:

The detergent extract samples (100 μg protein) were prepared by incubation with [^{125}I]-α-bungarotoxin (3 nM) in buffer B (final

volume 100 µl) for 90 min. at 23°C. Controls were incubated in an excess of unlabelled α-bungarotoxin (1.25×10^{-4} M). After incubation the samples were mixed with the standard marker enzymes, β-galactosidase (E. C. 3. 1. 2. 1. 2. 3; $S_{20,W} = 16$; 140 µg/28 µl), catalase (E. C. 1. 11. 1. 6 ; $S_{20,W} = 11.4$; 800 µg/32 µl) and yeast alcohol dehydrogenase (E. C. 1. 1. 1. 1. ; $S_{20,W} = 7.4$; 400 µg/40 µl) and the mixture (160 µl) was layered carefully on the gradients. Velocity sedimentation was performed in the SW : 50.1 rotor of a Beckman L5-65 ultracentrifuge and runs were carried out at 100,000 x g (av.rad.= 8.35 cm) for 6 h. at 23°C. After centrifugation, the sucrose gradients were pumped from the tube bottom with a Gilson peristaltic pump and 6-drop fractions (80 µl av.) were collected. Fractions were then counted in an LKB 1280 ultrogamma counter and assayed for marker enzyme activity (Bergmeyer, 1974).

b) The Partially Purified α-Bungarotoxin Binding Component:

The partially purified α-bungarotoxin binding component sample (200 µl) was carefully layered on the sucrose gradient. The marker enzymes (same concentrations as Methods 7(a)) were layered on another sucrose gradient. Velocity sedimentation was performed simultaneously for the sample and the marker enzymes, as described in Methods 7(a) (p.130). After centrifugation, the sucrose gradients were pumped from the tube bottom with a Gilson peristaltic pump and 6-drop fractions (80 µl av.) were collected. For the gradients containing the samples, all fractions were assayed for α-bungarotoxin binding activity and bound [^{125}I]-α-bungarotoxin separated from free by filtration through two DE 81 DEAE cellulose filters (Methods 4(c) p.128). Non-specific binding was accounted for by

assaying all fractions from a duplicate gradient in the presence of 10^{-3} M d-tubocurarine. Gradients containing the marker enzymes were assayed for enzyme activity (Bergmeyer, 1974).

8. 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 placed in a boiling water bath for 2 min. in 0.01 M potassium phosphate buffer, pH 7.4, containing 5% (w/v) sodium dodecyl sulphate and 2.5% (v/v) β-mercaptoethanol. A list of samples applied is given below and all contained 0.02% (w/v) bromophenol blue and 10% (w/v) sucrose:-

1. Molecular weight standards (10 µl of 1 mg/ml)
2. Membrane fragments (P_2) labelled with [^{3}H]-MBTA (100 µl)
3. Partially purified α-bungarotoxin binding component (20 µg protein/100 µl)

Electrophoresis was carried out in disc gels (8 cm x 0.5 cm) using 8% (w/v) acrylamide and 6 mA/gel at an operating voltage of 30 V for 5 h. at 23°C. Gels that were run with [^{3}H]-MBTA labelled membranes were frozen immediately (unstained), at -20°C, sliced at 1 mm intervals, placed in a scintillation cocktail (Methods 10 p.133) and left for 48 h. to solubilise. Gels that were run with the other samples 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 (approx. 14 h.). They were destained with 5% (v/v) methanol 17% (v/v) acetic acid by heating in a boiling water bath and scanned densitometrically at 620 nm. The molecular weight

standards used were: human I g M μ chain (74,000); human I g M γ chain (50,000); human immunoglobulin light chain (23,500); and ribonuclease A (13,700).

Relative mobilities of the stained protein bands were calculated from the following relationship

$$\text{Relative Mobility} = \frac{\text{Distance of Protein Migration}}{\text{Length of gel after staining}} \times \frac{\text{Length of gel before staining}}{\text{Distance of bromophenol blue migration}}$$

In the case of the unstained gels of labelled samples

$$\text{Relative Mobility} = \frac{\text{Distance of labelled peak migration}}{\text{Distance of bromophenol blue migration}}$$

9. The Preparation of the Partially Purified α-Bungarotoxin Binding Component for SDS-PAGE:

The partially purified α-bungarotoxin binding component eluted from the affinity column was dialysed against 4 x 2 l distilled water. The non-diffusible fraction (3 ml; ~30 μg protein) was lyophilised on an Edwards, Modulyo freeze-drier and the residue was taken up in 100 μl of 0.01 M potassium phosphate buffer, pH 7.4, containing 5% (w/v) sodium dodecyl sulphate and 2.5% (v/v) β-mercaptoethanol. The samples were then placed in a boiling water bath for 2 min., mixed with 10 μl bromophenol blue (final concentration 0.02% w/v) and sucrose (final concentration 10% w/v) and subjected to SDS-PAGE as described in Methods 8 (p.132).

10. Affinity Labelling of the P_2 Pellet Fraction:

The labelling procedure followed the method of Barrantes et al., (1975). The affinity ligand 4-(N-maleimido)-benzoyltrimethylammonium iodide (MBTA) was synthesised with a tritium label by Dr. R. Harrison

by the method of Karlin et al., (1971) to a specific activity of 126 μCi/ mol MBTA.

The membranes were reduced by resuspending the P_2 pellet fraction (~2 mg protein) in 0.5 ml of 1 mM dithiothreitol in 0.01 M Tris-HCl buffer, pH 7.3, containing 0.1 M NaCl, 1 mM EDTA and left at 23°C for 10 min. The samples were then centrifuged at 100,000 x g for 2 min. in a Beckman Airfuge. The pellets were resuspended in 0.01 M potassium phosphate buffer (pH 7.4, containing 0.15 M NaCl and 1 mM EDTA (total vol. 0.5 ml) and recentrifuged at 100,000 x g. Again the pellets were resuspended and α-bungarotoxin (1.25×10^{-4} M final concentration) was added to the control sample. The membranes were left at 23°C for 1 h. [^{3}H]-MBTA (1 nmol) dissolved in 0.1 mM HCl (25 μl) was added to each membrane sample and incubated at 23°C for 2 min. Excess β-mercaptoethanol (10 μl) was added and the membranes were centrifuged at 100,000 x g for 2 min. The pellets were dissolved in 0.01 M potassium phosphate buffer, pH 7.4, containing 5% (w/v) sodium dodecyl sulphate and 5% (v/v) β-mercaptoethanol and boiled for 5 min. Each sample was then subjected to polyacrylamide gel electrophoresis under denaturing conditions (Methods 8 p.132). After electrophoresis, the gels were frozen at -20°C for 30 min., sliced at 1 mm intervals and each slice was incubated for 48 h. at 23°C with a solubilisation/scintillation cocktail which contained toluene (1 l), hyamine hydroxide (10 ml), soluene 350 (10 ml), 2,5-diphenyloxazole (6 g), (Aloyo, 1979). The samples were then counted in a Packard scintillation spectrometer.

11. Assay for Acetylcholinesterase:

Acetylcholinesterase activity was measured in all fractions

by the method of Ellman et al. (1961).

12. Protein Determination:

Protein determination was by the method of Lowry et al. (1951). When fractions contained Triton X-100, sodium dodecyl sulphate (5% w/v) was added to reagent A. Bovine serum albumin was used as the standard protein.

RESULTS

1. A Comparison of [^{125}I]-α- Bungarotoxin Binding to Fractions Prepared from Locust Supra-Oesophageal Ganglia:

Binding to the fractions, as prepared in Fig.18, was carried out as described in the Methods 4 (p.127). [^{125}I]-α-bungarotoxin was at a final concentration of 15 nM. Non-specific binding was accounted for by incubation in the presence of excess (1.25×10^{-5}M) unlabelled α-bungarotoxin. Bound [^{125}I]-α-bungarotoxin was separated from free toxin by the ammonium sulphate precipitation assay for the total homogenate and the S_1 fraction, by the centrifugation assay for the P_1 and P_2 fractions and by the DEAE cellulose filtration assay for the S_2 fraction (Methods 4 p.127).

Table 23 shows the non-specific, total and specific binding for these fractions. It also shows the purification factor, taking binding in the total homogenate as unity. As can be seen the greatest specific activity was in the P_2 fraction. 60-70% of total specific binding activity of the initial homogenate was also routinely recovered in this fraction. In all fractions except the P_2 fraction the non-specific binding exceeds the specific binding. Binding in the S_2 fraction was inconsistent and represented 0-10% of total specific binding activity of the initial homogenate. Although the specific binding in the P_1 fraction is high, binding was difficult to assay accurately, because of the very fibrous nature of this fraction.

As the P_2 fraction appeared to be the fraction with the highest specific binding and had a high recovery of the total specific binding activity, it was used in subsequent experiments. A value of 80-85 pmol [^{125}I]-α-bungarotoxin binding sites/g wet weight of whole ganglia was calculated from specific binding to the total homogenate before filtration through the nylon mesh.

TABLE 23

Comparison of the Binding of [^{125}I]-α-Bungarotoxin to Various Fractions

FRACTION	BINDING (p.mol/mg protein)			PURIFICATION FACTOR
	Non-Specific Binding	Total Binding	Specific Binding	
T.H.	1.07 ± 0.0125	1.303 ± 0.03	0.233 ± 0.330	1
P_1	3.395 ± 0.079	4.63 ± 0.160	1.235 ± 0.173	5.3
S_1	0.733	1.205	0.473	2.0
P_2	1.24 ± 0.078	2.59 ± 0.065	1.35 ± 0.102	5.8
S_2	1.18 ± 0.035	1.72 ± 0.060	0.54 ± 0.069	2.3

Fractions were prepared as in Fig. 18 . Samples (30 - 200 µg protein) were incubated at 23°C for 90 min in the presence of [^{125}I]-α-bungarotoxin 15 nM (Methods 4 p.127). Non-specific binding was in the presence of 1.25×10^{-4} M unlabelled α-bungarotoxin. Results are means of quadruplicates ± S.E. except for S_1 fraction - mean of duplicates.

TABLE 24

Comparison of the GF/C Filtration Assay and the Centrifugation Assay to Separate [^{125}I]-α-Bungarotoxin Bound to the Total Homogenate or the P_2 Pellet Fraction from Free Toxin; Effect on Specific Binding

FRACTION	ASSAY USED	SPECIFIC BINDING (pmol/mg protein)
TH	Centrifugation	0.229 ± 0.033
TH	Filtration	0.211 ± 0.015
P_2	Centrifugation	1.348 ± 0.102
P_2	Filtration	1.281 ± 0.14

Samples (30 - 200 µg protein) were incubated with [^{125}I]-α-bungarotoxin (15 nM) for 90 min at 23°C. Non-specific binding was in the presence of unlabelled α-bungarotoxin (1.25×10^{-4} M). Results are the means of two experiments, each carried out in triplicate ± S.E.

2. Experiments with the P_2 Pellet Fraction:

A. The Binding Assay:

(i) Comparison of the GF/C Filtration Assay and the Centrifugation Assay:

Binding was carried out as described in the Methods 4 (p. 127). Separation of bound from free [^{125}I]-α-bungarotoxin (15 nM) was by either the GF/C filtration assay (Methods 4 p. 127) or the centrifugation assay (Methods 4 (a) p. 127). Non-specific binding was accounted for by incubation in the presence of an excess of unlabelled α-bungarotoxin (1.25×10^{-4}M).

Table 24 shows specific [^{125}I]-α-bungarotoxin binding to both the total homogenate and to the P_2 fraction as measured by both the GF/C filtration assay and the centrifugation assay. As can be seen there is no difference in the specific [^{125}I] -α-bungarotoxin binding of the fractions assayed by the two methods.

The centrifugation assay was adopted as the routine method for separation of bound from free [^{125}I]-α-bungarotoxin for the P_2 fraction.

(ii) Comparison of [^{125}I]-α-Bungarotoxin Binding in the Presence of Excess Unlabelled α-Bungarotoxin and of Excess d-Tubocurarine to Account for Non-Specific Binding:

[^{125}I]-α-Bungarotoxin was at a final concentration of 15 nM, unlabelled α-bungarotoxin 1.25×10^{-4}M and d-tubocurarine 1×10^{-3}M.

As Table 25 shows there is little difference in the non-specific binding as accounted for by unlabelled α-bungarotoxin or by d-tubocurarine and hence no difference in the specific binding.

In subsequent experiments d-tubocurarine (10^{-3}M) was used routinely to account for non-specific binding.

TABLE 25

Comparison of the Binding of [^{125}I]-α-Bungarotoxin to the P_2 Fraction, in the Presence of Excess Unlabelled α-Bungarotoxin and of Excess d-Tubocurarine to Account for Non-Specific Binding.

UNLABELLED LIGAND USED	BINDING (pmol/mg protein)		
	Non-Specific Binding	Total Binding	Specific Binding
d-Tubocurarine	1.24 ± 0.078	2.59 ± 0.065	1.35 ± 0.102
α-Bungarotoxin	1.35 ± 0.009	2.80 ± 0.09	1.45 ± 0.09

Experiments were carried out as described in the Methods 4 (a) (p.128). [^{125}I]-α-bungarotoxin was at a concentration of 15 nM. Non-specific binding was in the presence of either unlabelled α-bungarotoxin (1.25×10^{-4}M) or d-tubocurarine (1×10^{-5}M). Results are the means of two experiments, each carried out in triplicate ± S.E.

TABLE 26

The Effect of Preincubation with Excess d-Tubocurarine on [^{125}I]-α-Bungarotoxin Binding to the P_2 Fraction.

TIME (min)	BINDING (pmol/mg Protein)		
	Non-Specific Binding	Total Binding	Specific Binding
5	3.60	7.25	3.65
10	3.57	7.37	3.80
15	3.75	7.72	3.97
20	3.80	7.47	3.67

Experiments were carried out as described in the Methods 4 (p.127). [^{125}I]-α-Bungarotoxin was at a concentration of 12 nM. Non-specific binding was in the presence of d-tubocurarine (1×10^{-3} M). Results are the means of quadruplicates S.E. < 2%.

TABLE 27

The Effect of Incubation Time on Specific [^{125}I] -α-Bungarotoxin Binding to the P_2 Fraction.

CONDITIONS	SPECIFIC BINDING (pmol/mg protein)
90 min at 23°C	2.68
16 hours at 4°C	3.12

Samples (∿200 µg protein) were incubated with [^{125}I] -α-bungarotoxin (16 nM) for either 90 min at 23°C or for 16 hours at 4°C. Binding was assayed as described in Methods 4 (a) (p.127). Results are the means of quadruplicates. S.E. < 2%.

(iii) The Effect of Preincubation with an Excess of d-Tubocurarine on [^{125}I]-α-Bungarotoxin Binding:

Table 26 shows the effect of preincubation with 10^{-3} d-tubocurarine for the times shown on non-specific and hence specific binding of [^{125}I]-α-bungarotoxin (16 nM) to the P_2 fraction. As can be seen there is little difference in either non-specific or specific binding for the preincubation times shown.

(iv) The Effect of Incubation Time on Specific [^{125}I]-α-Bungarotoxin Binding:

Samples of the P_2 fraction were incubated in the presence of 16 nM [^{125}I]-α-bungarotoxin for either 90 min at 23°C or for 16 hours at 4°C and Table 27 shows the specific binding for the two conditions. As can be seen there is little difference in the specific binding under the two sets of conditions. Incubation for 90 min at 23°C was routinely used in subsequent experiments.

B. The Effect of Varying Protein Concentration on [^{125}I]-α-Bungarotoxin Binding:

Fig. 19 (a) and (b) shows the effect of varying the protein concentration on the binding of two concentrations of [^{125}I]-α-bungarotoxin to the P_2 fraction. Fig. 19 (b) shows binding at an [^{125}I]-α-bungarotoxin concentration of 24 nM over a protein range of 10-30μg. Fig 19 (a) shows binding at a concentration of 16 nM [^{125}I]-α-bungarotoxin over a protein range of 50-200μg.

Specific binding is directly proportional to the amount of protein present but is lower than the non-specific binding. In the graphical presentations of these data total and non-specific binding do not pass through zero because of apparent binding to the centrifuge

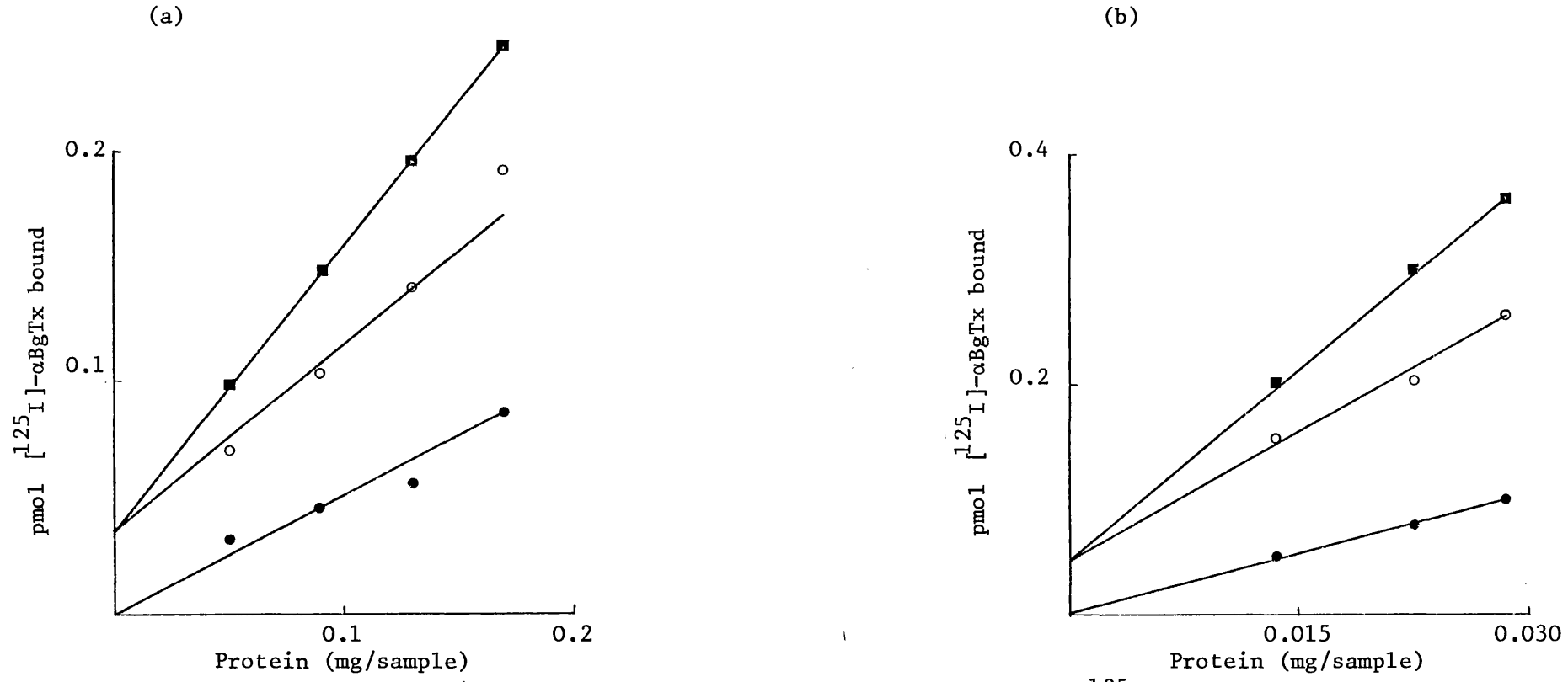


Fig. 19. The Effect of Varying the Protein Concentration on Binding of [^{125}I]-αBgTx Binding to the P_2 Membrane Fraction.

d-Tubocurarine 10^{-3}M; [^{125}I]-αBgTx (a) 16nM, (b) 24nM; ■ total binding; ○ non-specific binding; ● specific binding; bound [^{125}I]-αBgTx separated from free airfuge centrifugation. (Results are the means of 2 expt. each carried out in triplicate S.E. < 5%).

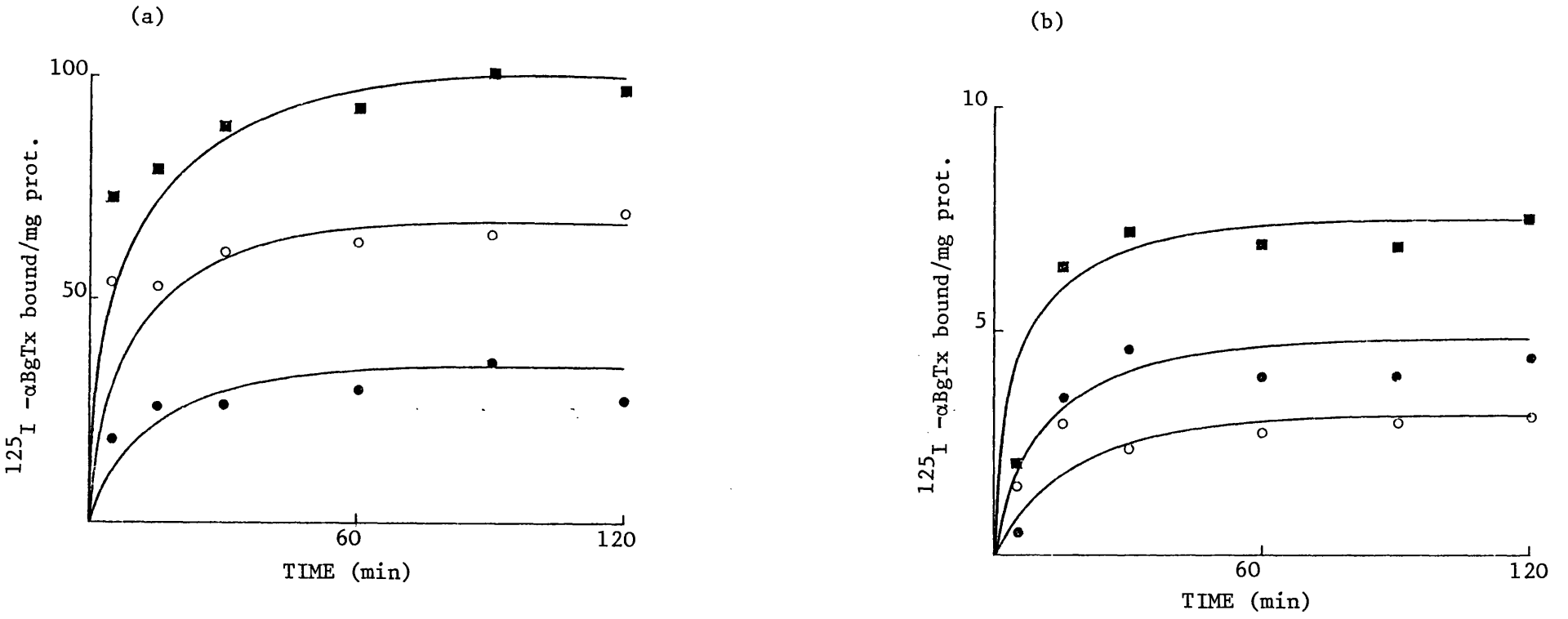


Fig. 20. Time Course of ^{125}I -αBgTx Binding to the P_2 Membrane Fraction.

d-Tubocurarine 10^{-3}M; ^{125}I -αBgTx (a) 600nM, (b) 12nM; bound ^{125}I -αBgTx was separated from free by airfuge centrifugation; ■ total binding; O non-specific binding; ● specific binding. (Results are the means of 2 expt. each in triplicate S.E. $<$ 5%).

tube and trapping of ligand in the "dead" space of the pellet. Standard error in both cases was less then 5%, determined from two separate series of experiments each carried out in triplicate.

C. Time Course of Specific [^{125}I]-α-Bungarotoxin Binding:

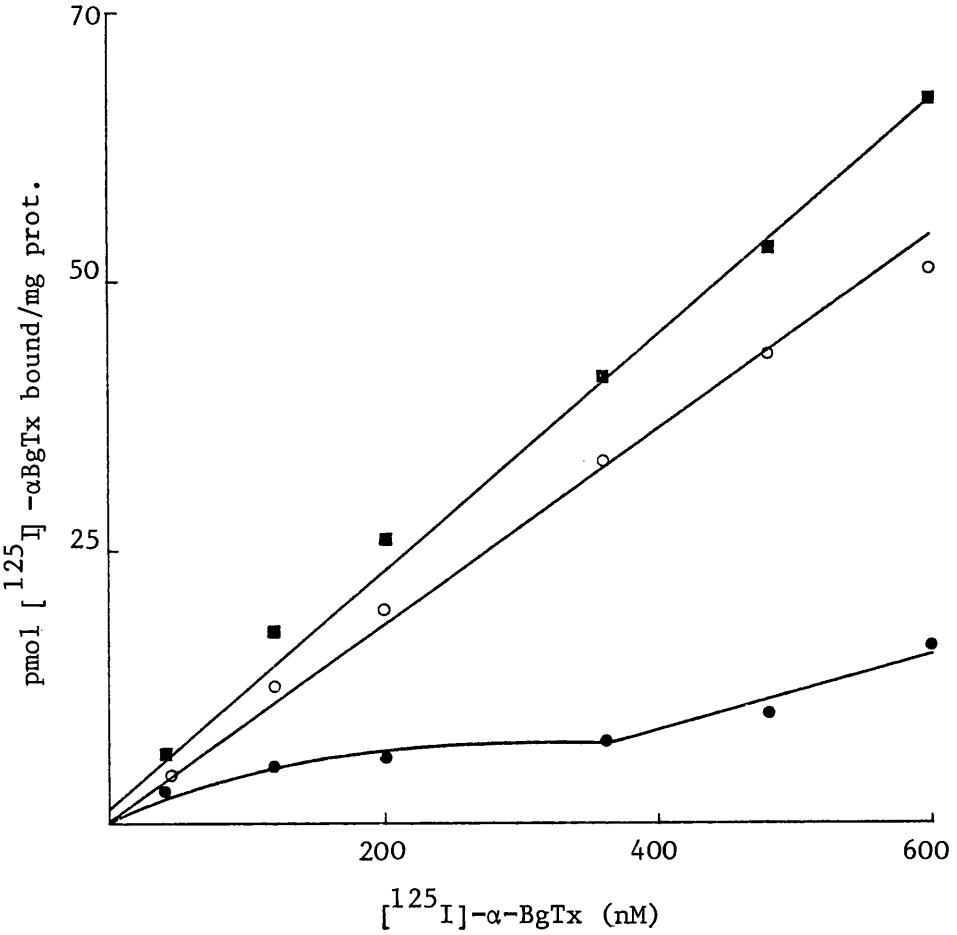
Incubation was at 23°C for up to 120 min. Incubation was terminated and bound [^{125}I]-α-bungarotoxin separated from free toxin by the centrifugation assay (Methods 4 (a) p.127). Two concentrations of [^{125}I]-α-bungarotoxin were used 12 nM (Fig. 20 (b)) and 600 nM (Fig. 20 (a)).

As can be seen from either graph, specific binding saturates with time and reaches equilibrium in 10-15 min. The standard error was less then 5% for two separate series of experiments each carried out in triplicate.

D. The Dependence of Binding of [^{125}I]-α-Bungarotoxin on Concentration (40-600 nM):

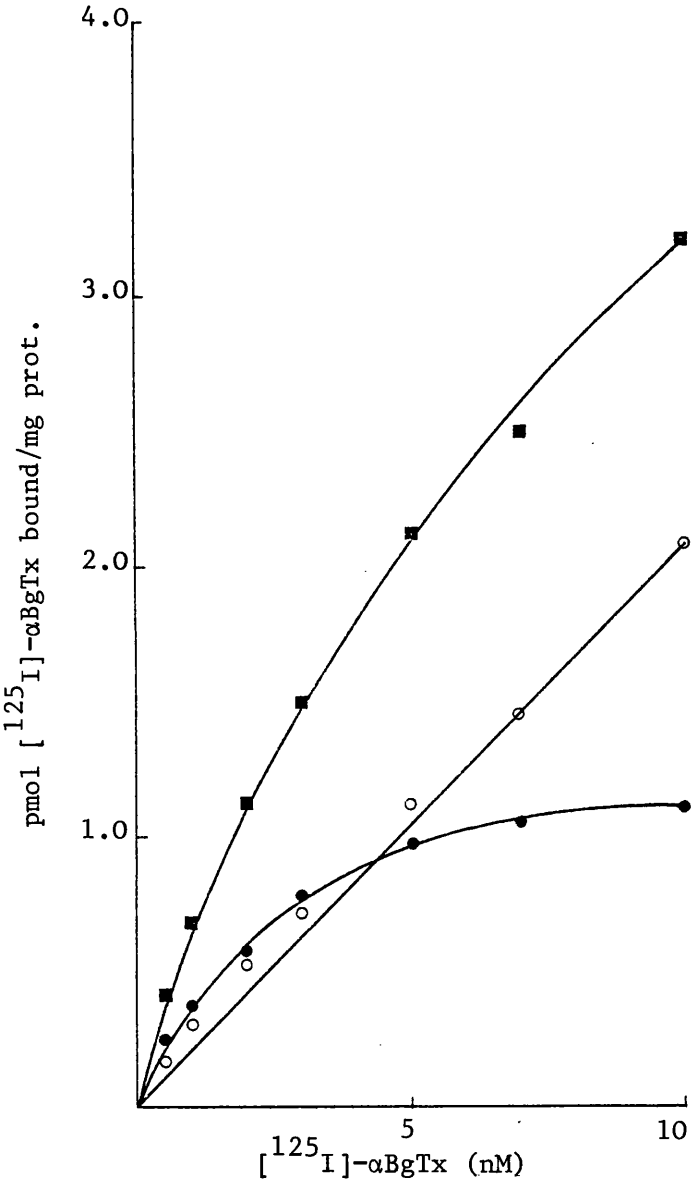
Experiments were carried out over a concentration range of 40 nM - 600 nM [^{125}I]-α-bungarotoxin. Fig. 21 is an example of a typical plot of pmol [^{125}I]-α-bungarotoxin bound/mg protein against concentration for total, non-specific and specific binding (1 expt. in triplicate, S.E. < 5%). Specific binding appears to be approaching saturation up to an [^{125}I]-α-bungarotoxin concentration of 360 nM but then doubles between this concentration and 600 nM. In other preparations specific binding was found to increase linearly within this concentration range of [^{125}I]-α-bungarotoxin and in all preparations specific binding was lower than non-specific binding.

Fig. 21. The Dependence of Binding of [^{125}I]-αBgTx Binding to the P_2 Membrane Fraction on Concentration (40nM - 600nM).



d-Tubocurarine 10^{-3}M; bound [^{125}I]-αBgTx was separated from free by airfuge centrifugation; ■ total binding; ○ non-specific binding; ● specific binding. (Results are the means of triplicates S.E. < 5%).

Fig. 22 (a). The Dependence of Binding of [^{125}I]-αBgTx to the P_2 Membrane Fraction on Concentration (0.5 - 10nM)



d-Tubocurarine 10^{-3}M; bound [^{125}I]-αBgTx was separated from free by airfuge centrifugation; ■ total binding; ○ non-specific binding; ● specific binding (Results are the means of triplicates S.E.< 4%).

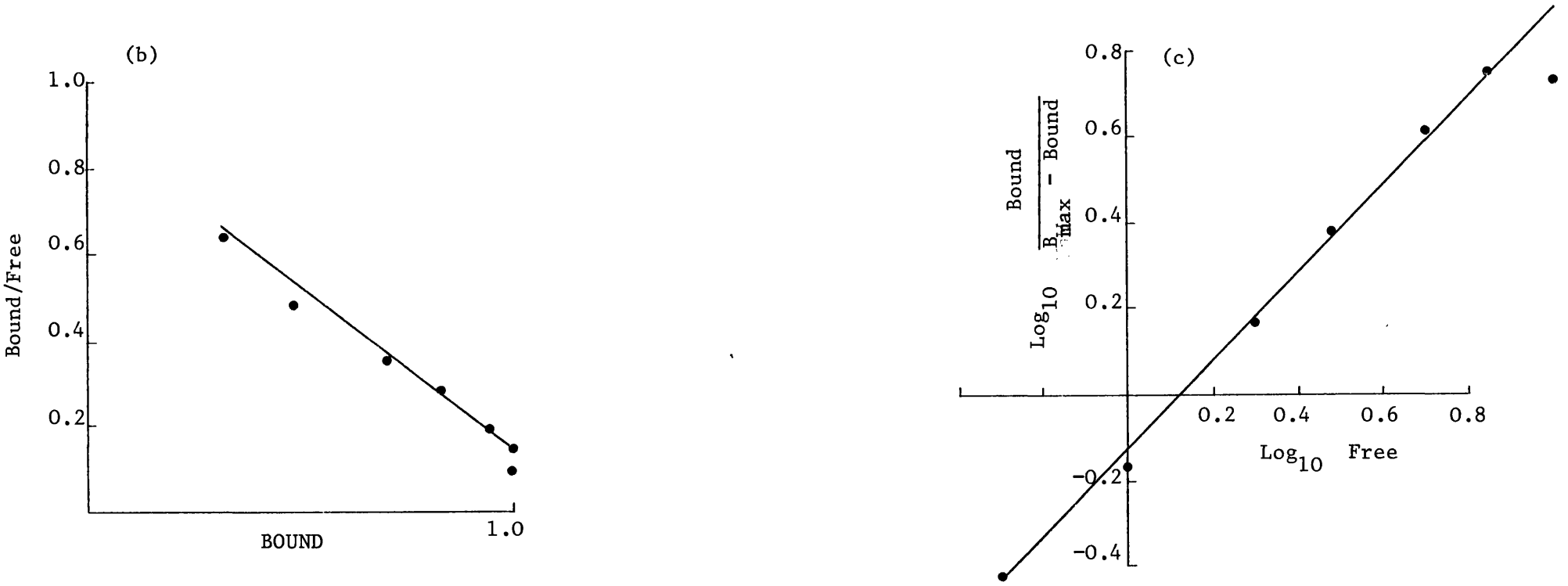


Fig. 22. (b) Scatchard Analysis of Specific [^{125}I]-αBgTx Binding to the P_2 Membrane Fragments (K_D = 1.38nM; B_{max} = 1.18 pmol/mg prot).

(c) Hill Plot of Specific [^{125}I]-αBgTx Binding to the P_2 Membrane Fragments (taking B_{max} as 1.18 pmol/mg prot)

(Results are the means of 3 expt. each in triplicate S.E. < 5%).

E. The Dependence of Binding of [^{125}I]-α-Bungarotoxin on Concentration (0.5 - 10 nM):

Samples were incubated with [^{125}I]-α-bungarotoxin over a concentration range of 0.5 - 10 nM. Fig. 22 (a) shows the binding profile for total, non-specific and specific binding for one experiment carried out in triplicate (S.E. < 4%). From this graph it can be seen that specific binding saturates in the region of 5 nM [^{125}I]-α-bungarotoxin.

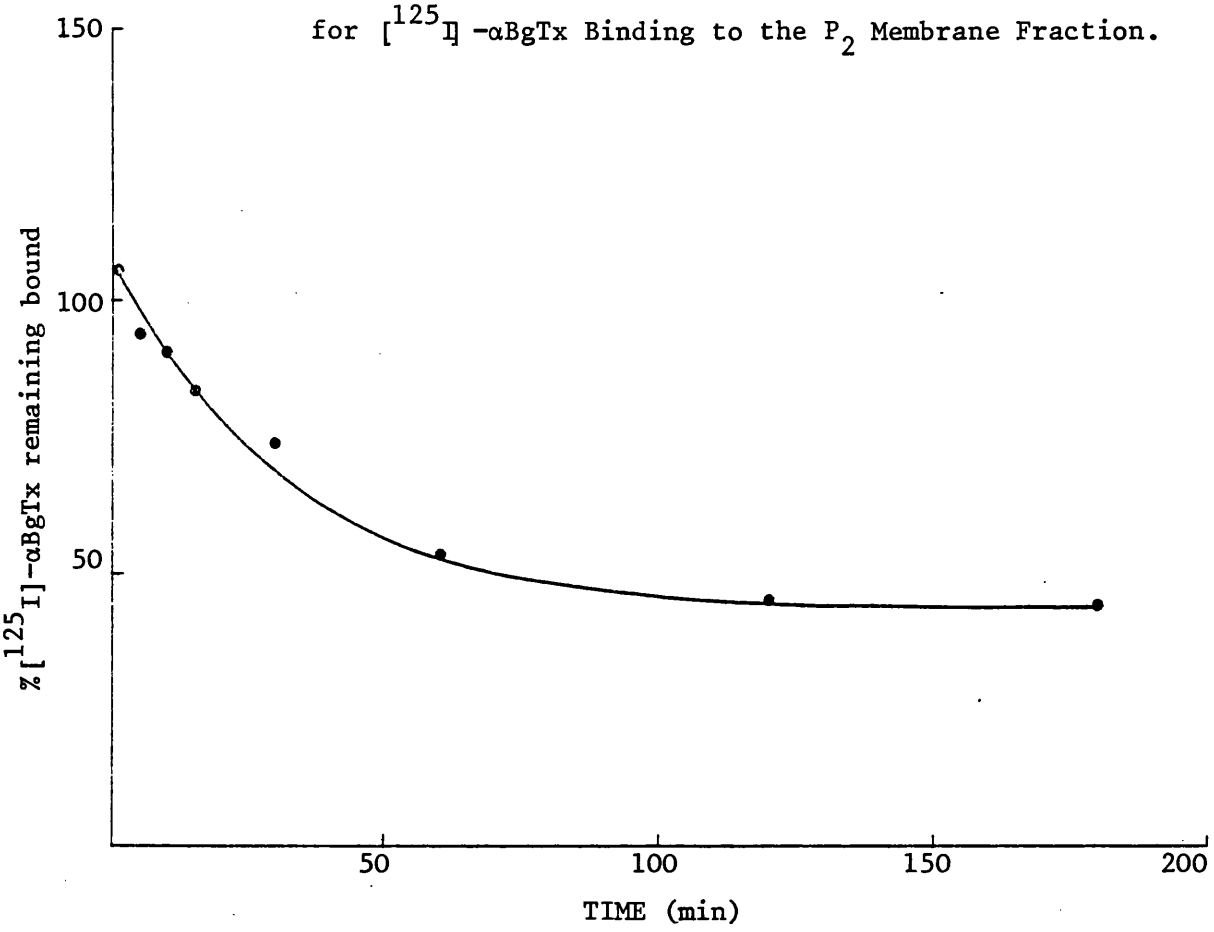
Fig. 22 (b) is a Scatchard Plot of the mean of three experiments each carried out in triplicate. From this plot a dissociation constant (K_d) of 1.38 nM and a maximum number of binding sites (B_{max}) of 1.18 pmol [^{125}I]-α-bungarotoxin bound/mg protein was obtained. The correlation coefficient (r) was 0.993.

Fig. 22 (c) is a Hill plot of the same data as Fig. 22(b). The B_{max} obtained from the Scatchard plot (B_{max} = 1.18 pmol/mg protein) was used in fitting the saturation data to the Hill equation. The points lie on a straight line (r = 0.991) with a slope of 0.97 and therefore the Hill number (n_H) is considered to be 1.0 and so specific [^{125}I]-α-bungarotoxin binding obeys classic mass action laws and no cooperativity is taking place.

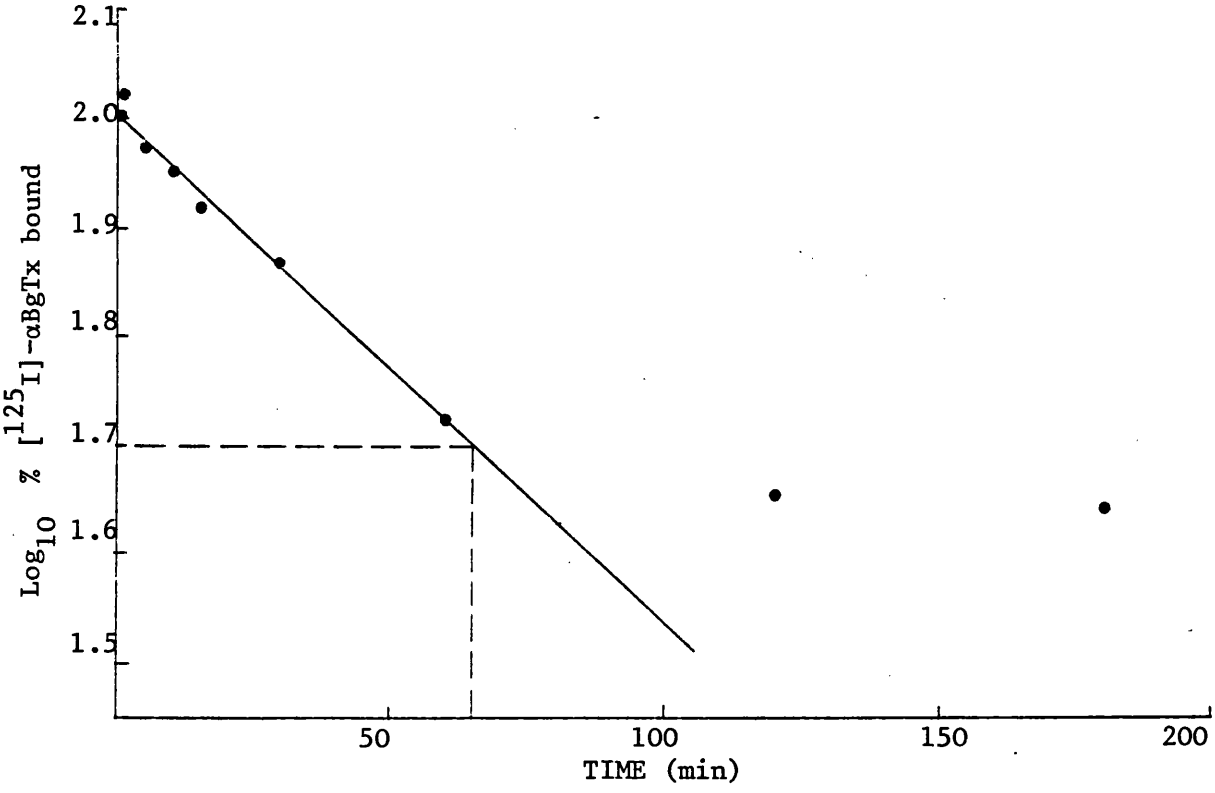
F. Determination of the Rate of Dissociation (k_{-1}) for [^{125}I]-α-Bungarotoxin Binding:

The experiment was carried out as outlined in the Methods 5 (p129). [^{125}I]-α-bungarotoxin was at a final concentration of 3 nM. Fig. 23 (a) shows a plot of % [^{125}I]-α-bungarotoxin specifically bound versus time, for one experiment carried out in duplicate. Fig. 23 (b) is a

Fig. 23 (a) Determination of the Rate of Dissociation (k_{-1}) for [^{125}I]-αBgTx Binding to the P_2 Membrane Fraction.



(b) Secondary Plot (of the above data)



d-Tubocurarine 10^{-3}M; [^{125}I]-αBgTx 3nM; bound [^{125}I]-αBgTx separated from free by airfuge centrifugation. Results are the means of triplicates).

plot of Log [% ^{125}I-α BT] bound versus time for the same experiment.

The dissociation followed first order kinetics until more than 50% of the [^{125}I]-α-bungarotoxin had dissociated (Fig.23 (b). From this a $t_{0.5}$ (time take for 50% of specifically bound [^{125}I] -α-bungarotoxin to be displaced) was calculated to be 65 min. Using the relationship

$$k_{-1} = \frac{0.693}{t_{\frac{1}{2}}}$$

k_{-1} was calculated as $1.7 \times 10^{-4} S^{-1}$. The experiment was repeated twice more and k_{-1}'s of $3.6 \times 10^{-4} S^{-1}$ and $3.04 \times 10^{-4} S^{-1}$ were obtained.

From the equation:

$$[R] + [L] \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} [RL]$$

Where [R] = the concentration of binding sites

[L] = the concentration of [^{125}I] -α-bungarotoxin

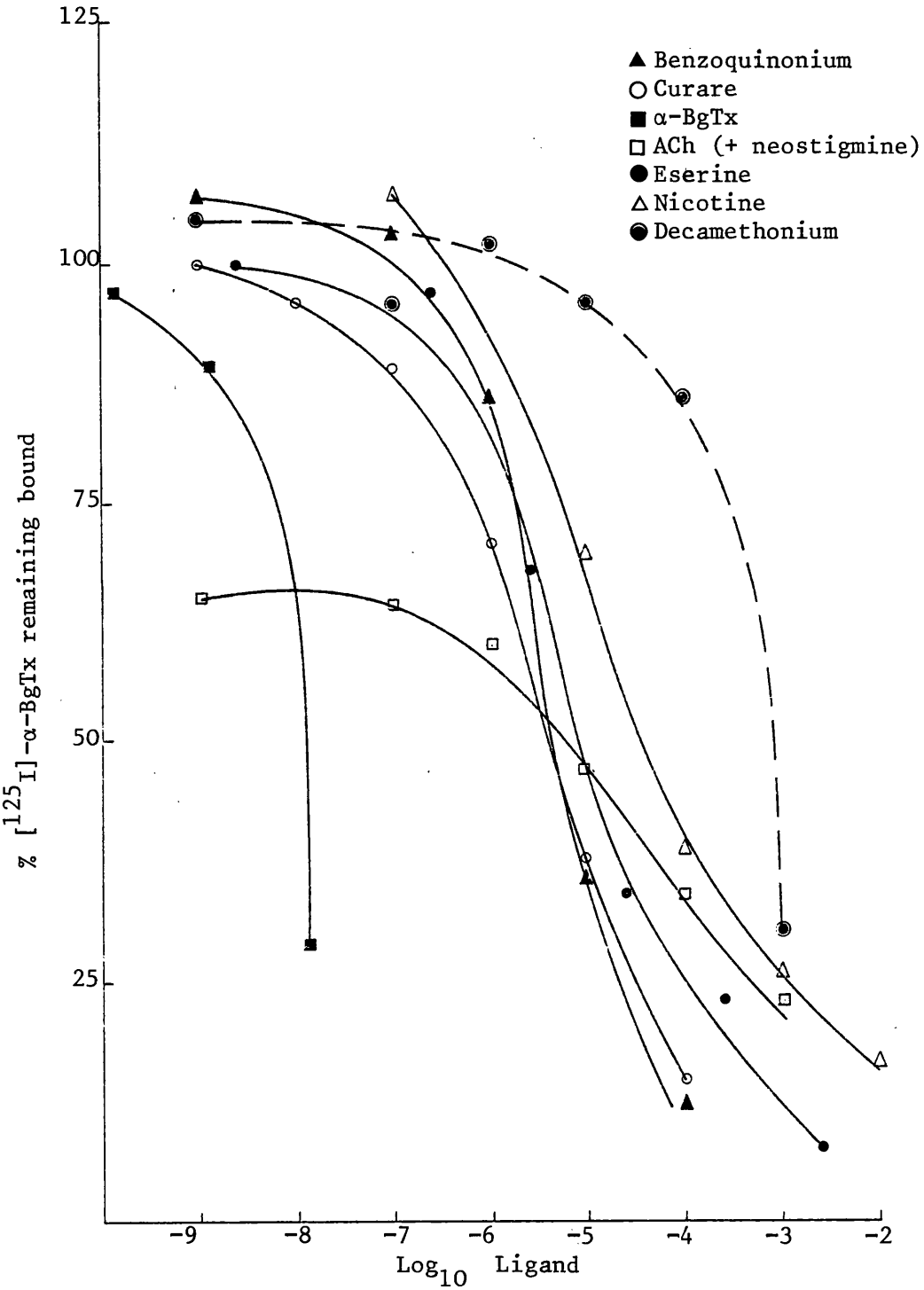
[RL] = the concentration of receptor-ligand complex

k_{+1} = the association rate constant

the intrinisic dissociation constant $K_D = \frac{k_{-1}}{k_{+1}}$

The on-rate (k_{+1}) for most neurotransmitter binding has been calculated to be of the order of 10^6 M^{-1} S^{-1}. Assuming this value applies to [^{125}I]-α-bungarotoxin binding to the P_2 fraction, and using the mean off-rate (k_{-1}) calculated from these experiments (2.78×10^{-4} S^{-1}), a K_D of 2.78×10^{-10} M was calculated; this is a factor of ten times smaller than the K_D obtained from equilibrium studies (1.38×10^{-9} M). The dissociation is biphasic; after dissociation of approximately 60%, the rate of dissociation in much slower, with only approximately 5% [^{125}I]-α-BT dissociating in the next 120 minutes.

Fig. 24. Determination of the IC_{50}'s for Various Ligands Displacing [^{125}I]-αBgTx Binding to the P_2 Membrane Fraction.



d-Tubocurarine 10^{-3}M; [^{125}I] -αBgTx 2nM; binding sites 0.4nM; bound [^{125}I]-αBgTx separated from free by airfuge centrifugation. (Results are the means of 2 expt. each carried out in triplicate S.E. <4%).

TABLE 28

IC_{50} Values of Various Ligands for Inhibition of Specific [^{125}I]-α-Bungarotoxin Binding to the P_2 Fraction.

LIGAND	IC_{50} (M)
α-Bungarotoxin	8.9×10^{-9}
d-Tubocurarine	3.2×10^{-6}
Benzoquinonium	4.11×10^{-6}
Eserine	4.73×10^{-6}
* Acetylcholine	6.68×10^{-6}
Nicotine	3.98×10^{-5}
Atropine	1.12×10^{-4}
Dexetimide	3.50×10^{-4}
Carbamoylcholine	4.47×10^{-4}
Decamethonium	6.31×10^{-4}
Hexamethonium	$> 10^{-3}$
Neostigmine	$> 10^{-3}$

* Acetylcholine was assayed in the presence of 10^{-3} M neostigmine.

The experiment was carried out as described in the Methods 6 (p.130). [^{125}I]-α-Bungarotoxin was at a concentration of 0.2 nM. Results are the means of two experiments each carried out in triplicate. S.E.< 4%.

G. Determination of the IC_{50} Values of Various Ligands Inhibiting [^{125}I]-α-Bungarotoxin Binding:

The IC_{50} for a given unlabelled ligand is defined as that concentration of unlabelled ligand at which maximum specific binding of labelled ligand is displaced by 50%. Under certain conditions and allowing for a number of assumptions (Discussion p.164) the IC_{50} for a particular ligand very closely approximates to the equilibrium dissociation constant for binding(K_i) for that ligand.

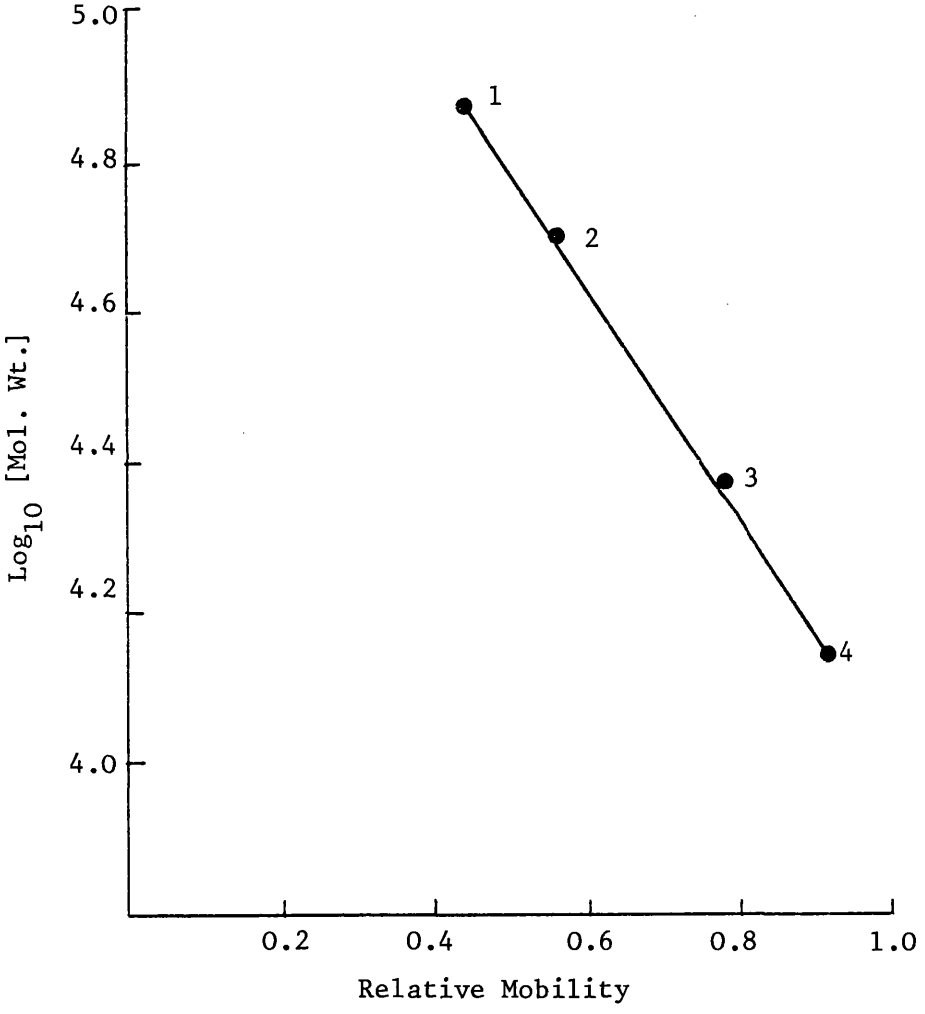
In these experiments the concentration of [^{125}I]-α-bungarotoxin used was 2.0 nM and the concentration of protein was ∿ 50µg/150 µl (taking B_{max} as 1.18 pmol [^{125}I]-α-bungarotoxin binding sites/mg protein, 50µg protein/150µl ≡ 0.4 nM of [^{125}I]-α-bungarotoxin binding sites). Fig.24 shows the displacement of [^{125}I]-α-bungarotoxin by different ligands. Table 28is a summary of the IC_{50} values and hence under the above conditions (Discussion p.164)∿the K_i's of the ligands.

H. [^{3}H]-MBTA Labelling of the Membrane Fragments:

Membranes were prepared and labelled with [^{3}H]-MBTA as outlined in the Methods ↄO (p.133). Control samples were preincubated with excess (1.25×10^{-4}M) unlabelled α-bungarotoxin before the labelling procedure. Molecular weight markers were run on a separate disc gel simultaneously, the calibration line for which is represented in Fig.25 .

Only one major peak of [^{3}H]-MBTA label was obtained after counting (Fig.26) and this corresponded to a molecular weight of 58,000. This peak of radioactivity was not apparent on the control sample.

Fig. 25 Calibration Curve of Standards for SDS-PAGE (^{3}H -MBTA).



1 = human IgM μ chain (74,000)

2 = human IgG γ chain (50,000)

3 = human immunoglobulin light chain (25,500)

4 = ribonuclease A (13,700)

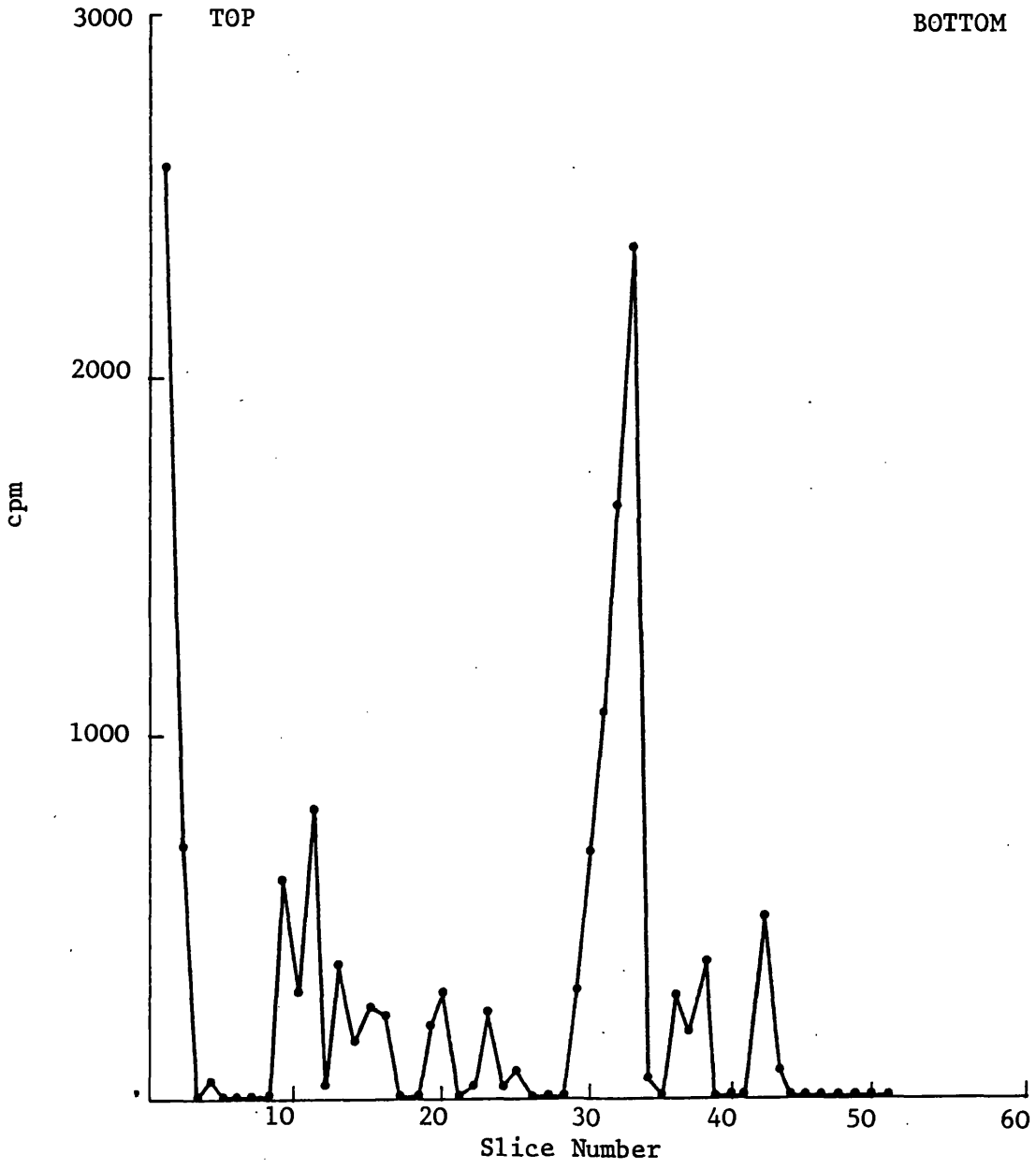


Fig. 26 [^{3}H]-MBTA Labelling of the P_2 Membrane Fragments

Distribution of Radioactivity after SDS-PAGE

3. Experiments on the Detergent Extract:

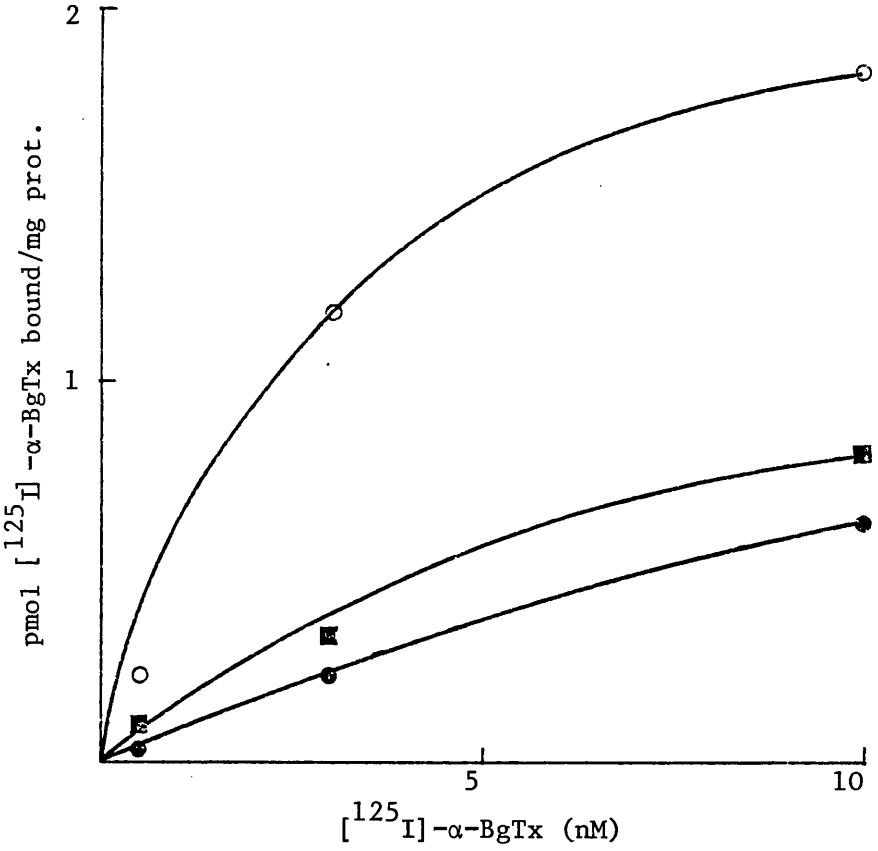
A. Comparison of Specific [^{125}I]-α-Bungarotoxin Binding to the P_2 Fraction Solubilised in Various Concentrations of Triton X-100:

The P_2 fraction was resuspended in buffer B (Table22) containing either 0.1%, 0.5% or 1% (v/v) Triton X-100 (Methods 2 (b) p.125). [^{125}I]-α-bungarotoxin binding was assayed at three concentrations, 0.5 nM, 3 nM and 10 nM. The ammonium sulphate precipitation assay (Methods 4 (b) p.128) was used to separate bound [^{125}I]-α-bungarotoxin from free toxin in this and all subsequent experiments on the detergent extract.

Fig. 27shows specific binding of [^{125}I]-α-bungarotoxin to the solubilised fraction, at the different concentrations of Triton X-100 used. As can be seen from this, more [^{125}I]-α-bungarotoxin binding protein was solubilised in buffer containing 1% Triton X-100 (at 10 nM [^{125}I]-α-BT, 1.82 pmol bound/mg prot.) than either 0.1% or 0.5% (v/v) Triton X-100 (at 10 nM [^{125}I]-α-BT, 0.815 and 0.655 pmol bound/mg protein, respectively). The experiment was carried out in quadruplicate (S.E. < 3%). For subsequent experiments buffer B containing 1% (v/v) Triton X-100 was used and the solubilised P_2 fraction will henceforth be called the "detergent extract".

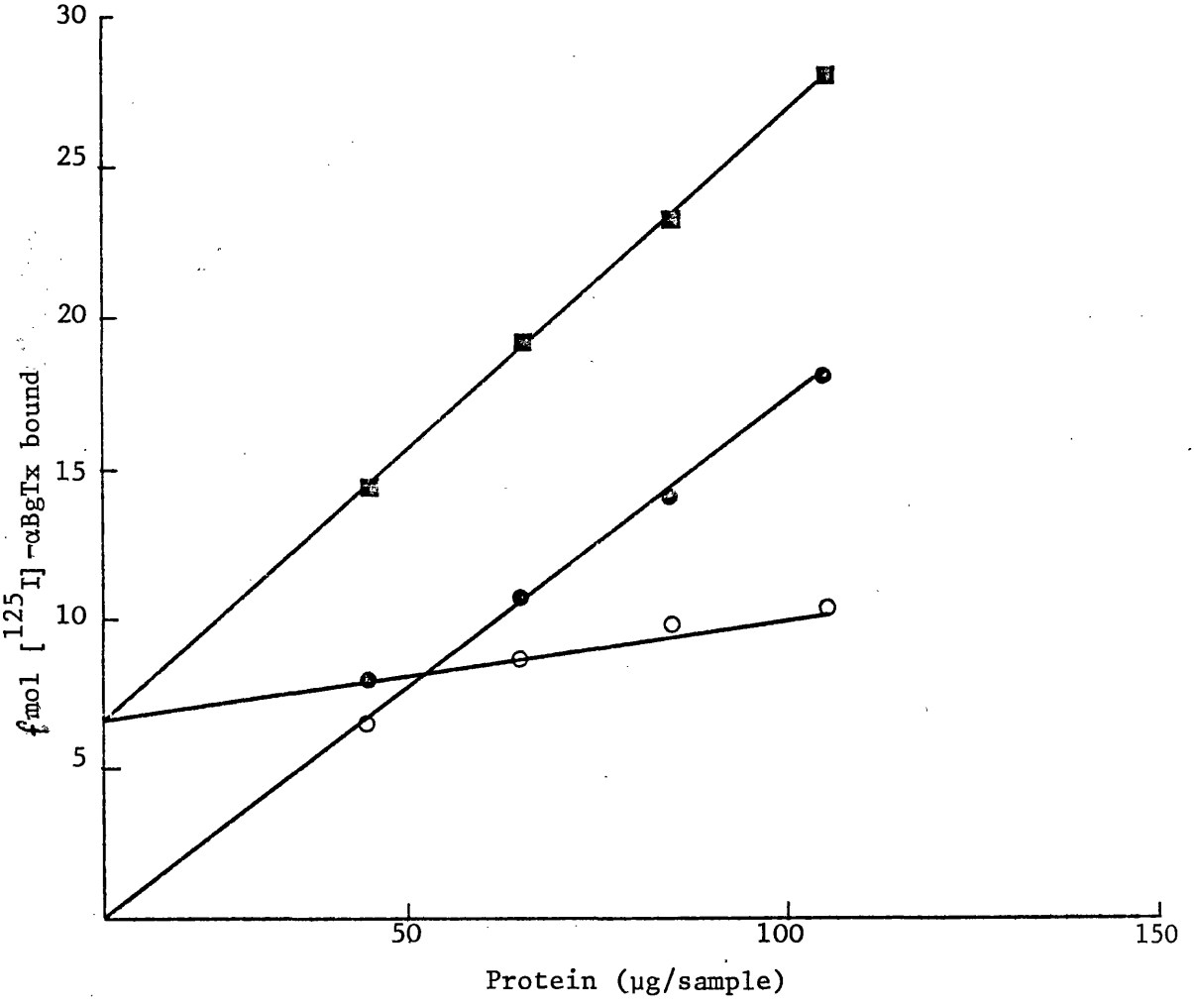
After solubilisation 90% of the [^{125}I]-α-bungarotoxin binding activity in the P_2 fraction was routinely recovered in either a 100,000 x g or 200,000 x g supernatant. This is equivalent to 50-60% recovery of the total specific [^{125}I]-α-bungarotoxin binding.

Fig. 27 Comparison of Specific [^{125}I]-αBgTx Binding to the P_2 Membrane Fraction Solubilised in Various Concentrations of Triton X-100.



d-Tubocurarine 10^{-3}M; bound [^{125}I]-αBgTx was separated from free by $(NH_4)_2 SO_4$ ppt. and filtration; ○ 1% (v/v) Triton; ● 0.5% (v/v) Triton; ■ 0.1% Triton. (Results are the means of quadruplicates S.E. < 3%).

Fig. 28 The Effect of Protein Concentration on [^{125}I]-αBgTx Binding to the Detergent Extract.



[^{125}I]-αBgTx, 2nM; d-tubocurarine 10^{-3}M; bound [^{125}I]-αBgTx was separated from free by $(NH_4)_2 SO_4$ ppt. and filtration; ■ total binding; ○ non-specific binding; ● specific binding (Results are the means of 2 expt. each in triplicate S.E. < 5%).

B. The Effect of Protein Concentration on Binding of [^{125}I]-α-Bungarotoxin:

Fig. 28 shows the effect of increasing the protein concentration (40-150µg per 150µl) on total, non-specific and specific [^{125}I]-α-bungarotoxin (2 nM) binding to the detergent extract. Specific binding is proportionate to protein concentration and is greater than non-specific binding at this concentration of [^{125}I]-α-bungarotoxin. Total and non-specific binding do not go through zero because of apparent binding to the filters. The experiment was carried out twice in triplicate (S.E. < 5%).

C. The Dependence of Binding of [^{125}I]-α-Bungarotoxin on Concentration (0.5 - 10 nM):

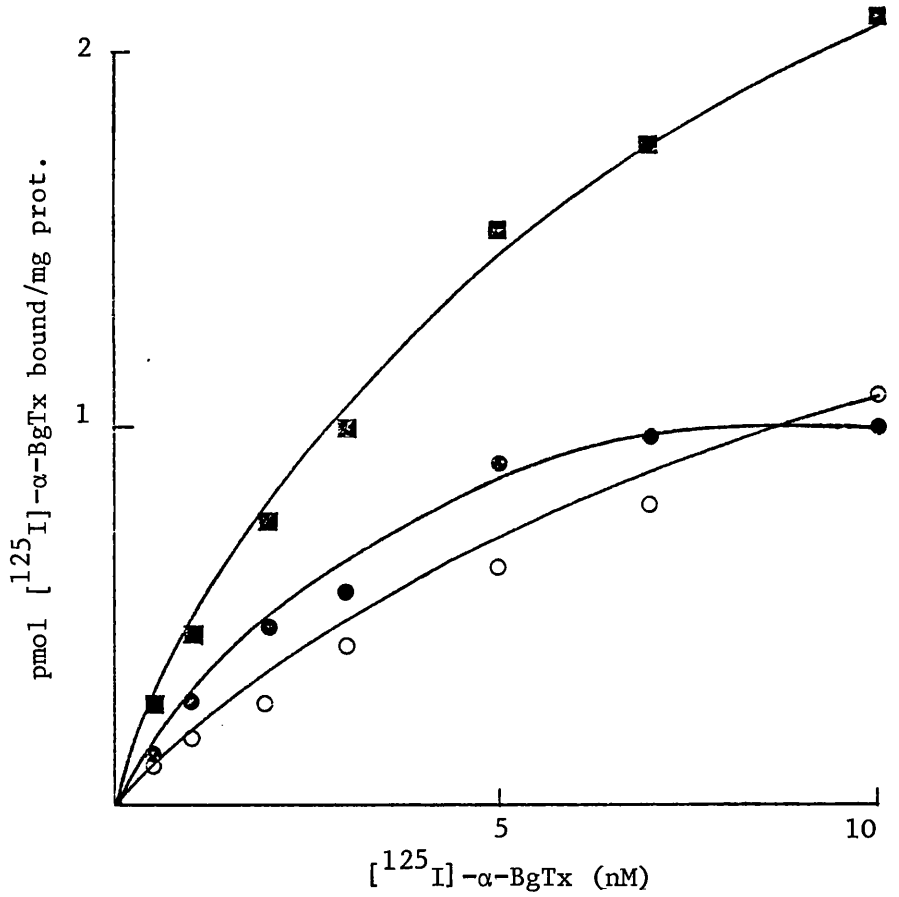
Binding was carried out over a range of [^{125}I]-α-bungarotoxin concentration of 0.5 - 10 nM.

Fig. 29 (a) shows the binding profile for total, non-specific and specific [^{125}I]-α-bungarotoxin binding (mean of two experiments, each in triplicate, S.E. < 4%). As can be seen specific binding shows a tendancy to saturate and it is only at 10 nM [^{125}I]-α-bungarotoxin that non-specific binding exceeds specific binding.

Fig. 29 (b) shows a Scatchard analysis of the specific binding. A K_D of 5.6 nM and a B_{max} of 1.714 pmol [^{125}I]-α-bungarotoxin bound/mg protein were obtained. The points lie on a straight line with a correlation coefficient of 0.950.

Fig. 29 (c) shows a Hill plot of the same data taking B_{max} as 1.714 pmol/mg protein. The points lie on a straight line (r = 0.991) the slope of which is 0.972 giving a Hill number (n_H) of 1.0 and hence it can be assumed that no cooperativity is taking place.

Fig. 29 (a) The Dependence of [^{125}I]-αBgTx Binding to the Detergent Extract on Concentration.



d-Tubocurarine 10^{-3}M; bound [^{125}I]-αBgTx was separated from free by $(NH_4)_2\ SO_4$ ppt and filtration; ■ total binding; ○ non-specific binding; ● specific binding. (Results are the means of 2 expt. each in triplicate S.E. < 4%).

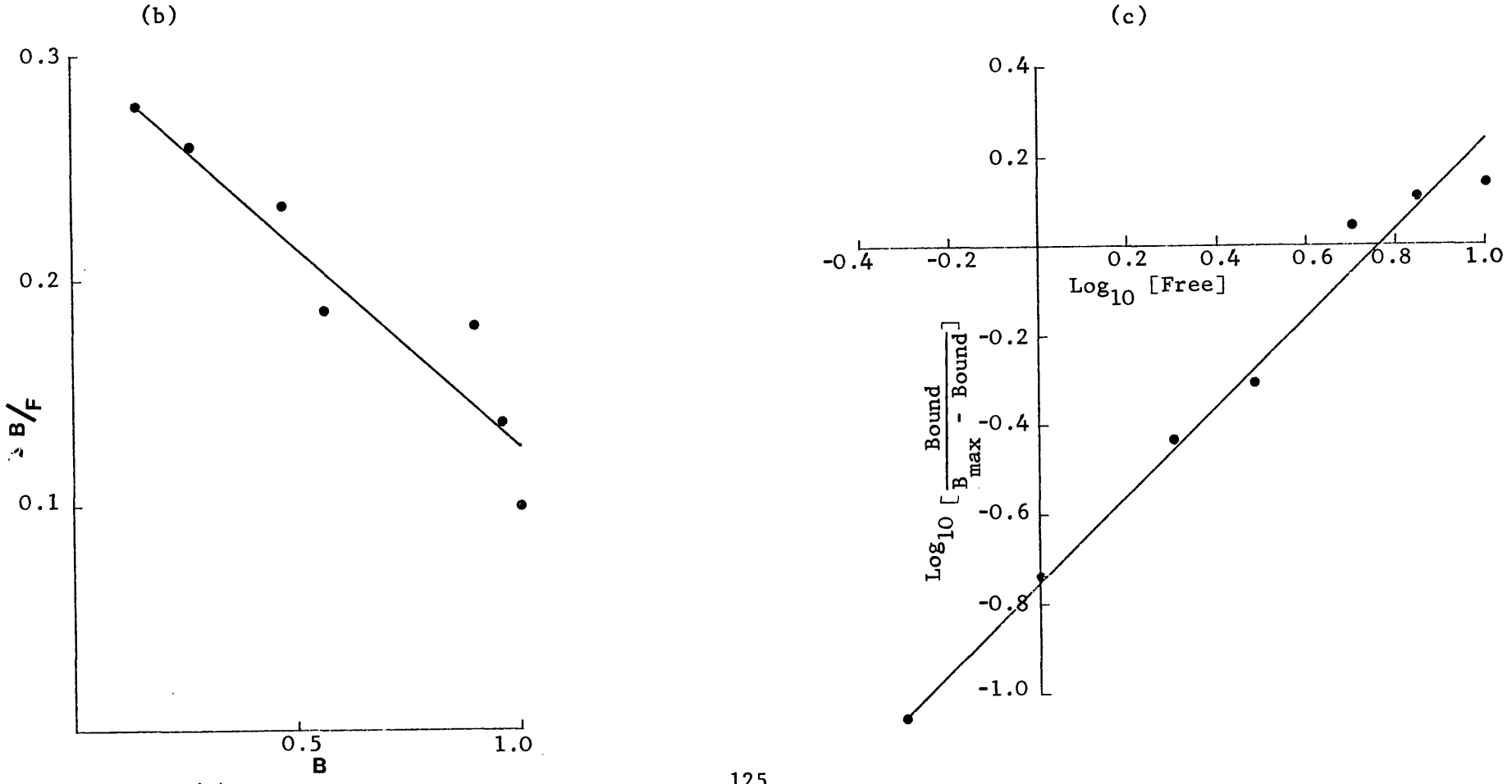


Fig. 29 (b) Scatchard Analysis of Specific [^{125}I]-αBgTx Binding to the Detergent Extract (K_D = 5.6 nM; B_{max} = 1.7 14 pmol/mg prat.)

(c) Hill Plot of Specific [^{125}I]-αBgTx Binding to the Detergent Extract (taking B_{max} as 1.7 14 pmol/mg prot.)

Fig. 30 (a) Sedimentation in a Sucrose Density Gradient of the Detergent - Extract - [^{125}I]-α-BgTx Complex.

(b) Sedimentation in a Sucrose Density Gradient of [^{125}I]-α-BgTx.

Fig. 30 (a)

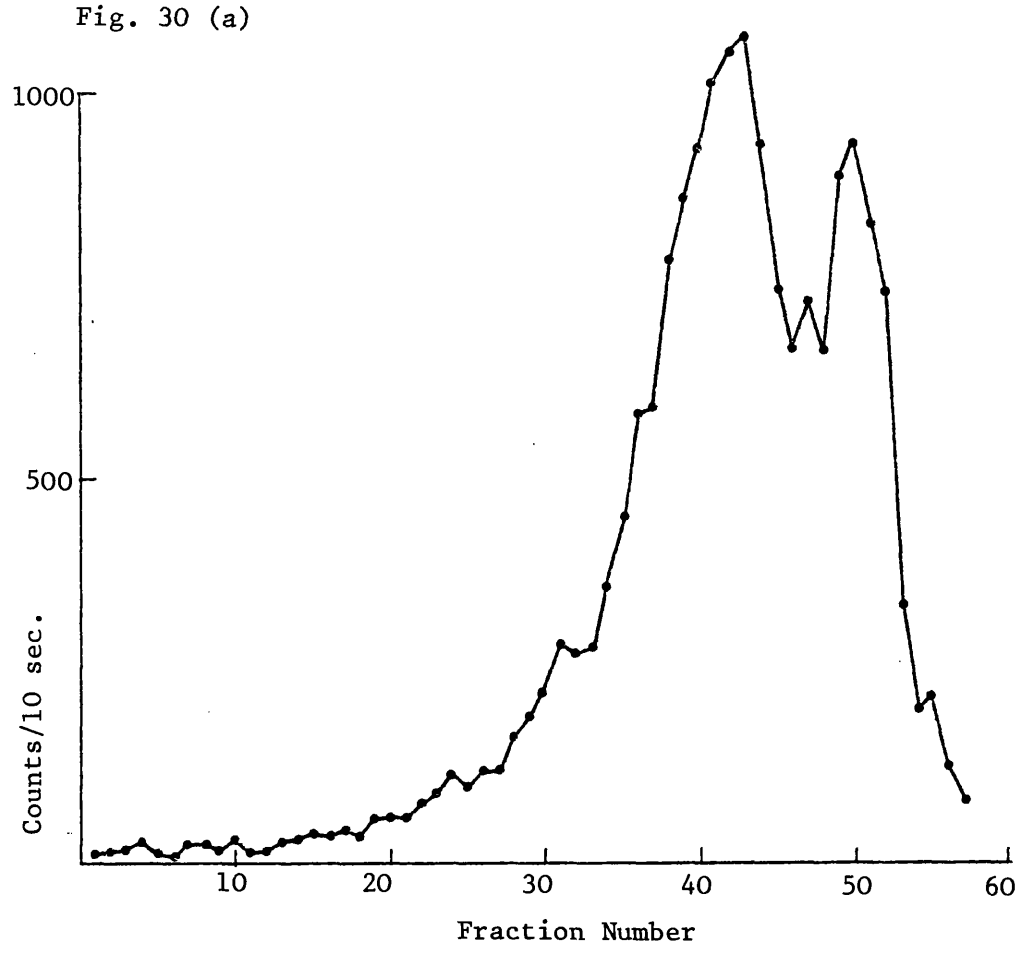
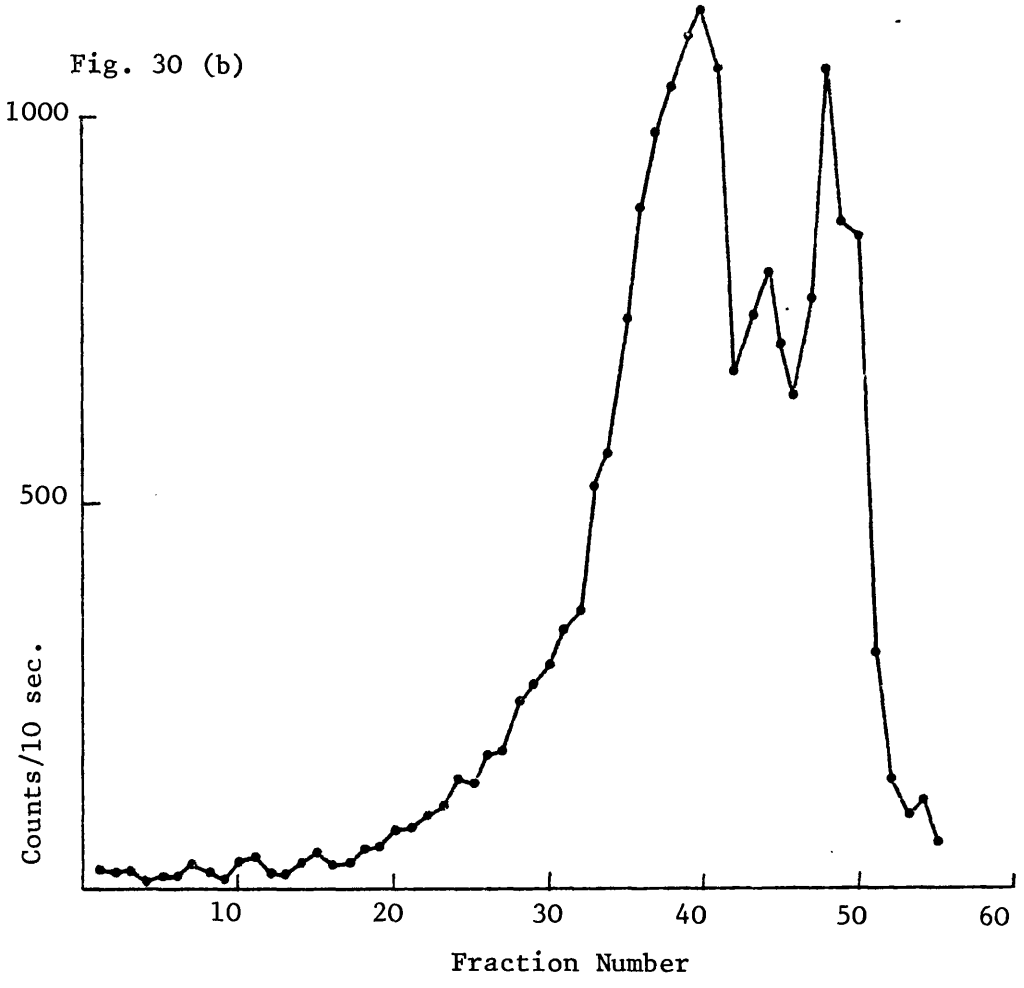


Fig. 30 (b)



D. Sedimentation in a Sucrose Density Gradient:

Sedimentation in a sucrose density gradient was carried out as described in Methods 7 (a) (p.130).

Fig. 30(a) shows the distribution of radioactivity after sedimentation of the [^{125}I]-α-bungarotoxin-detergent extract complex and Fig. 30(b) shows the radioactive distribution of free [^{125}I]-α-bungarotoxin. The profiles are the same, suggesting that the detergent extract - toxin complex has completely dissociated under these conditions.

4. Experiments on the Partially Purified α-Bungarotoxin Binding Component:

The α-bungarotoxin binding component was partially purified as outlined in Methods 2 (c) (p.125). Binding was as described in Methods 4 (c) (p.128) and non-specific binding was accounted for by incubating in the presence of an excess of d-tubocurarine (10^{-3} M).

A. The Extent of Purification:

Tables 29(a) and (b) show the specific binding ([^{125}I]-α-bungarotoxin; 10 nM) and the purification factor (total homogenate = 1.0), at four stages in the fractionation scheme for two separate preparations. It can be seen, firstly, that both the specific activity and the extent of purification are different for the two preparations although the percentage recovery of binding activity (taking the total homogenate as 100%) is similar. Secondly, it appears that in both preparations, most of the protein is lost between the S_1 fraction and the solubilisation in Triton X-100. Accompanying this loss of protein there is a loss of total pmol of specific binding activity. So it would appear that at this stage not only is a lot of non-specific

TABLE 29

The Extent of Purification at Various Stages in the Preparation.

PREPARATION (a)

Fraction	Protein Conc. (mg/ml)	Tot. Prot (mg)	Sp.Act (pmol/ mgprot	Purifi-cation	Tot. Binding (pmol)	% Recovery of Binding
Tot.Hom.	6.8	54.4	0.207	1	11.1	100
S_1	3.825	30.6	0.414	2	12.67	114
Detergent Extract	4.725	4.82	1.526	19	7.35	66
*PPαBTBC	0.004	0.0264	230.0	1100	6.07	55

PREPARATION (b)

Tot.Hom	5.37	37.62	0.412	1	15.5	100
S_1	4.35	30.232	0.702	1.7	21.22	137
Detergent Extract	5.27	5.27	1.694	2.4	8.93	58
*PPαBTBC	0.01575	0.0819	71.91	174.5	5.89	38

P.P.αBT.BC = Partially Purified αBT Binding Component

[^{125}I]-α-BgTx was at a concentration of 10 nM. The fraction was prepared as described in the Methods 2 (c) (p. 125). Binding was carried out as described in Methods 4 (c) (p. 128).

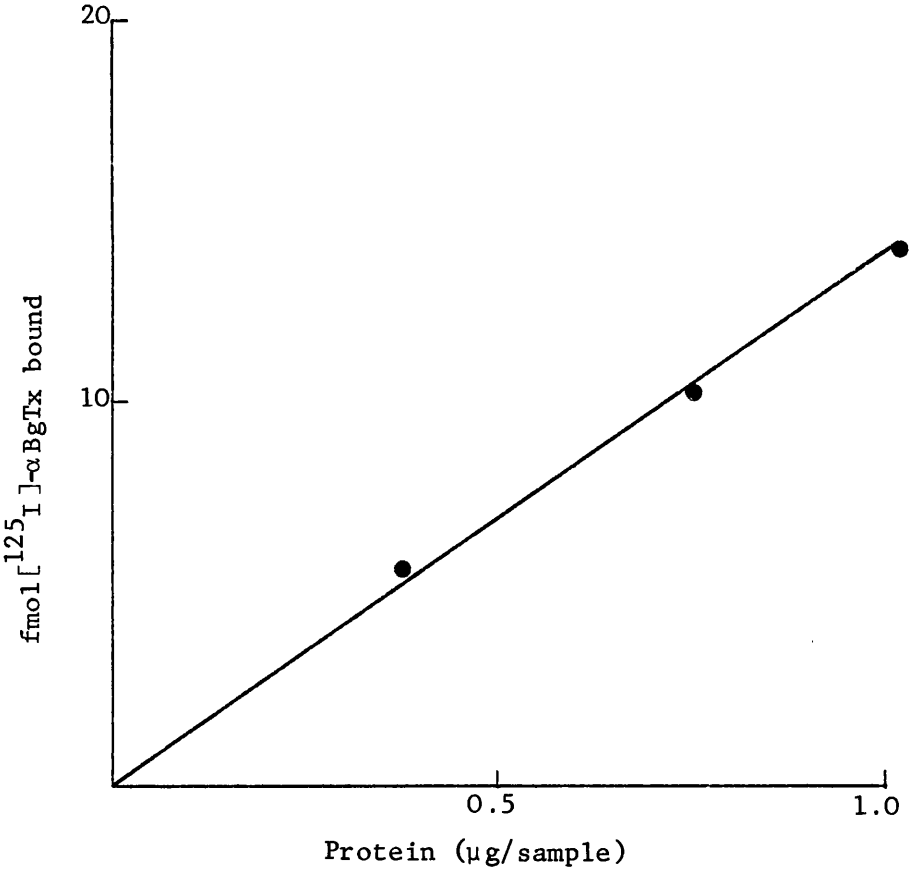
TABLE 30

Summary of the Extent of Purification for Six Preparations.

Prep.	Protein /Conc. (mg/ml)	Specific Binding (pmol/ mg prot)	% Recovery Total Binding Acitivity (Tot.Hom. = 100%)	Purification (Tot. Hom. = 1)
1	0.0075	13.96	9	56
2	0.0030	75.88	26	404
3	0.0040	230.00	48	1100
4	0.0030	6.68	-	50
5	0.0012	26.53	-	190
6	0.0160	71.91	38	174

[^{125}I]-αBgTx was at a concentration of 10 nM. The fraction was prepared as described in the Methods 2 (c) (p. 125). Binding was carried out as described in Methods 4 (c) (p. 128).

Fig. 31 The Effect of Varying the Protein Concentration on the Specific Binding of [^{125}I]-αBgTx to the Partially Purified Binding Component.



[^{125}I]-αBgTx 10 nm; d-tubocurarine 10^{-3} M; bound [^{125}I]-αBgTx was separated from free by DEAE cellulose filtration.

binding protein lost (purification factor 19 and 3 for preparations (a) and (b) respectively) but also there is a loss of specific [^{125}I]-α-bungarotoxin binding protein. This is not the case for the next step in the purification procedure i.e. passage through the affinity column. Although the total protein recovered at this stage is greatly reduced, the loss of total specific binding activity is minimal and hence the purification maximal.

Table 30 is a summary of the protein concentrations, specific binding and purification factors for six preparations. This table shows how the specific binding (6.68 - 230 pmol bound/mg protein) and the extent of purification (50 - 1100) varies considerably from preparation to preparation.

B. The Effect of Varying the Protein Concentration on Specific [^{125}I]-α-Bungarotoxin Binding:

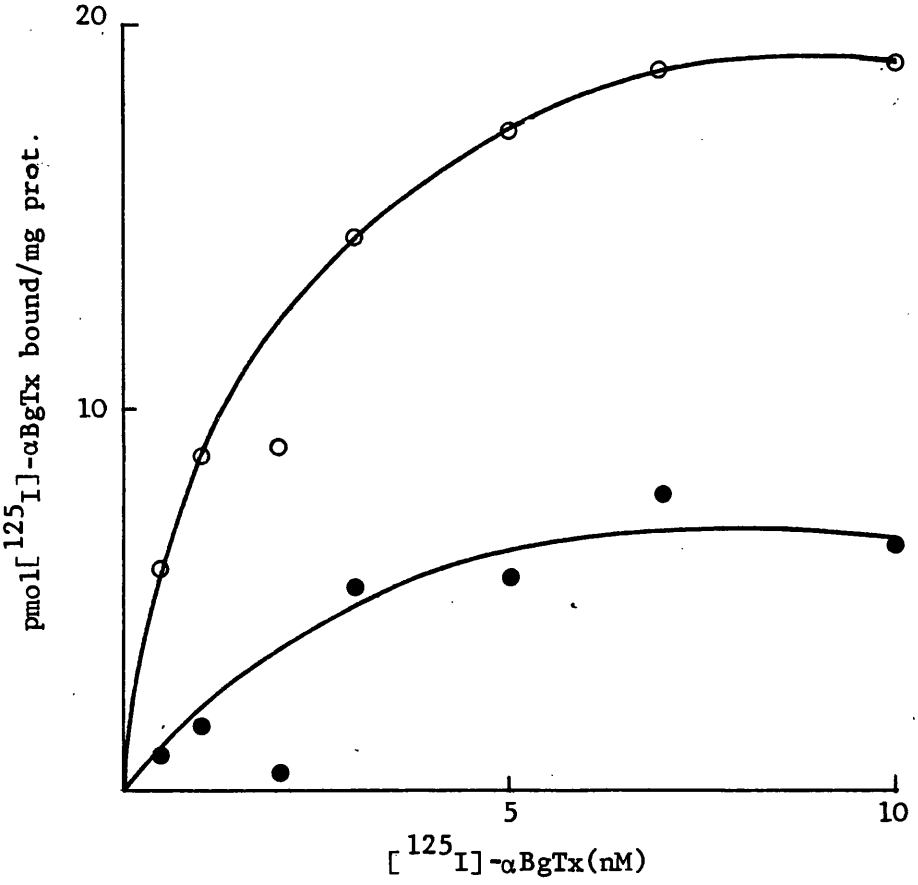
Specific binding of [^{125}I]-α-bungarotoxin (10 nM) was found to vary proportionally with protein concentration (0.37 - 1.12 µg per 150 µl).The experiment was repeated and the same result was obtained. (Fig. 31)

C. The Dependence of Binding of [^{125}I]-α-Bungarotoxin on Concentration (0.5 - 10 nM):

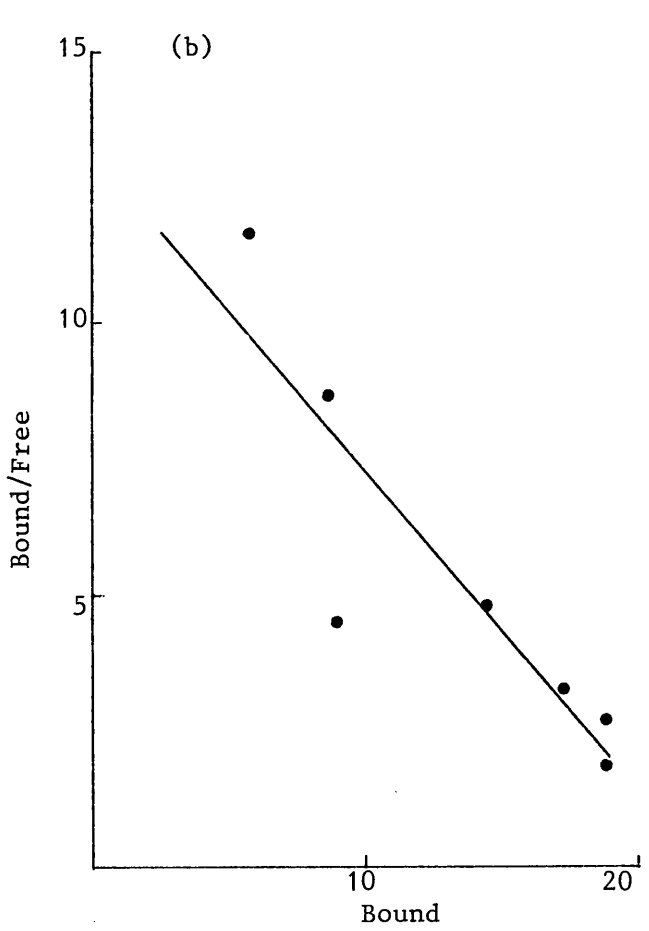
Binding was carried out over a concentration range of 0.5 - 10 nM [^{125}I]-α-bungarotoxin. Fig. 32(a) shows specific and non-specific binding. Non-specific binding is shown after subtraction of the filter blanks, and as can be seen it is much lower than specific binding.

Fig. 32(b) is a Scatchard plot of the specific binding data and from this a K_D of 1.71 nM and a B_{max} of 22.4 pmol bound/mg protein was obtained, with a correlation coefficient of 0.89. Each point is a mean of quadruplicates (S.E. <5%).

Fig. 32 (a) The Dependence of [^{125}I]-αBgTx Binding to the Partially Purified Binding Component on Concentration.



d-tubucurarine 10^{-3} M; bound [^{125}I]-αBgTx was separated from free by DEAE cellulose filtration; o specific binding; ● non-specific binding.



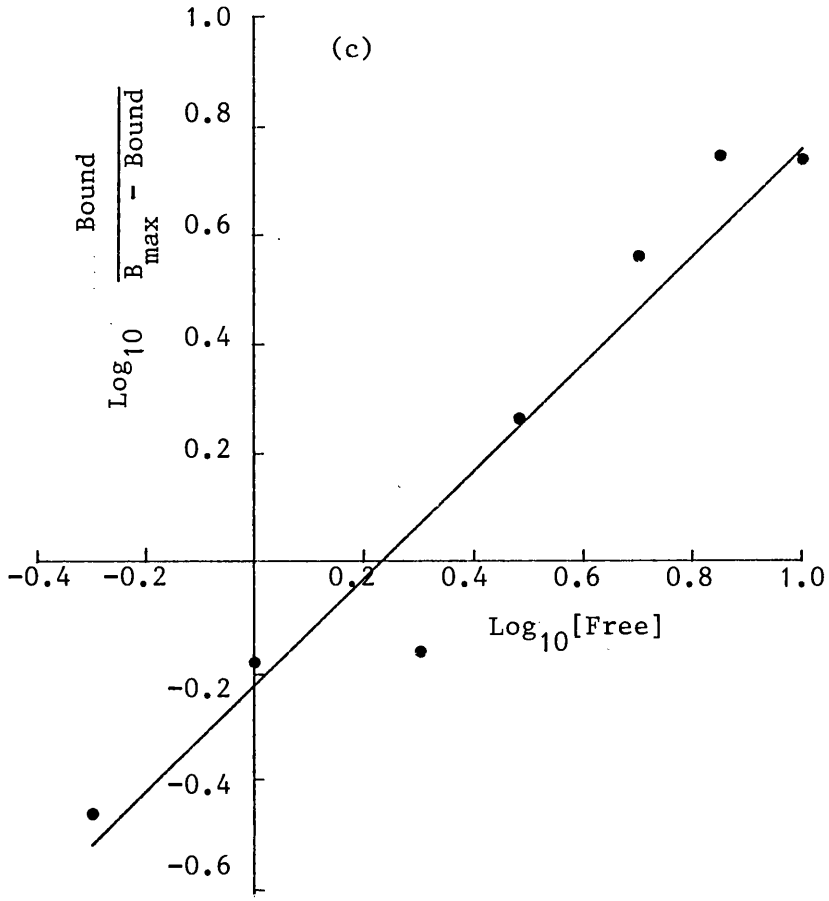


Fig. 32 (b) Scatchard Analysis of Specific [^{125}I]-α-BgTx Binding to the Partially Purified Binding Component (K_D = 1.71 nM; B_{max} = 22.4 pmol/mg prot).

(c) Hill Plot of the same data (taking B_{max} as 22.4 pmol/mg prot).

(Results are the means of quadruplicates S.E. < 5%).

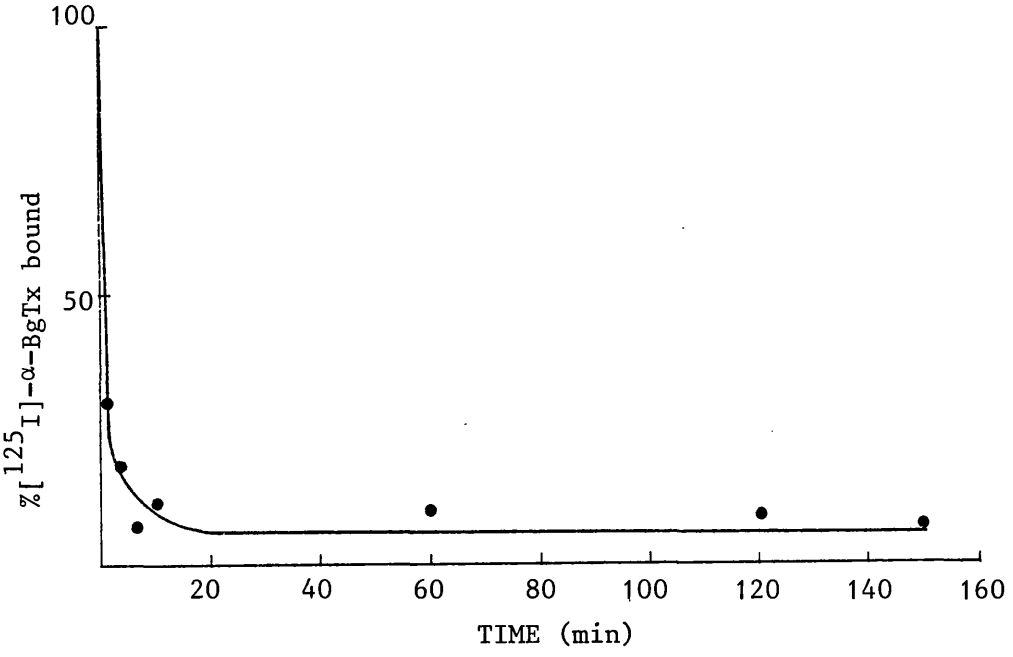
Fig. 32 (c) is a Hill plot of the same data taking B_{max} as 22.4 pmol bound/mg protein. This plot gives a Hill number (n_H) of 0.992, (r = 0.969) which can be taken as unity and hence it would appear that no cooperativity is taking place.

D. The Determination of the Rate of Dissociation (k_{-1}) for [^{125}I]-α-Bungarotoxin Binding:

The k_{-1} was determined as described in the Methods 5 (p.129). The experiment was carried out in triplicate and samples were taken at the time intervals indicated after the addition of excess unlabelled α-bungarotoxin (x 1000 molar excess). Samples incubated in the presence of an excess of d-tubocurarine (10^{-3} M) were also taken at the same intervals, to account for non-specific binding. Results are expressed as a percentage of total specific [^{125}I]-α-bungarotoxin binding i.e. taking the amount bound before the addition of the excess unlabelled α-bungarotoxin as 100% and taking into account the volume dilution.

Fig. 33 shows a plot of percent [^{125}I]-α-BgTx bound against time (1 experiment in triplicate S.E. < 3%). After 1 minute only 30% of the [^{125}I]-α-bungarotoxin remained bound and so the rate of dissociation was too fast to obtain an accurate measurement of k_{-1} by calculating $t_{0.5}$. Therefore a visual estimate from the time course of $t_{0.9}$ and $t_{0.8}$ was made. The term "greater than", must always be employed because as the time course is exponential, the actual $t_{0.5}$ will not be directly proportional to either $t_{0.9}$ or $t_{0.8}$. It will always be faster and an under estimation of k_{-1} will be obtained using $t_{0.9}$ or $t_{0.8}$.

Fig. 33 The Determination of the Dissociation Rate Constant (k_{-1}) for [^{125}I]-α-BgTx Binding to the Partially Purified Binding Component



[^{125}I]-α-BgTx, 2mM; d-tubocurarine 10^{-3}M; bound [^{125}I]-α-BgTx was separated from free by DEAE cellulose filtration. (Results are the means of triplicates S.E. < 3%).

Using the equation:

$$\log \left({}^{A_o}/_{A}\right) = k_{-1}.t$$

where A_o = Amount bound at zero time

A = Amount bound at time t

k_{-1} = the off rate

t = time in sec.

∴ Estimation of k_{-1} from $t_{0.9}$ (i.e. when 90% of labelled toxin has dissociated), where $t_{0.9}$ is 600 s, k_{-1} is calculated to be $> 3.8 \times 10^{-3}$ S^{-1}, from $t_{0.8}$ (150 s), $k_{-1} > 10.7 \times 10^{-3}$ S^{-1}. The values are of the same order of magnitude and the mean of k_{-1} 's estimated from $t_{0.9}$ and $t_{0.8}$ is $> 7.25 \times 10^{-3}$ S^{-1}.

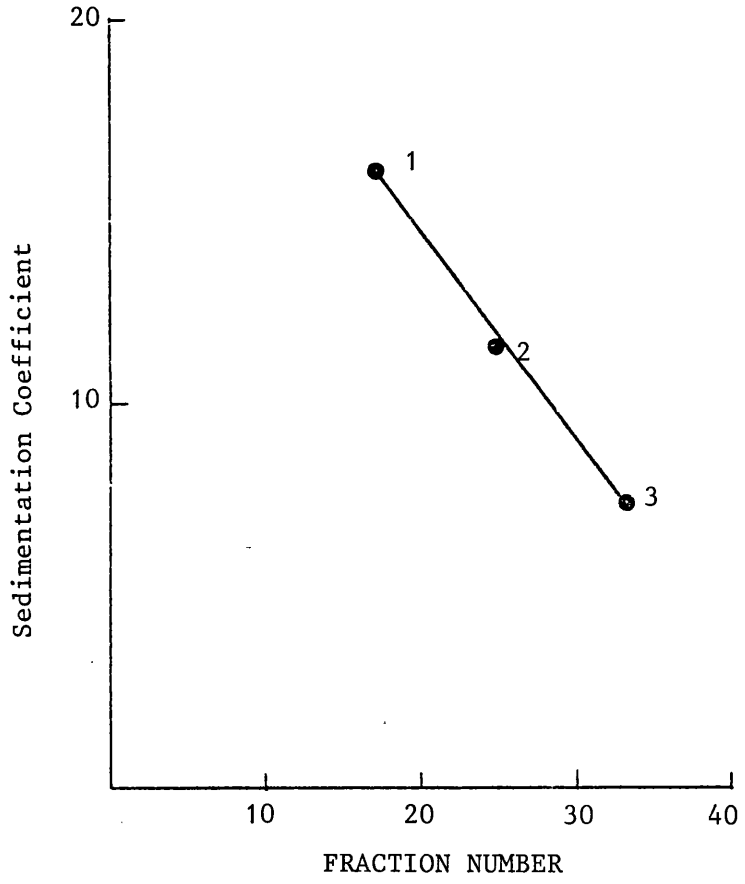
Using the relationship $K_D = \frac{k_{-1}}{k_{+1}}$ at equilibrium (where K_D is the intrinsic dissociation constant and k_{+1} is the on-rate) and assuming that k_{+1} is equal to 1×10^6 M^{-1} S^{-1}, which is very close to experimentally determined values for most neurotransmitter receptors, K_D was calculated to be $> 7.25 \times 10^{-9}$ M, a value which is very close to the K_D estimated from equilibrium binding studies (1.71 nM).

E. Sedimentation in a Sucrose Density Gradient:

Sedimentation in a sucrose density gradient was carried out as described in the Methods 7 (b) (p.131). A plot of sedimentation coefficient against the fraction number with peak enzyme activity for each standard marker enzyme gave a straight line ($r = 0.993$) Fig.34

Every fraction from the gradient containing the sample was assayed for [^{125}I]-α-bungarotoxin binding activity. The activity sedimented as a single component (Fig. 35) with a $S_{20,W} = 14.2$ S, corresponding to a molecular weight of 400,000 - 450,000 using the approximation (Schachman, 1959):-

Fig. 34 The Calibration Curve for the Marker Enzymes after Sedimentation in a Sucrose Density Gradient

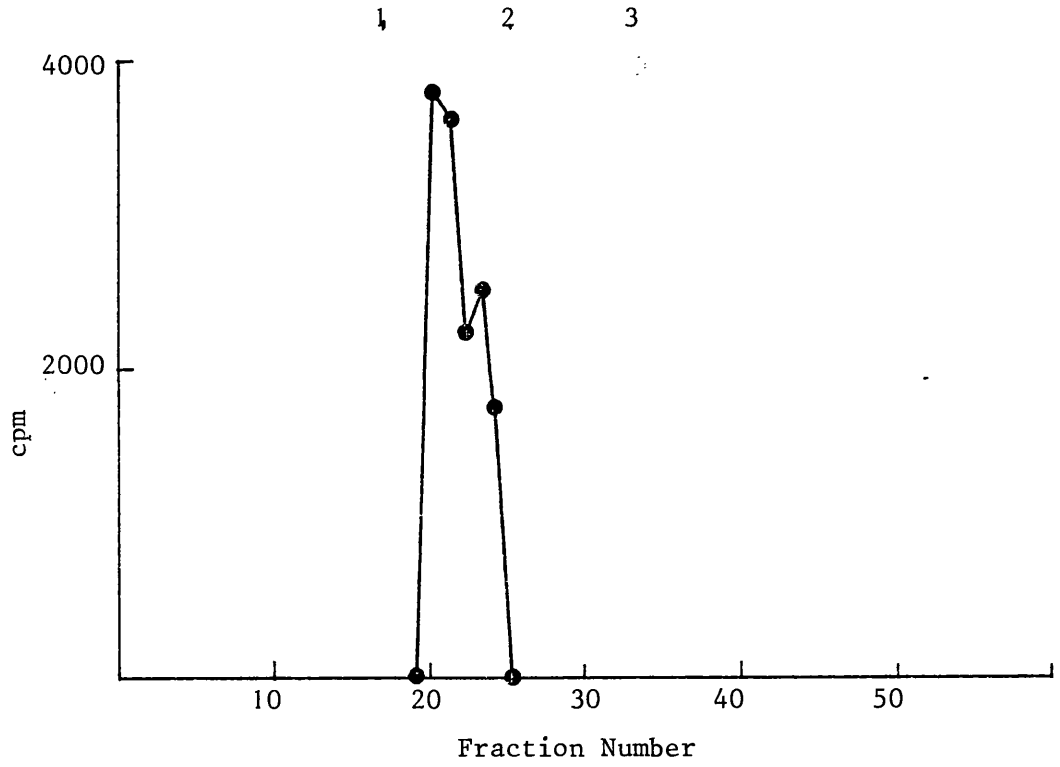


1 = β galactosidase (16.0s; 520,000M_r)

2 = catalase (11.4s; 232,000 M_r)

3 = yeast alcohol dehydrogenase (7.4s; 150,000 M_r)

Fig. 35 The Distribution of Specific [^{125}I]-αBgTx Binding Activity after Sedimentation in a Sucrose Density Gradient of the Partially Purified Binding Component.



Centrifugation was at 100,000 x g for 6 h.; every fraction was assayed for αBgTx binding activity; [^{125}I]-αBgTx 10 nM; d-tubocurarine 10^{-3} M; bound [^{125}I]-αBgTx was separated from free by DEAE cellulose filtration. Marker enzymes: 1 = β galactosidase (16.05); 2 = catalase (11.45); 3 = yeast alcohol dehydrogenase (7.45).

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

The contribution of bound detergent to the Stokes' radius was not taken into account. The experiment was repeated and a value of 14.1 S was obtained.

F. SDS Polyacrylamide Gel Electrophroesis:

SDS polyacrylamide gel electrophoresis of the partially purified α-bungarotoxin binding component was carried out under denaturing conditions as described in the Methods 8 and 9 (p.132). Fig. 25 shows the calibration line for the standard markers. (Log_{10} Molecular Weight) vs distance of Protein Migration).

The partially purified α-bungarotoxin binding component gave three faint but distinct bands on this gel system. The bands correspond to molecular weight of 60,000, 41,000 and 25,000. Fig. 37 shows densitometric analysis of the same gel and shows clearly the three bands as peaks.

5. Determination of Acetylcholine Esterase Activity in All Fractions:

Table 31 shows the relative specific activities (RSA's) of acetylcholine esterase in the various fractions.

$$\text{RSA} = \frac{\%\text{ Enzyme activity recovered in the fraction}}{\%\text{ Total protein recovered in the fraction}}$$

where enzyme activity and total activity in the total homogenate is taken as 100%. As can be seen no fraction has a RSA value greater than one but activity is greatest in the S_1 and P_2 fractions. No enzyme activity was recorded in the partially purified fraction.

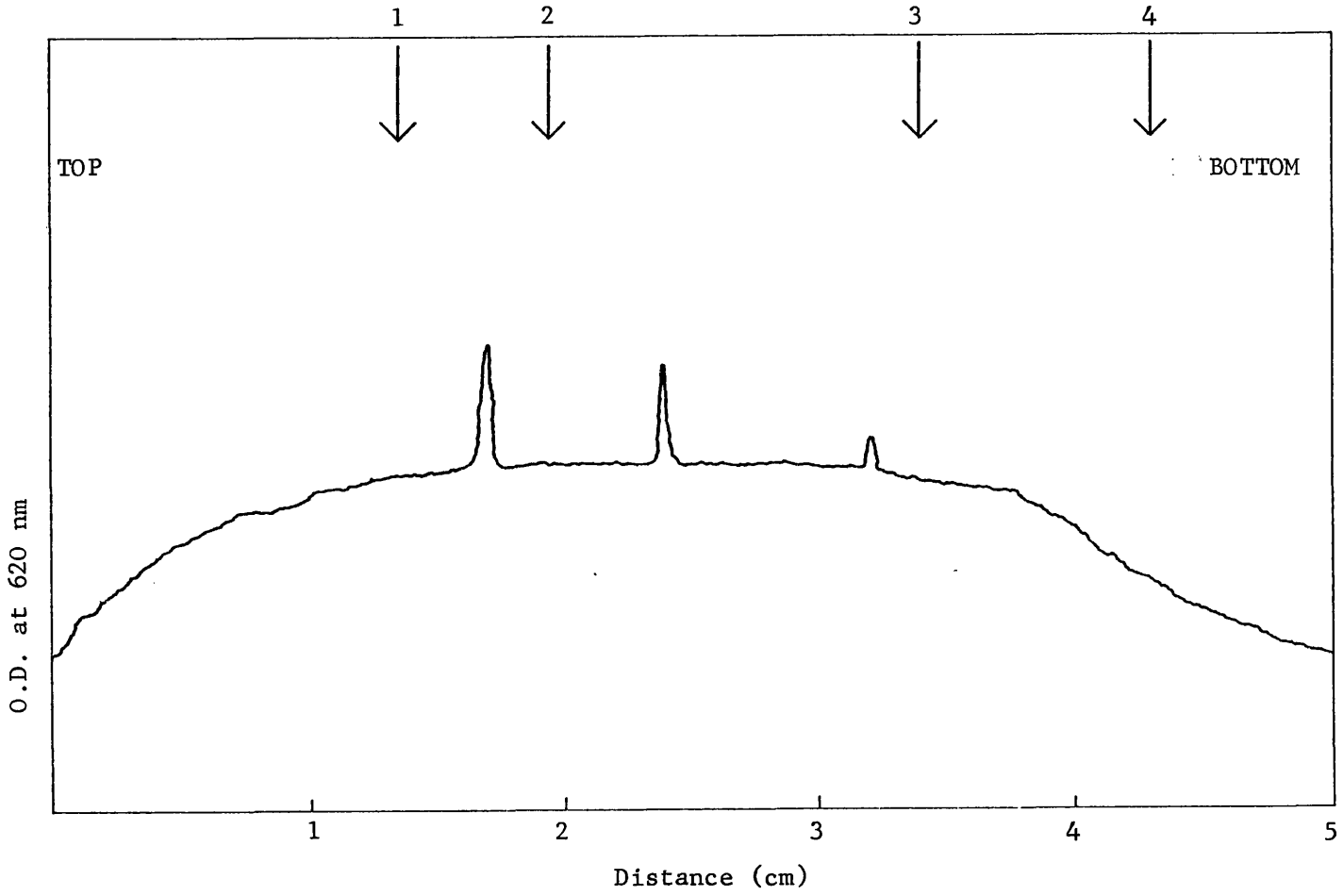


Fig. 36 Densitometric Analysis of the Partially Purified Binding Component after SDS-PAGE.

Markers : 1 = human IgM chain (74,000 Mr); 2 = human IgG chain (50,000 Mr); 3 = human immuno lobulin light chain (23,500); 4 = ribonuclease A (13,700).

TABLE 31

RSA Values for Acetylcholine Esterase Activity in Various Fractions.

FRACTION	RSA
Total Homogenate	1
S_1	0.679
S_2	0.130
P_2	0.588
Detergent Extract	0.461
Partially Purified Binding Component	0

RSA = Relative Specific Activity

$$= \frac{\% \text{ Total Enzyme Activity}}{\% \text{ Total Protein}}$$

Enzyme activity was assayed as described in the Methods 11 (p.134).

DISCUSSION

Radiolabelled ligand binding studies have played a major role in the identification, purification and characterisation of putative receptors. Initial binding studies can be made on very crude preparations which are often little more than a whole tissue homogenate. Once specific, high affinity binding of a specified ligand has been identified, purification can proceed and the binding characteristics can be monitored through the various stages of purification. Finally, when purification to homogeneity has been achieved, complete characterisation can be carried out. The ultimate aim of all such studies is to assign a physiological receptor role to what are initially no more than specific ligand binding sites (Criteria, Introduction p.15). To date, of the three putative acetylcholine receptors in insect CNS (Introduction p.20), only in the case of the putative nAChR has enough complementary evidence accumulated to warrant its definition as a synaptic acetylcholine receptor. The characterisation of this particular receptor has been critically dependent on the snake venom α-toxins as diagnostic receptor probes. The procedures employed in the isolation and purification of the insect nAChR have been developed from the well established methodology associated with the nAChR of fish electric organ and vertebrate skeletal muscle (see, Fulpius, 1976). The studies reported in this thesis sought to apply this expertise to the characterisation and purification of the nAChR of the supra-oesophageal ganglion of the desert locust, Schistocerca gregaria.

The Binding Assay:

The primary objective in receptor binding studies is to quantitate the specific binding of a ligand to the tissue in question. In the

great majority of cases detection of the ligand depends on radiolabelling and the first consideration must be the choice of a technique which will efficiently separate bound radiolabelled ligand from free radiolabelled ligand. The choice of a separation technique is largely dependent on whether the putative receptor is in a particulate or in a soluble form. The α-bungarotoxin binding component studied in this thesis was investigated in both states. For preparations in which the binding component remained in the membrane-bound form a centrifugation assay was chosen. Although there was little difference in binding between this procedure and a vacuum filtration method, the centrifugation method was adopted for the routine assay of particulate fractions. It offered advantages in terms of reproducibility of separation times with an ensuing improvement in the extent of variation of experimental results.

For the soluble S_2 fraction an ammonium-sulphate precipitation assay, derived from Meunier et al (1972), was chosen in preference to an ion-exchange separation by vacuum filtration on DEAE cellulose discs because the fraction contained large amounts of soluble proteins which could easily saturate the rather limited capacity DEAE filter discs; indeed such a problem was encountered in studies on the nAChR from human skeletal muscle (Stephenson, 1980). On the other hand, the ammonium-sulphate procedure results in the precipitation of all proteins, soluble and particulate, specific and non-specific. However free toxin is not precipitated under these conditions. The precipitated bound toxin is then separated from free toxin by a simple physical filtration. Therefore this precipitation method was also suitable for fractions which contained both soluble and particulate material, such as the total homogenate and the S_1 fractions.

Ion-exchange on DEAE-cellulose filter discs was used for the assay of the preparation of the partially purified α-bungarotoxin binding component because such preparations contained very low amounts of protein, the majority of which was the specific binding component. The ion-exchange assay also offers considerable advantages in that the entire procedure takes only about 2 hours compared with the 24 hours required for the ammonium sulphate procedure.

The major constraint on separation techniques is the rate of dissociation of the receptor-ligand complex at the temperature at which the separation is carried out. Dissociation will commence immediately the equilibrium is changed either by removal of the free radiolabelled ligand, such as occurs during separation, or by dilution as will occur during washing of the receptor-ligand complex. Thus complexes that dissociate rapidly will require separation and washing techniques that minimise the amount of dissociation.

It has been estimated that a separation and washing time of approximately 0.15 x $t_{0.5}$ (where $t_{0.5}$ is the half-time for dissociation of the complex) results in less than 10% dissociation. In the bulk of the work described here separation and washing procedures were routinely completed in less than one minute for the centrifugation method and about 6 seconds for the filtration methods, thus ensuring minimal dissociation of membrane-toxin-complex ($t_{0.5}$ = 30-65 minutes) and the partially-purified-binding-component-toxin-complex ($t_{0.5}$ > 1 minute), respectively. In the case of the ammonium sulphate precipitation assay samples are left for at least 16 hours for the precipitate to form, however, it appears that free toxin is still in equilibrium with the receptor-toxin precipitate (Stephenson, 1980). The efficiency of the separation techniques used will be

inactivation of preparations (Schmidt-Nielson et al., 1977). The rationale for such a procedure is that heating the tissue destroys the tertiary structure of proteins and hence the specific binding conformation of the receptor but that binding to non-physiological sites will not be impaired. Schmidt-Nielson et al., (1977) have compared this method with the more conventional displacement procedures and concluded that they are equally effective, both in membrane-bound and in detergent-treated preparations of housefly heads.

In work reported in this thesis non-specific [^{125}I]-αBgTx binding to all fractions was routinely estimated by incubation in the presence of an excess of d-tubocurarine (10^{-3}M). Closely similar results were obtained with excess αBgTx.

Subcellular Localisation of the α-Bungarotoxin Binding Component:

Most reports of α-BgTx binding to insect CNS have shown the α-BgTx binding component to be associated with particulate sub-fractions of the tissue (see Introduction p.26). The work reported in this thesis shows that αBgTx binding is associated with a 100,000 x g pellet fraction of homogenates of locust supra-oesophageal ganglia and is in agreement with the majority of reports of other preparations of insect CNS (see Sattelle, 1980a). Contrary to these findings however is the recent report of March et al., (1980) which suggests that the αBgTx binding component from housefly heads readily behaves as a soluble protein. They found an enrichment of α-BgTx binding activity in a 150,000 x g supernatant of a preparation of whole housefly heads homogenised in buffered sucrose. However, an earlier report from these workers showed that α-BgTx binding was associated with a 20,000 x g pellet prepared under apparently similar conditions (Cattell et al., 1980). This apparent

discussed further when the rate of dissociation of bound [^{125}I] αBgTx from the membrane fragments and from the partially purified fraction is considered (p. 162).

In all the fractions studied a proportion of the total [^{125}I]-αBgTx binding was non-specific. Non-specific binding may be composed of several components:

1. Binding to tissue components other than the site under investigation.
2. Free radiolabelled ligand not removed by washing.
3. Binding to separation materials.

The most widely used method to account for non-specific binding is the "unlabelled ligand excess" method, where specific binding of the radiolabelled ligand is defined as the difference between the total binding and the binding that occurs in the presence of an excess of unlabelled ligand. The method depends on displacement of labelled ligand only from sites having a high affinity and as such can be subject to interference from binding to sites with high affinity but which are of no physiological relevance (Turner, 1980). This problem can, to some extent, be circumvented by adopting procedures that depend on displacement of radiolabelled ligand by a chemically different ligand, that is a potent agonist or antagonist. In this case the probability of competition between labelled and unlabelled ligand for any common high-affinity but non-specific site is less and in general the use of well characterised, structurally distinct unlabelled ligands is to be preferred in the displacement of specific binding (Bennett, 1978).

A quite different approach to the problem of distinguishing between specific and non-specific binding relies on the heat

discrepancy could perhaps result from the method of assay of the binding activity. The earlier study was mainly concerned with an investigation of [^{125}I]-α-BgTx binding to a total homogenate of housefly heads, in which filtration through Whatman GF/C filters was used to separate bound from free toxin. It must be assumed that the same method was employed to measure binding activity in the various subcellular fractions as the report gives no indication of any variations in the binding assay. If indeed this is the case, then an under-estimation of binding activity in the soluble fractions would be expected as it is unlikely that Whatman GF/C filters retain soluble receptor [^{125}I]-α-BgTx complex. The later study (March et al., 1980) in which the binding component is greatly enriched in the soluble fraction, uses the DEAE filtration assay and it may well be that this soluble component was not detected in the earlier studies that used the GF/C filtration procedure.

There are as yet insufficient data to provide an explanation for the ready solubility of the flyhead binding protein. In the case of nAChR from vertebrate muscle there is an extensive literature concerned with the proteolytic degradation of the receptor during purification and it is generally agreed that proteolysis is a major problem (Lindstrom et al., 1979; Lindstrom et al., 1980a; Shorr et al., 1981). Rather less attention has been paid to this matter in the insect work and certainly there are no reports of systematic studies of the effects of proteolysis and/or the effects of anti-protease agents on the various putative AChR's from insect material. March et al (1980) included a number of anti-proteases, that have been employed in studies on the nAChR from mammalian muscle (Stephenson, 1980), in the homogenisation

buffer but it is not established that the agents used are effective inhibitors of the particular proteases encountered in the flyhead. There is the possibility that the behaviour of the fly binding proteins represents a true species variation but other workers have reported a particulate localisation for [^{125}I]-α-BgTx binding activity in housefly heads (Jones & Sumikawa, 1981;Eldefrawi & Eldefrawi, 1980), however in these studies no quantitative assessment of overall recovery of binding activity in the various fractions was given.

In none of the reports of identification of an α-BgTx binding component in insect CNS, is there a detailed account of the homogenisation procedure used. It would seem that release of the putative nAChR into a soluble fraction would depend not only on the nature of the association of the receptor with the post-synaptic membrane but also on the degree of disruption of that membrane by the homogenisation procedure used. The procedure used in this thesis was relatively harsh in comparison to methods used in the preparation of synaptic membranes from mammalian brain (see Jones, 1975). As shown in Table 23 the behaviour of the [^{125}I]-α-BgTx binding component is indicative of a protein tightly associated with its membrane.

The Membrane Fragments (P_2 Fraction)

Specific [^{125}I]-α-BgTx binding to the membrane fragments increased linearly with tissue concentration indicative of specific binding and thus is in agreement with one of the criteria for defining a physiological receptor (Introduction p. 15).

Specific binding was saturable with respect to time, reaching equilibrium in 10 to 15 minutes. To demonstrate that a putative receptor has a high affinity and finite number of binding sites for a particular

ligand it is necessary to obtain estimates of the intrinsic dissociation constant, K_D, and the maximum number of binding sites per unit weight of tissue, B_{max}. These values are usually calculated from equilibrium binding studies over a saturating concentration range of ligand.

Initially such studies with the membrane fragments were carried out over a concentration range of 40 nM - 600 nM [^{125}I]-α-BgTx. This range was chosen because preliminary results from Cattell et al (1980a) suggested the presence of both a high (K_D, 3.1 nM; B_{max} 0.204 nmol/g prot.) and a low (K_D 549.6 nM; B_{max} 14146 nmol/g prot.) affinity binding site for α-BgTx binding to an homogenate of housefly heads. At these concentrations specific binding of [^{125}I]-α-BgTx to the membrane fragments of locust supra-oesophageal ganglia was inconsistent between preparations. In some experiments specific binding appeared to approach saturation but Scatchard analysis of the data gave no reliable estimates of K_D or B_{max}. It was thought that perhaps this concentration range of [^{125}I]-α-BgTx was masking a possible high affinity binding site which would saturate within a lower concentration range. Specific binding was investigated over a concentration range of 0.5 nM - 10 nM [^{125}I]-α-BgTx. Specific, saturable binding was observed and was consistent from preparation to preparation. Scatchard analysis of the binding data gave values of K_D, 1.38 nM and B_{max}, 1.18 pmol/mg protein. The binding data showed no evidence of cooperativity as indicated by a Hill number of one. A K_D of this magnitude indicates high affinity binding of [^{125}I]-α-BgTx to the membrane fragments; although higher than the K_D's quoted for α-BgTx binding to the nAChR's of the electroplax and of vertebrate skeletal muscle, 10^{-10}M - 10^{-12}M, (see, Karlin, 1980) the value of 1.38 nM is in agreement with the K_D's calculated for

TABLE 32

The [^{125}I]-α-Bungarotoxin Binding Parameters for Different Fractions Prepared from Various Insects CNS

Fraction	Species	K_D (nM)	k_{+1} ($M^{-1}S^{-1}$)	k_{-1} (S^{-1})	B_{max} (pmol/mg prot)
Total homogenate[1]	Housefly	3.1	-	-	0.204
Total homogenate[2]	Locust	1.11	6.75×10^5	4.25×10^{-4}	1.775
500 x g supernanant	Fruitfly	1.9	2.4×10^5	1.4×10^{-4}	0.3 - 0.8
		0.6*			
20,000 x g pellet[4]	Housefly	5.6	2×10^5	-	0.0229
20,000 x g pellet[5]	Fruitfly	1.8	-	-	0.19
20,000 x g pellet[6]	Fruitfly	1.1			0.887
40,000 x g pellet[7]	Fruitfly	0.16			
		0.14*	5.5×10^5	7.7×10^{-5}	-
100,000 x g pellet[8]	Housefly	3.0			2.0
100,000 x g pellet[9]	Locust	1.38	-	2.78×10^{-4}	1.18
		0.278*			
Deoxycholate solubilised[7]	Fruitfly	0.23	4.6×10^5	8.3×10^{-5}	-
Triton solubilised[6]	Fruitfly	0.21			-
Triton solubilised[9]	Locust	5.6	-	-	1.714
Partially purified[10]	Fruitfly	0.15*	7.8×10^5	1.2×10^{-4}	600-1100
Partially purified[9]	Locust	1.71	-	$> 10.7 \times 10^{-3}$	7-230
		> 7.25*			

* K_D calculated from on- and off-rate constants, otherwise from equilibrium studies

1. Cattell et al., 1980 a
2. Breer, 1981
3. Dudai, 1980
4. Jones & Sumikawa, 1981
5. Rudloff, 1978
6. Schmidt-Neilson, et al., 1977
7. Jimenez & Rudloff, 1980
8. Eldefrawi & Eldefrawi, 1980
9. This thesis
10. Hall, 1980

[^{125}I]-α-BgTx binding to other insect preparations (Table 32) and to preparations of mammalian CNS, 10^{-9}M - 10^{-8}M, (see, Oswald & Freeman, 1981). A B_{max} of 1.18 pmol [^{125}I]-α-BgTx bound/mg protein agrees well with a value of 2 pmol/mg protein reported by Eldefrawi and Eldefrawi (1980), for a similar preparation from housefly heads. Other workers have reported values of B_{max} of approximately an order of magnitude lower for the binding of α-BgTx to crude homogenates of fruitfly heads (Dudai, 1980) or of whole flyheads (Cattell 1980a) suggesting that these preparations are comparable in specific binding activity to the total homogenate of locust supra-oesophageal ganglia (Table23).

A concentration of 80 - 85 pmol [^{125}I]-α-BgTx binding sites/g wet weight locust supra-oesophageal ganglia, was estimated from measurements of specific binding to the total homogenate, a figure comparable to the value of 88 pmol/g fruitfly heads reported by Hall (1980). Thus both locust supra-oesophageal ganglia and fruitfly heads represent a slightly richer source of toxin binding activity than Electrophorus electroplax (Raftery et al., 1971) and denervated rat diaphragm (Berg et al., 1972), both of which have in the order of 20 - 66 pmol of toxin binding activity/g tissue, and a much richer source than rat brain, which contains about 6 pmol binding activity/g wet weight (see, Schmidt et al., 1980). However, a major problem of working with insects is their size, a single locust supra-oesophageal ganglion weighs on average 4 mg (wet weight) and so although a relatively rich source of α-BgTx binding activity, hundreds of ganglia must be used to obtain sufficient material on which to commence potential purification schemes.

As an alternative to binding studies at equilibrium, K_D can be estimated from measurements of the individual rate parameters, the on-rate constant (k_{+1}) and the off-rate constant (k_{-1}), where $K_D = k_{-1}/k_{+1}$. The k_{+1} for binding of neurotransmitters to their receptors, is usually about $10^6 M^{-1}S^{-1}$ (Bennett, 1978). In practice little variation in k_{+1} is seen between widely differing preparations. Furthermore, the conditions chosen for most binding experiments ensure that equilibrium is reached and therefore the precise value of k_{+1} is usually of little practical relevance. On the other hand, k_{-1} is of great relevance to the choice of technique for the separation of bound from free ligand or indeed to any procedure that may perturb the equilibrium. Thus investigations that may result in separation of free ligand from receptor-ligand complex, such as sedimentation in a sucrose density gradient, electrophoresis in non-denaturing systems or gel filtration will necessarily be constrained.

The k_{-1} is most commonly measured by one of two methods of "infinite" dilution of the radiolabelled ligand when equilibrium has been reached. Either a 100-fold volume-excess of incubation buffer is added or alternatively a 1000 fold molar excess of unlabelled ligand is added; radiolabelled ligand that remains bound is then measured at intervals. The second method,referred to as the molar-excess method, was chosen in preference to the volume dilution method because apparent negative cooperativity may arise artifactually at high receptor and low ligand concentrations due to the "retention effect". As the receptor site concentration approaches or exceeds the K_D of the radiolabelled ligand, diffusion of dissociated radiolabelled ligand away from free receptor sites is progressively hampered, probably because of the very

high local concentrations of unoccupied binding sites. This does not happen with the molar excess method, as the total concentration of ligand, (labelled and unlabelled) is high and only labelled ligand is infinitely diluted.

Dissociation of [^{125}I]-α-BgTx from the membrane fragments was found to be biphasic; a fast phase in which 50% dissociated in∿ 70 minutes followed by a very slow phase where only 5% dissociated in the next 120 minutes. A similar biphasic pattern of dissociation was observed by Eldefrawi and Eldefrawi (1980) for a preparation from housefly heads; 37% dissociated in the first 10 minutes and 23% in the next 110 minutes. The slower dissociation rate does not represent non-specific binding because the data refer exclusively to those sites that are protectable with 10^{-3}M d-tubocurarine. The faster phase of dissociation follows first-order kinetics (Fig.23) and a half-time of dissociation of 65 minutes was calculated; from the mean of three experiments a k_{-1} of 2.78 x 10^{-4}S was obtained. This value agrees well with values obtained by other workers both for particulate fractions and for a partially purified preparation (Table32) from insect CNS. Half-times of dissociation of around 30 minutes have been calculated for locust, Locusta migratoria, (Breer, 1981) and flyhead homogenates (Jones & Sumikawa, 1981). There is little difference between 65 minutes and 30 minutes when compared to the $t_{0.5}$ for the electroplax receptor-toxin complex which is of the order of days (Rang, 1975; Cohen & Changeux, 1975). A half-time of dissociation of tens of minutes gives a value of 0.15 $t_{0.5}$ of about 9 minutes (see p.154) and therefore rather less than 10% of the bound [^{125}I]-α-BgTx will have dissociated during the separation of free from bound ligand by the centrifugation method,

as the separation time using this method is less than one minute.

These relatively short half-timesof dissociation indicate that α-BgTx binding to the various insect preparations investigated to date, is slowly reversible and in this respect differs markedly from the nAChR of the electroplax and vertebrate skeletal muscle where binding of α-BgTx is essentially irreversible ($t_{0.5} > 100h$), but resembles the putative nAChR from mammalian CNS from which α-BgTx dissociates more rapidly with a $t_{0.5}$ of 5-15 hours (see, Oswald and Freeman, 1981). Furthermore, dissociation of [^{125}I]-α-BgTx from preparations of mammalian brain has been shown to be biphasic for toad (Oswald, 1979) and goldfish (Oswald and Freeman, 1979), a further similarity with the particulate form of the putative nAChR from locust supra-oesphageal ganglia.

In defining a putative cholinergic receptor, demonstration of, high-affinity, specific binding of α-bungarotoxin is in itself inadequate evidence to justify the designation, nicotinic acetylcholine receptor. It must also be shown that the specific binding of α-BgTx is inhibited by other nicotinic ligands. A widely used convention is the expression of inhibition data as IC_{50}'s. The IC_{50} of a particular ligand is that concentration of ligand which will either inhibit 50% of maximum specific binding of another ligand at equilibrium or will retard the initial rate of binding of that ligand by 50%. The former method of estimating the IC_{50}'s of various ligands was chosen for this membrane fragment preparation because less sample is required to obtain a displacement curve than measurement of initial rates over a range of ligand concentrations.

Under certain conditions and allowing for a number of assumptions the IC_{50} for a particular ligand closely approximates to the equilibrium dissociation constant for binding (K_i) for that ligand. It must be assumed that the displacing ligand behaves at the binding site very similarly to the bound ligand and that binding phenomena under investigation follow classic mass action laws. The experimental conditions for $IC_{50} \simeq K_i$ for a particular ligand are related to both the concentration of the labelled ligand i.e. [^{125}I]-α-BgTx in these experiments and the concentration of binding sites. As the concentration of radiolabelled ligand utilized in displacement studies is increased, the difference between the IC_{50} of the unlabelled displacing ligand and the binding dissociation constant of that ligand (K_i) progressively widens, at fixed tissue concentration, according to the following equation:

$$K_i = \frac{IC_{50}}{1 + [L]/K_D}$$

where K_i = binding dissociation constant for unlabelled ligand

IC_{50} = IC_{50} measured for the same unlabelled ligand

[L] = Concentration of radiolabelled ligand, i.e. [^{125}I]-α-BgTx

K_D = Dissociation constant for bound radiolabelled ligand i.e. [^{125}I]-α-BgTx.

The concentration of receptor sites in the incubation medium can also influence the relationship between IC_{50} and the true K_i. Jacobs et al (1975) point out that the inter-relationship between IC_{50}, K_i and K_D and receptor site concentration at steady state is:

$$IC_{50} = n(K_D + L_T + R_T - {}^3/_2\ RL)$$
$$= K_i + n(L_T + R_T - {}^3/_2\ RL)$$

where L_T = Total concentration of radiolabelled ligand [^{125}I]- α-BgTx.

R_T = Total concentration of receptor sites.

RL = Concentration of receptor-radiolabelled ligand complex formed in the absence of unlabelled ligand i.e. maximum specific binding of [^{125}I]-α-BgTx.

and $K_i = n\ K_D$

Therefore if $(L_T + R_T) \ll K_D$, then the IC_{50} will approximate to K_i.

In practice the concentration of total receptor sites should thus not exceed approximately 10% of the K_D for [^{125}I]-α-BgTx binding. Also the concentration of [^{125}I]-α-BgTx present should be not more than a quarter of the concentration of unlabelled ligand giving 50% displacement. The latter point was fulfilled ([^{125}I]-α-BgTx was at 2nM) but carrying out experiments at a receptor site concentration of less than 10% of the K_D for [^{125}I]-αBgTx binding was not possible because at this concentration of receptor sites(<0.2nM) the low amounts of [^{125}I]-αBgTx bound would be very difficult to detect. Therefore a compromise was made and a concentration of 0.4nM of binding sites was chosen as the lowest at which acceptable determination of binding could be made. This may have resulted in an overestimation of K_i. This point of standardising the IC_{50} values is important if one is to compare values quoted from different groups of workers.

The order of potency of ligands in inhibiting [^{125}I] -α-BgTx binding will be the same from studies carried out under differing conditions provided that the conditions are the same within any one study. However, the absolute values cannot be compared between different studies unless the previously mentioned conditions of [^{125}I] -α-BgTx and of binding site concentration are met or if corrections are made to the results.

The most effective inhibitor of specific [^{125}I] -α-BgTx binding to the membrane fragments were nicotinic in nature, α-bungarotoxin

TABLE 33

The Pharmacological Specificity of the High Affinity α-Bungarotoxin Binding Component in Insect Head/Ganglia Subcellular Fractions:

Ligand	IC_{50} (M)				
	LOCUST[1] 100,000 x g pellet	HOUSEFLY[2] 500 x g Supernatant	HOUSEFLY[3] 150,000 x g Supernatant	FRUITFLY[4] Partially Purified	FRUITFLY[5] 500 x g Supernatant
α-Bungarotoxin	8.9×10^{-9}	1.0×10^{-9}	5.01×10^{-10}	-	5×10^{-10}
d-Tubocurarine	3.2×10^{-7}	6.2×10^{-8}	7.94×10^{-9}	6.6×10^{-7}	2×10^{-6}
Benzoquinonium	4.11×10^{-6}	-	-	-	-
Eserine	4.73×10^{-6}	-	-	-	6×10^{-6}
Acetylcholine	6.68×10^{-6}	-	3.6×10^{-6}	3×10^{-6}	2×10^{-5}
Nicotine	3.98×10^{-5}	1.0×10^{-8}	1.99×10^{-8}	3.5×10^{-7}	8×10^{-7}
Atropine	1.12×10^{-4}	-	-	2×10^{-5}	5×10^{-5}
Dexetamide	3.5×10^{-4}	9.5×10^{-3}	1.8×10^{-5}	6×10^{-5}	$> 10^{-4}$
Carbamoylcholine	4.47×10^{-4}	-	-	2×10^{-5}	$> 10^{-4}$
Decamethonium	6.31×10^{-4}	-	3.38×10^{-5}	6×10^{-5}	$> 10^{-4}$
Hexamethonium	$> 10^{-3}$	-	-	-	$> 10^{-4}$

[1]. This Thesis
[2]. Cattell et al., 1980a
[3]. March et al., 1980
[4]. Hall, 1980
[5]. Haim et al., 1978

having an IC_{50} value very close to its K_D measured from equilibrium binding studies (8.89×10^{-9}M and 1.38×10^{-9}M respectively). Of the ligands tested, d-tubocurarine was next to α-bungarotoxin in potency, followed closely by benzoquinonium, eserine, acetylcholine and nicotine in that order. The muscarinic ligands atropine and dexetimide were not very strong inhibitors, having IC_{50}'s of $> 10^{-4}$M. Decamethonium, the ligand used to identify the putative nm AChR was relatively ineffectual, thus lending credence to the hypothesis that there are at least two distinct cholinergic receptors in insects. Hexamethonium, a potent ganglionic blocking agent in vertebrate CNS, also had little effect, as did carbamoylcholine. These results strongly suggest the presence of a binding component of nicotinic specificity and are in general agreement with those for membrane fragments or total homogenates from other insect species (Cattell, 1980a & b; Dudai, 1978; Gepner, 1979; Rudloff, 1978), although in some cases nicotine was found to be more potent an inhibitor than d-tubocurarine (Dudai, 1978; Gepner, 1979).

The absolute values of the IC_{50}'s quoted by different workers for different insect species are also comparable for any one ligand (see Table33). Any differences may be due to the preparations being at different stages of purity such that high levels of non-specific binding could lead to an overestimate of the concentration of ligand necessary to give 50% displacement. Alternatively, the conditions mentioned previously to ensure that $IC_{50} \simeq K_i$, may not have been met. Unfortunately, the great majority of workers do not give sufficient experimental details to allow such an assessment of these data to be made.

Affinity reagents have been designed to specifically label the nAChR in vertebrate skeletal muscle and in electroplax (Karlin, 1969).

These reagents combine the features of two molecules. One consists of a moiety resembling a cholinergic ligand, including a quaternary ammonium ion, which reacts at the negative subsite of the active site of the receptor and confers specificity on the reagent. The other portion of the molecule contains a reactive centre that alkylates sulphydryl groups. If the geometry of the reagent is such that the alkylating centre comes into juxaposition with a reduced sulphydryl on the receptor, when the quaternary ammonium group is bound at the negative subsite of the nAChR, the reagent will alkylate reduced nAChR's with much higher specificity, and at a faster rate than it will react non-specifically with "non-receptor" reduced sulphydryls. Such a reagent is maleimidobenzyltrimethylammonium (MBTA), which alkylates reduced electroplax nAChR's with high affinity, antagonises the flux of ions through the receptor coupled channels and perturbs nicotinic-like neurotoxin binding (Karlin, 1977; Karlin et al., 1971). If the α-BgTx binding component of insects is similar at the molecular level to the nAChR of electroplax, then [^{3}H]MBTA would serve as a useful tool in its further characterisation and, if the α-BgTx binding component of insects reacts in the same way with MBTA as does the nAChR of the electroplax, this would suggest that they bear resemblances at the active site and would provide further supportive evidence for the identity of the α-BgTx binding component of insect CNS as a nAChR.

Affinity labelling also enables the identification of the acetylcholine binding site by electrophoresis on polyacrylamide gels under denaturing conditions of either membranes or the purified receptor, prelabelled with [^{3}H]-MBTA. To date, there have been no reported attempts of using affinity labelling to identify the acetylcholine binding subunit from insect tissue or from mammalian CNS.

In the case of the electroplax and vertebrate muscle nAChR [^{3}H]MBTA has been shown to label a single subunit of about M_r 40,000 (Sobel et al., 1977; Weill et al., 1974; Rubsamen et al., 1978), similar results were obtained for the nAChR from human skeletal muscle (Stephenson, 1980). It has been reported that in the case of rat denervated (Froehner et al., 1977) and innervated muscle (Nathanson and Hall, 1979) two subunits are labelled.

In a preparation of membranes from locust supra-oesophageal ganglia labelled with [^{3}H]MBTA and subsequently examined by SDS-PAGE, only one major peak of radioactivity of M_r 58,000 was apparent. This peak was eliminated by preincubation of the membranes with unlabelled α-BgTx prior to the affinity labelling procedure, thereby indicating a common binding site for α-BgTx and MBTA. This apparent binding site however differs from both electroplax and vertebrate skeletal muscle, with respect to the size of the subunit.

The Solubilised Membranes (Detergent Extract):

A prerequisite for purification of a putative receptor is the presence of that receptor in a soluble form. This requirement would appear to present no problems to March et al. (1980), who achieved solubilisation of the putative receptor from housefly heads upon homogenisation. Other workers (Dudai, 1978; Jimenez and Rudloff, 1980) reported problems with the solubilisation of particulate fractions of fruitfly heads enriched in α-BgTx binding activity. Dudai(1980) found that procedures which totally solubilised the nAChR of vertebrate CNS (Lowry et al., 1976) were inefficient when applied to fruitfly heads. A combination of high ionic strength and non-ionic detergents released 40% of the α-BgTx binding activity into a 100,000 x g supernatant but

90% of this binding activity was sedimented after further centrifugation at 200,000 x g suggesting that it was in the form of small membrane fragments or supramolecular aggregates. The authors suggest that solubilisation is very sensitive to homogenisation procedures and appears to depend upon mechanical parameters involved in tissue disruption. Jimenez and Rudloff (1980) demonstrated complete solubilisation of 30 - 40% of α-BgTx binding activity by treatment of a particulate fraction of fruitfly heads with sodium deoxycholate at pH 9.0. Inclusion of binding activity on a Sepharose 4B gel filtration column was used as a criterion of solubility.

Other workers have reported studies on Triton X-100 solubilised binding components from housefly heads (Eldefrawi and Eldefrawi, 1980) and from fruitfly heads (Hall, 1980). The latter group of workers used release of binding activity into a 30,000 x g supernatant as the criterion for solubilisation but neither study reports the percentage of total binding activity that is recovered in a soluble form.

It was found that approximately 90% of the α-BgTx binding activity from locust supra-oesophageal ganglion membrane fragments was released into either a 100,000 x g or 200,000 x g supernatant after treatment with 1% (v/v) Triton X-100, with approximately a 60% recovery of total binding activity. The amount of specific binding protein released was greater with 1% (v/v) Triton X-100 than with either 0.1% (v/v) or 0.5% (v/v) Triton X-100.

Specific [^{125}I]-α-BgTx binding was proportional to tissue concentration, providing that toxin was in excess. From equilibrium binding studies a K_D of 5.6 nM and a B_{max} of 1.714 pmol/mg protein were calculated. The concentration of binding sites is in agreement with

that for the membrane fragments (1.18 pmol/mg protein) and although of the same order of magnitude, there is a slight increase in the K_D for the detergent extract compared to the membrane fragments. This could be due to the different methods of assay or to interference of the detergent with α-BgTx binding. It has been noted that both ionic and non-ionic detergents can have a marked effect on the affinity for α-BgTx of the nAChR from the electroplax (Sugiyama and Changeux, 1975). Nevertheless the decrease in affinity for α-BgTx of the membranes after solubilisation is relatively small and it is reasonable to assume that the binding kinetics of the membrane fragments are relatively unchanged after treatment with Triton X-100.

Sedimentation in a continuous sucrose gradient of the [^{125}I]-α-BgTx detergent extract complex was attempted but no difference in the distribution of radioactivity was found between the complex and free [^{125}I]-α-BgTx (Fig.30). The most likely explanation for this is dissociation of the binding component-toxin complex occasioned by the change in equilibrium when the complex enters the gradient, thus after the five hour centrifugation, undetectable amounts of [^{125}I]-α-BgTx remain bound. This is plausible in view of the $t_{0.5}$ of approximately 65 minutes calculated for dissociation of the membrane fragments - toxin complex.

The Partially Purified Binding Component:

Purification of the solubilised nAChR from electroplax and from vertebrate skeletal muscle entails adsorption onto an affinity column, washing to remove non-bound material and finally specific elution of the receptor. The most popular affinity columns for such preparations have been of Naja-naja α-toxin coupled to Sepharose 4B. Naja-naja

α-toxin is used in preference to α-BgTx because the latter binds to these receptors essentially irreversibly ($t_{0.5}$ of days) and early studies using affinity columns of α-toxin from N.nigricollis venom, which has a similar $t_{0.5}$ as α-bungarotoxin, failed to achieve any recovery of the bound receptor (Meunier et al., 1971). The acetylcholine analogue carbomoylcholine in high concentrations (1 M) is usually used as an elutant because it binds with relatively low affinity and is therefore easily removed from the receptor by dialysis. However, with membrane fragments from locust supra-oesophageal ganglia, it has been shown firstly, that α-BgTx binds with a much lower affinity to this preparation than to either electroplax or vertebrate skeletal muscle receptors and secondly, that carbamoylcholine has an IC_{50} value of greater than 10^{-4}M and therefore is relatively ineffectual in displacing bound α-BgTx. For these reasons an affinity column of α-BgTx coupled to Sepharose 4B was chosen for further purification of the α-BgTx binding component from the detergent extract of locust supra-oesophageal ganglia and the column was eluted with 1 M acetylcholine which, although it has a lower IC_{50} (6.68×10^{-6}M) than carbamoylcholine, is still easily removed by dialysis. Using this method, the α-BgTx binding component from locust supra-oesophageal ganglia, in the solubilised form, was further purified.

The specific [^{125}I]-α-BgTx binding of activity of the affinity-purified material ranged from 7 - 230 pmol/mg protein and the purification factor (taking the total homogenate as one) varied from 32 - 1100. This figure may be compared with values of 0.19 - 12 nmol/mg protein for the purified nAChR from electroplax and vertebrate skeletal muscle (see Karlin, 1980). However, until more detailed experiments are made for the locust receptor, with respect to molecular weight, subunit

composition and stoichiometry of binding subunits, no meaningful comparison can be made with these well characterised binding receptor proteins.

Although the experimental conditions were identical for every preparation of locust supra-oesophageal ganglia, a consistent specific binding activity and purification factor was not achieved. This may be due to a number of factors, some of which are outlined below, along with possible remedies:

1. To date no study has been made of the extent of proteolysis during purification of the α-BgTx binding component from insect CNS tissue. Although, in this thesis and in the partial purification of a preparation of fruitfly heads, (Hall, 1980) a range of anti-protease agents are included in the buffers used, it is not known whether they are sufficient to completely prevent proteolysis.

Shorr et al (1981) made two major observations with respect to proteolysis when working with purified nAChR from denervated cat skeletal muscle. First, in addition to the 43,000 M_r polypeptide always demonstrated by SDS-PAGE of the purified receptor, a polypeptide of 39,000 M_r was sometimes apparent. Rapid preparation of the receptor in the presence of a wide range of protease inhibitors eliminated this smaller polypeptide which was therefore assumed to be a degradation product of the 43,000 M_r subunit. Secondly, sedimentation in a sucrose density gradient of the native purified receptor yielded two bands of $S_{20,W}$ of 9S and 4S. Both were composed of the same subunits, 43,000 M_r, when subjected to SDS-PAGE but the 4S oligomer disappeared if either the muscle protease inhibitor,

iodoacetamide, was added early in the preparative stages or if fresh rather than frozen muscle was used. These two points exemplify the subtle effects of proteolysis on the behaviour of both the native protein and its constituent subunits. Questions therefore arise, with respect to both fruitfly and locust preparations, as to the extent of proteolysis during purification and whether the binding capacity and/or affinity of the putative receptor is impaired. Therefore, proteolysis could be partly responsible for the variability of specific binding activities from preparation to preparation of locust supra-oesophageal ganglia. A detailed systematic study, using such a preparation, of the effects of different combinations of anti-proteases and preparation times on binding capacity, sedimentation coefficient and subunit composition is necessary before any definite conclusions can be drawn about the effects of proteolysis. This problem seems to highlight yet again our almost total lack of knowledge of many aspects of the biochemistry of insect tissues in that no information is available on the nature of the proteases presumed to be present in these tissues.

2. The overall recovery of total specific [^{125}I]-α-BgTx binding activity after the affinity column stage ranged from 9% - 48%, which is equivalent to a recovery of 18% - 83% of the specific binding activity applied to the column. Therefore, a portion of the binding activity is lost in the affinity chromatography and subsequent dialysis steps, a loss which may be partly due to inefficient elution of the putative

receptor from the affinity column and/or incomplete removal of the acetylcholine by dialysis. The former problem has been minimised in some cases (Shorr et al., 1981) by eluting the affinity column with a combination of eluting ligand and anti-toxin immunoglobulin. The rationale for this procedure is that immunoglobulin will mask any unoccupied toxin and will therefore prevent reassociation of displaced receptor. Constraints are imposed by the difficulty and cost of raising sufficient anti-toxin antisera to routinely adopt this method.

In the case of preparations of AChR from Torpedo electroplax and vertebrate skeletal muscle extensive dialysis of the elutant appears to remove a large proportion but not all of the eluting ligand from the putative receptor. Thus, preparations that are subjected only to dialysis do not show such high specific activities as the preparations in which dialysis is followed by ion-exchange chromatography on DEAE cellulose (Fulpius, 1976; Stephenson, 1980). The eluting ligand not removed (in the case of the work reported in this thesis, acetylcholine) will consequently interfere with the binding assay, giving an underestimation of the concentration of binding sites. However, because the amount of protein was so small in the preparations described here an ion-exchange step was not included in the purification procedure.

3. The amount of protein recovered was low and just fell within the lower limits of detection by the Lowry method. Ideally a more sensitive method of protein estimation or

recovery of greater amounts of protein would eliminate any errors in the calculation of specific activity which may result from inaccuracies in protein determination.

An additional step in the purification procedure of the nAChR from denervated vertebrate skeletal muscle (Shorr et al., 1981) has exploited the glycoprotein nature of the receptor. The eluate from an α-toxin affinity column was chromatographed on lentil lectin immobilised on Sepharose 4B. An increased specific activity resulted but the yield was decreased by more than one half. Herein, lies the problem of applying the partially purified α-bungarotoxin binding component from locust supra-oesophageal ganglia to a lectin affinity column but such procedures should be borne in mind for future use, when the yield has been improved.

Specific binding to the partially purified α-BgTx binding component from locust supra-oesophageal ganglia increased linearly with protein concentration and showed the same binding kinetics as the P_2 membrane fragments, K_D's 1.71 nM and 1.38 nM respectively, as calculated from binding studies at equilibrium. Binding, as before, was non-cooperative (Hill number = 1). These binding data suggest that little change in the conformational structure of the α-BgTx binding component has occurred during the purification procedure. However, the dissociation of [^{125}I]-α-BgTx from this partially purified preparation is monophasic and faster ($t_{0.5}$ < 1 min) than from the membrane fragments ($t_{0.5}$ ≃ 65 minutes). This suggests, perhaps, that the biphasic nature of dissociation of [^{125}I]-α-BgTx from the membrane fragments could be an inherent property of the putative receptor in situ, a characteristic which is lost on removal of the receptor from its membrane environment.

The difference in $t_{0.5}$ for the partially purified preparation and the membrane fragments is not so large when compared to the $t_{0.5}$ for electroplax and vertebrate skeletal muscle receptor-toxin complexes which is of the order of days. Nonetheless, such a fast $t_{0.5}$ imposes restrictions on using the [^{125}I]-α-BgTx labelled binding component in further characterisation. Vacuum filtration on DEAE cellulose discs was used to separate bound [^{125}I]-α-BgTx from free toxin and as filtration and washing was complete within 6 seconds little dissociation will have occurred in this time.

The K_D calculated using k_{-1} was $> 7.25 \times 10^{-9}$M, (p.148) which is a factor of ten times higher than the K_D calculated in the same way for the membrane fragments (2.78×10^{-10}M) but is in close agreement with the K_D calculated from equilibrium binding studies for either preparation (1.38×10^{-9}M and 1.71×10^{-9}M for the membrane fragments and the partially purified binding components respectively). For this reason the rate of dissociation of [^{125}I]-α-BgTx from the partially purified receptor as opposed to the membrane fragments was taken as the more reliable estimate of k_{-1}.

Sedimentation in a continuous secrose density gradient of the paritally purified α-BgTx binding component was carried out without pre-incubation with [^{125}I]-α-BgTx (see p.161). Instead, the unlabelled α-BgTx binding component was sedimented and each fraction was assayed for [^{125}I]-α-BgTx binding activity. A sedimentation coefficient of 14.2s was obtained which is higher than the value measured for solubilised receptors from electroplax and from vertebrate skeletal muscle (9S) (Shorr et al., 1981; Stephenson, 1980). However, sedimentation coefficients higher than 9s have also been reported for putative receptors from both mammalian CNS (11.4S - 12.9S)

(Seto et al., 1977; Salvaterra and Mahler, 1976) and from preparations of fruitfly heads (11.4S) (Gepner, 1979) and therefore similarities can be drawn between these putative receptors and the partially purified α-BgTx binding component from locust supra-oesophageal ganglia.

The solubilised Torpedo nAChR has been found to exist in both a 9S and 13S form and the 13S is apparently a dimer of the 9S form (Raftery et al., 1979). A high sedimentation coefficient for the α-BgTx binding component prepared from locust supra-oesophageal ganglia could be due to the presence of a dimer. However, no smaller form of the α-BgTx binding component, which could be attributed to a possible monomer, was detected. Clearly, until further detailed characterisation of this binding component is made such questions must remain open.

A M_r of 400,000 - 450,000 was estimated from the value of 14.2S for the α-BgTx binding component by the approximation of Schachman (1959). It is probable that this value is an under-approximation of the molecular weight of the binding species due to the presence of bound detergent. Tanford et al (1974), have shown that there is a significant decrease in buoyant density of protein following the binding of either lipid material or detergents. Thus, it follows that there is an increase in partial specific volume of the protein, and as the molecular weight of a protein is related to the sedimentation coefficient by the formula:-

$$M = \frac{RTS}{D\ (1 - \bar{\gamma}\ p)}$$

Where $\bar{\gamma}$ is the partial specific volume, it follows that an apparent increase in $\bar{\gamma}$ would yield an erroneously low value for S and hence a low molecular weight with respect to marker proteins that do not bind detergent. The problem can be circumvented with no knowledge of the

percentage detergent bound to a protein by adjusting the solvent density with D_2O to blank out the contribution of bound detergent to the sedimentation coefficient (Reynolds and Tanford, 1976).

Polyacrylamide gel electrophoresis under denaturing conditions of the partially purified α-BgTx binding component from locust supra-oesophageal ganglia yielded three faint but distinct protein bands. These bands corresponded to M_r's of 60,000, 41,000 and 25,000. The 60,000 M_r subunit, allowing for experimental error, closely corresponds to the subunit that is labelled by the affinity reagent [^{3}H]-MBTA. A recent report from Hall (1981) tentatively suggests that a 60,000 M_r subunit from a preparation of fruitfly heads carries the AChR binding site, on the basis of studies in which this subunit was photoaffinity labelled with a deri vative of α-BgTx. In electroplax and in vertebrate skeletal muscle preparations, however, it is the subunit of ∿ 40,000 M_r which binds both MBTA and α-BgTx. Herein lies a possible major difference between the nAChR from either electric fish electroplax or vertebrate skeletal muscle and the putative nAChR from insect CNS.

The 24,000 M_r subunit revealed by SDS-PAGE of the partially purified α-BgTx binding component may be due to proteolytic degradation of one of the larger subunits. In the well-characterised receptor from electroplax and vertebrate skeletal muscle it is generally agreed that subunits with molecular weights lower than 40,000 are the results of proteolysis (Bartfeld and Fuchs, 1979; Lindstrom et al., 198Cb). The purified nAChR from Torpedo electroplax is reported to have four different subunits of M_r's 40,000 (α); 50,000 (β); 57,000 (γ); 64,000 (δ), present in the ratio 2:1:1:1 (see Heidmann and Changeux, 1978) whereas the existence of only two subunits of ∿ 40,000 and ∿ 60,000 M_r's has been reported for human (Stephenson, 1980) and rat (Kemp et al., 1980)

skeletal muscle receptors. The latter observations are in general agreement with the subunit patterns reported here for the partially purified α-BgTx binding component from locust supra-oesophageal ganglia. The subunit composition of the vertebrate muscle nAChR is however still the subject of considerable debate. Thus the group of Barnard and Dolly have produced compelling evidence that the nAChR from cat muscle contains a single subunit of $M_r \sim 40,000$ (Shorr et al., 1981) whereas Lindstrom and co-workers find that foetal calf muscle has an identical subunit pattern to Torpedo electroplax AChR (Lindstrom et al., 1980a). It is possible that acetylcholine receptors from all sources have a unique quaternary subunit structure and that the observed differences simply reflect differing degrees of proteolysis and this is the view expressed by Lindstrom and co-workers (Lindstrom et al., 1979; 1980_a). However may workers have taken stringent precautions to minimize such effects (Shorr et al., 1981, Stephenson et al., 1981) and it seems premature to reach such a conclusion. Our appreciation of exactly how the insect receptors fit into the patterns of structural composition of nAChR's must await further detailed studies of subunit composition.

Acetylcholine Esterase Activity:

A slight enrichment of acetylcholine esterase activity was apparent in the particulate fractions of the preparation. The partially purified α-BgTx binding component exhibited no enzyme activity thereby confirming that the binding component for α-BgTx and the enzyme are distinct. This is in agreement with the well established findings in the electroplax (Meunier et al., 1971).

In conclusion, an α-BgTx binding component has been partially purified from locust supra-oesophageal ganglia. Binding of [^{125}I]-α-BgTx

increased linearly with protein concentration in the particulate, in the solubilised and in a partially purified preparation. The binding was in each case of high affinity, specific and saturable. The pharmacological characteristics of the membrane fragments were predominantly nicotinic. Taking all these points into consideration with respect to the criteria for defining a receptor (p.15), the α-BgTx binding component of locust supra-oesophageal ganglia can tentatively be termed a nicotinic acetylcholine receptor. The partially purified nAChR from locust supra-oesophageal ganglia sediments as a single component $S_{20,W}$ = 14.2S, from which an approximate value of M_r 400,000 - 450,000 has been calculated. SDS-PAGE revealed two major subunits, M_r 41,000 and 60,000, of which only the 60,000 subunit was labelled with [^{3}H]-MBTA.

Further work on this putative nAChR from locust supra-oesophageal ganglia should be primarily concerned with improvement of the purification procedure and with increasing the yield, thereby facilitating a more intensive and thorough characterisation.

The problem of detection and analysis of very small amounts of nAChR has been overcome by some workers by radioiodination of the purified receptor (Stephenson, 1980; Shorr et al., 1981). The same technique may also be applied to the partially purified nAChR from locust supra-oesophageal ganglia which may enable detection of not only small amounts of the native receptor but also small amounts of its constituent subunits.

Further characterisation and comparison with other nAChR's should include investigation of the immunological properties of this putative insect nAChR. A preliminary study by Eldefrawi and Eldefrawi (1980) demonstrated that there was no cross-reactivity between a detergent solubilised α-BgTx binding component from housefly heads and antibodies

raised in sheep against purified Torpedo nAChR. The authors concluded that the α-BgTx binding components of Torpedo electroplax and housefly heads differed with regard to the antigenic site(s). However, it should be borne in mind that the cross reactivity between Torpedo and other nAChR from vertebrate muscle is very low (Lindstrom et al., 1979; Garlepp et al., 1981). It would be interesting, therefore, to compare the cross-reactivity and hence antigenic similarities of putative insect AChR's with antibodies raised against receptors from vertebrate skeletal muscle and mammalian CNS. In addition a different antigenic pattern for the three putative acetylcholine receptors from insect CNS would be strong evidence for three distinct receptors.

A novel approach to producing the nAChR in large quantities would be the insertion of the gene responsible for acetylcholine receptor synthesis into the DNA of a prolific bacterium. The difficulty in such a study is the identification of the AChR gene. Hall et al (1978;Hall, 1980) have approached the problem of gene identification by investigating drug resistant mutants of fruitfly on the premise that resistance to the cholinergic drug nicotine may be indicative of a change in AChR structure. This they found to be the case and observed a change in the isoelectric point of the solubilised α-BgTx binding component from the resistant strain compared to the wild type. Their conclusion was that the mutated gene of nicotine resistant fruitfly coded for a structural polypeptide in the α-BgTx binding complex, i.e. the putative nAChR. However, it is highly probable that more than one gene codes for the AChR, therefore considerably more work is necessary to identify all the genes required for a complete AChR. This study however serves to demonstrate the ease of genetic manipulation of fruitfly in comparison

to higher organisms. In this respect, insects, especially the fruit-fly, are an extremely useful tool in specific gene identification.

Recently Sumikawa et al (1981) have reported the extraction of AChR mRNA from Torpedo and have shown it to be translated in a cell-free reticulocyte system and also in Xenopus oocytes. The latter efficiently assembled intact, multi-subunit AChR molecules which showed properties characteristic of the native AChR, including the binding of α-BgTx. The suggested use of such a system is in the elucidation of mechanismsby which the receptor molecule is assembled and inserted into the membrane. Such an investigation could provide evidence in support of the "signal hypothesis" of orientation of proteins in the membrane (see Lodish and Rothman, 1979; Harrison and Lunt, 1980). It is thought that the N-terminal section of the nascent peptide carries a "signal"sequence which directs the insertion of the peptide into the endoplasmic reticulum whereupon the signal sequence is removed, initial sugar residues are added and the C-terminal section of the polypeptide is completed. The membrane glycoprotein is now transferred to the Golgi apparatus, where carbohydrate incorporation is largely completed. Finally, the glycoprotein, still membrane bound, migrates to and fuses with the plasma membrane. Throughout the entire process the asymmetric orientation of the glycoprotein, with respect to the cytoplasm, is maintained. In the Xenopus oocyte system (Sumikawa et al., 1981) the AChR is both synthesised and inserted into the membrane and may therefore prove to be a particularly appropriate system in which to investigate the possible functions of carbohydrate residues of the receptor. Wonnacott et al (1980a) have shown that removal of the carbohydrate from the purified Torpedo nAChR has no effect on the binding of α-BgTx. Further work by Wonnacott et al (1980b) suggests

that the carbohydrate residues may have only a minor role as antigenic determinants. It is possible therefore that the major function of the glycosyl residues is to maintain the orientation of the receptor in the membrane during assembly of the synaptic membrane.

The major problem in the study in vitro of AChR synthesis is identification of the AChR, mRNA. Sumikawa et al (1981) identified the specific mRNA by screening of all the proteins produced by the cell-free reticulocyte system; this is very laborious work but these same techniques could be applied to the mRNA from insects.

If the AChR from insect CNS could be produced in large quantities, from cloning studies, this would greatly accelerate its characterisation and may also provide a novel system for the screening of potential insecticides.

REFERENCES

Anderson, C.R., Cull-Candy, S.G., & Miledi, R. (1976) Nature (London) 261, p. 151.

Anderson, C.R., Cull-Candy, S.G. & Miledi, R. (1977) Nature (London) 268, p. 663.

Anderson, C.R., Cull-Candy, J.G. & Miledi, R. (1978) J. Physiol. 282, p. 219.

Anderson-Cedergren, E. (1959) J. Ultrastr. Res. (suppl.) 1, p. 1.

Anholt, R., Lindstrom, J. & Montal, M. (1980) Eur. J. Biochem., 109, p. 481.

Anwyl, R. (1975) In: "Perspectives in Experimental Biology, Vol. 1, Zoology" (Ed. P. Spencer Davies) p. 361, Pergamon, Oxford.

Anwyl, R. (1977a) J. Physiol. (London) 273, p. 367.

Anwyl, R. (1977b) J. Physiol. (London) 273, p. 389.

Anwyl, R. & Usherwood, P.N.R. (1974a) Nature (London) 252, p. 591.

Anwyl, R. & Usherwood, P.N.R. (1974b) J. Physiol. (London) 242, p. 86P.

Anwyl, R. & Usherwood, P.N.R. (1975a) Nature (London), 257, p. 410.

Anwyl, R. & Usherwood, P.N.R. (1975b) J. Physiol. (London), 254, p. 46P.

Axelsonn, J. & Thesleff, S. (1959) J. Physiol. (London), 147, p. 178.

Balerna, M., Fosset, M., Chicheportiche, R., Romey, G. & Lazdunski, M. (1975) Biochemistry, 14, p. 5500.

Barnard, E.A., Dolly, J.O., Lang, B., Lo, M. & Shorr, R.G. (1979) In: "Advances in Cytopharmacology" (Ed. Ceccarelli, B. & Clementi, F.) Vol. 3, pp. 409-436, Raven Press, New York.

Barrantes, F.J., Changeux, J-P., Lunt, G.G. & Sobel, A. (1975) Nature, 256, p. 325.

Bartfeld, D. & Fuchs, S. (1979) Biochem. Biophys. Res. Comm., 89 (2), p. 512.

Baudry, M. & Lynch, G. (1979) Nature (London), 282, p. 748.

Baudry, M. & Lynch, G. (1980) Proc. Natl. Acad. Sci. USA., 77 p. 2298.

Baudry, M. & Lynch, G. (1981) J. Neurochem., 26, p. 811.

Baudry, M., Oliver, M., Creager, R., Weirasko, A. & Lynch, G. (1980) Life Sci., 27, p. 325.

Bennett, J.P. (1978) In: "Neurotransmitter Receptor Binding", (Ed. Yamamura, H.I., Enna, S.J. & Kuhar, M.J.), Raven Press, New York, p. 57.

Berg, D.K., Kelly, R.B., Sargent, P.B., Williamson, P. & Hall, Z.W. (1972) Proc. Natl. Acad. Sci. USA, 69, p. 147.

Bergmeyer, H.U. (1974) In: " Methods of Enzymic Analysis", Academic Press, London and New York.

Biesecker, G. (1973) Biochem., 12, p. 4403.

Birdsall, N.J.M. & Hulme, E.C. (1976) J. Neurochem., 27, p. 7.

Biziere, K., Thompson, H. & Coyle, J.T. (1980) Brain Res., 183, p. 421.

Bradford, H.F. (1972) In: "Methods in Neurochemistry" (Ed. Freid, R.) Vol. 3 p. 155, Marcel Dekker Inc., New York.

Breer, H. (1981) Neurochem. Int., 3, p. 43.

Briley, P.A. (1981) Ph.D. Thesis, University of Bath, Bath.

Briley, P.A., Filbin, M.T., Lunt, G.G. & Turner, P.D. (1981) Molec. Cell Biochem. (in press).

Bullock, T.H. & Horridge, G.A. (1965) In: "Structure and Function in the Nervous Systems of Invertebrates". W.H. Freeman & Co., London.

y Cajal, S.R. (1888) Rev. Trimest. Histol. Norm. Patal., 1, p. 305.

y Cajal, S.R. (1890) Z. Wiss. Mikrosk., 7, p. 332.

Callec, J.-J. & Sattelle, D.B. (1973) J. Exp. Biol., 59, p. 725.

Cariello, L. & Zanetti, L. (1977) Comp. Biochem. Physiol., 57C, p. 169.

Cariello, L. & Zanetti, L. (1979) Comp. Biochem. Phsyiol., 64C p. 15.

Cattell, K.J. & Donnellan, J.F. (1972) Biochem. J., 128, p. 187.

Cattell, K.J., Harris, R. & Donnellan, J.F. (1980a) In: "Receptors for Neurotransmitters, Hormones and Pheromones in Insects", (Ed. Sattelle, D.B., Hall, L.M. & Hildebrand, J.G.) p. 71.

Cattell, K.J., Harris, R. & Donnellan, J.F. (1980b) In: "Insect Neurobiology and Pesticide Action Neurotox 79", p. 209. London: Society of Chemical Industry.

Chance, B. & Sacktor, B. (1958) Archs. Biochem. Biophys., 76, p. 509.

Chang, H.H. & Michaelis, E.K. (1980) J. Biol. Chem., 255, p. 2411.

Changeux, J.-P. (1975) In: "Handbook of Psychopharmacology" (Ed. Iverson, L.L.), Vol. 6., pp. 235-301, Plenum Press, New York.

Changeux, J.-P., Benedetti, L., Bourgeois, J.P., Brisson, A., Cartaud, J., Devaux, P., Grunhagen, H., Moreau, M., Popot, J.L., Sobel, A. & Weber, M. (1976) In: "The Synapse, Cold Spring Harbor Symp. on Quantitative Biology", Vol. XL, p. 211.

Chen, P.S., Kubli, E. & Manimann, F. (1968) Rev. Suisse Zool., 75, p. 509.

Clarke, B.S. & Donnellan, J.F. (1974) Biochem. Soc. Trans., 2, p. 1373.

Clark, R.B., Gration, K.A.F. & Usherwood, P.N.R. (1979a) Br. J. Pharmac., 66, p. 267.

Clark, R.B., Gration, K.A.F. & Usherwood, P.N.R. (1979b) J. Physiol., 290, p. 551.

Cleworth, J.F., Robinson, N.L. & Usherwood, P.N.R. (1980) In: "Insect Neurobiology and Pesticide Action Neurotox 79" p. 280 London: Society of Chemical Industry.

Clements, A.N. & May, T.E. (1974) J. Exp. Biol., 60, p. 673.

Cochrane, D.G., Elder, H.Y. & Usherwood, P.N.R. (1972) J. Cell. Sci., 10, p. 419.

Cohen, J.B. & Changeux, J-P. (1975) A. Rev. Pharmac., 15, p. 83.

Colhoun, E.H. (1963) In: "Advances in Insect Physiology" (Ed. Beament, J.W.L., Treherne, J.E. & Wigglesworth, V.W.) 1, p. 1, Academic Press, London and New York.

Creese, I. (1978) In: "Neurosciences Res. Prog. Bull.", 16, p. 498.

Cull-Candy, S.G. (1975) Nature (London), 258, p. 449.

Cull-Candy, S.G. (1976) J. Physiol. (London), 255, p. 449.

Cull-Candy, S.G. (1978) J. Physiol., 276, p. 165.

Cull-Candy, S.G. & Usherwood, P.N.R. (1973) Nature (London), New Biol., 246, p. 62.

Cull-Candy, S.G., James, R.W. & Lunt, G.G. (1976) Nature (London), 262, p. 408.

Dale, H.H. (1914) J. Pharmacol. Exp. Ther., 6, p. 147.

Dale, H.H., Feldberg, W. & Vogt, M. (1936) J. Physiol. (London), 86, p. 353.

Daoud, A. & Miller, R. (1976) J. Neurochem., 26, p. 119.

Davies, J., Evans, R.H., Francis, A.A., Jones, A.W. & Watkins, J.C. (1980) In: "Neurotransmitters and their Receptors". (Ed. Littauer, U.Z., Dudai, Y., Silman, I., Teichberg, V.I. & Vogel, Z.). John Wiley & Sons Ltd. p. 333.

Del Castello, J. & Katz, B. (1955) J. Physiol. (London) 128, p. 157.

De Robertis, E. (1967) In: "Nerve as a Tissue". (Ed. Rodahl, K. & Issekutz, B.), Harper & Row, New York, Ch. 4.

De Robertis, E. (1975) In: "Synaptic Receptors", Marcel Dekker, New York.

De Robertis, E. & Fiszer de Plazas, S. (1976) J. Neurochem., 26, p. 1237.

Dolly, J.O. (1979) In: "Physiological and Pharmacological Biochemistry" (Ed. Tipton, K.) Vol. 26 pp. 257-309, University Park Press, Baltimore.

Dolly, J.O. & Barnard, E.A. (1974) FEBS Lett., 46, p. 145.

Donnellan, J.F. & Harris, R. (1977) Biochem. Soc. Trans., 5, p. 852.

Donnellan, J.F., Jenner, D.W. & Ramsey, A. (1974) Insect Biochem., 4, p. 243.

Donnellan, J.F., Jewess, P.J. & Cattell, K.J. (1975) J. Neurochem., 25, p. 623.

Donnellan, J.F., Barker, M.D., Wood, J. & Beechey, R.B. (1970) Biochem. J., 120, p. 467.

Dowson, R.J. & Usherwood, P.N.R. (1973) J. Insect Physiol., 19, p. 355.

Dudai, Y. (1977) FEBS Lett., 76, p. 211.

Dudai, Y. (1978) Biochim. Biophys. Acta., 539, p. 505.

Dudai, Y. (1980) In: "Receptor for Neurotransmitters, Hormones and Pheromones in Insect". (Ed. Sattelle, D.B., Hall, L.M. & Hildebrand, J.G.) p. 71. Elsevier/North Holland, Amsterdam.

Dudai, Y. & Amsterdam, A. (1977) Brain Res., 130, p. 551.

Dudai, Y. & Ben-Barak, J. (1977) FEBS Lett., 81, p. 134.

Eccles, J.C. & Jaeger, J.C. (1958) Proc. Roy. Soc. B. (London), 148, p. 38.

Edwards, G.A. (1959) J. Biophys. Biochem. Cytol. (5), 241, p. 65.

Edwards, G.A., Ruska, H. & de Harven, E. (1958) J. Biophys. Biochem. Cytol., 4, p. 107.

Eldefrawi, A.T. & Eldefrawi, M.E. (1980) In: "Receptors for Neurotransmitters, Hormones and Pheromones in Insects".(Ed. Sattelle, D.B., Hall, L.M. & Hildebrand, J.G.) p. 59. Elsevier/North Holland, Amsterdam.

Eldefrawi, A.T. & O'Brien, R.D. (1970) J. Neurochem., 17, p. 1287.

Eldefrawi, M.E., Eldefrawi, A.T. & O'Brien, R.D. (1970) J. Agri. Food. Chem., 18, p.1113.

Eldefrawi, M.E., Eldefrawi, A.T. & O'Brien, R.D. (1971) Mol. Pharmacol., 7, p. 104.

Elder, H.Y. (1975) In: "Insect Muscle" (Ed. Usherwood, P.N.R.) Academic Press, New York and London.

Ellman, G.L., Courtney, D., Andres, V. & Featherstone, R.M. (1961) Biochem. Pharmac., 7, p. 88.

Faeder, I.R. & Salpeter, M.M. (1970) J. Cell Biol., 46, p. 300.

Faeder, I.R., Matthews, J.A. & Salpeter, M.M. (1974) Brain Res. 80, p.53.

Fain, J.N. (1978) In: "Receptors and Recognition". Series A, No. 6 (Ed. Cuatrecasas, P. & Greaves, M.F.) p. 1, Chapman and Hall, London.

Foster, A.C. & Roberts, P.J. (1978) J. Neurochem., 31, p. 1467.

Froehner, S.C., Reiness, C.G. & Hall, Z.W. (1977) J. Biol. Chem., 252, p. 8589.

Frontali, N. (1958) J. Insect Physiol., 1, p. 319.

Frontali, N., Piazzi, R. & Scopelliti, R. (1971) J. Insect Physiol., 17, p. 1833.

Fulpius, B.W. (1976) In: "Motor Innervation of Muscle", (Ed. Thesleff, S.) p. 1. Academic Press, London, New York and San Francisco.

Garlepp, M.J.H., Kay, P.H., Dawkins, R.L., Buchnall, R.C. & Kemp. A. (1981) Muscle and Nerve, 4, p. 282.

Gepner, J.I. (1979) Ph.D. Thesis, Massachusetts Institute of Technology, Cambridge, Mass., USA.

Gerschenfeld, H.M. (1973) Physiol. Rev., 53, p. 1.

Gilmour, D. & Robinson, P.M. (1964) J. Cell Biol., 21, p. 385.

Ginetzinsky, A.G. & Shamarina, N.M. (1942) Usp. Sovrem. Biol., 15, p. 283.

Goodman, C.S. & Spitzer, N.C. (1979) Nature (London), 280, p. 208.

Gration, K.A.F., Clark, R.B. & Usherwood, P.N.R. (1979) Brain Res., 171, p. 267.

Gray, E.G. & Whittaker, V.P. (1962) J. Anat. (London), 96, p. 79.

Grubbs, R.D. & Michaelis, E.K. (1979) Soc. Neurosci. Abst., 5, p. 304.

Haim, N., Nahum, S. & Dudai, Y. (1979) J. Neurochem., 32, p. 543.

Hall, L.M. (1980) In: "Receptors for Neurotransmitters, Hormones and Pheromones in Insects". (Ed. Sattelle, D.B., Hall, L.M. & Hildebrand, J.G.) p. 111. Elsevier/North Holland, Amsterdam.

Hall, L.M. (1981) In: "Neuropharmacology of Insects CIBA Found. Symp. 88" (in press).

Hall, L.M. & Teng, N.N.H. (1975) In: "Developmental Biology - Pattern Function - Gene Regulation" ICN - UCLA Symposia on Molecular and Cellular Biology (Ed. McMahon, D. & Fox, C.F.) Vol. 2 p. 282. Benjamin, Menlo Park, California.

Hall, L.M., Von Borstel, R.W., Osmond, B.C., Hoeltzli, S.D. & Hudson, T.H. (1978) FEBS Lett., 95, p. 243.

Hanson, J. (1956) Biochim. Biophys. Acta., 20, p. 289.

Harris, R., Cattell, K.J. & Donnellan, J.F. (1979) Biochem. Soc. Trans., 7, p. 136.

Harris, R., Cattell, K.J. & Donnellan, J.F. (1980) In: "Insect Neurobiology and Pesticide Action Neurotox 79". London: Society of Chemical Inudstry p. 213.

Harrison, R. & Lunt, G.G. (1980) In "Biological Membranes". 2nd Edn. pp. 124-128. Blackie, Glasgow and London.

Heidman, T. & Changeux, J-P. (1978) Ann. Rev. Biochem., 47, p. 317.

Heidmann, T., Sobel, A., Popet, J.L. & Changeux, J-P. (1980) Eur. J. Biochem., 110, p. 35.

Hess, A. (1972) Brain Res., 46, p. 287.

Hildebrand,J.G., Hall, L.M. & Osmond, B.C. (1979) Proc. Natl. Acad. Sci. USA, 76, p. 499.

Hoyle, G. (1957) In: "Comparative Physiology of the Nervous Control of Muscular Contraction", Pergamon Press, London

Huxley, A.F. & Niedergerke, R. (1954) Nature, 173, p. 971.

Huxley, H.E. & Hanson, J. (1954) Nature, 173, p. 973.

Huxley, H.E. & Hanson, J. (1956) Proc. Stockholm Conf. on Electron Microscopy, p. 202.

Irving, S.N. & Miller, T.A. (1980) In: "Insect Neurobiology and Pesticide Action Neurotox 79". p. 227, London: Society of Chemical Industry.

Irving, S.N. & Osborne, M.P. (1976) Nature (London), 263, p. 431.

Jacobs, S., Chang, K.J. & Cuatrecasas, P. (1975) Biochem. Biophys. Res. Commun., 66 , p. 687.

James, R.W. (1977) Ph.D. Thesis, Bath University, Bath.

James, R.W., Lunt, G.G. & Donnellan, J.F. (1977) Insect Biochem., 7, p. 247.

Jewess, P.J., Clarke, B.S. & Donnellan, J.F. (1975) Croat. Chem. Acta., 47, p. 459.

Jimenez, F. & Rudloff, E. (1980) FEBS Lett., 113, p. 183.

Johnston, G.A.R., Kennedy, S.M.E. & Twitchin, B. (1979) J. Neurochem., 32, p. 121.

Jones, D.G. (1975) In: "Synapses and Synaptosomes", Chapman & Hall, London.

Jones, S.W. & Sumikawa, K. (1981a) J. Neurochem., 36, p. 447.

Jones, S.W. & Sumikawa, K. (1981b) J. Neurochem., 36, p. 454.

Karlin, A. (1969) J. Gen. Physiol., 54, p. 245s.

Karlin, A. (1977) In: "Pathogenesis of the Human Muscular Dystrophies" (Ed. Rowland, L.P.) p. 73. Excerpa Medica, Amsterdam.

Karlin, A. (1980) In: "The Cell Surface and Neuronal Function". Ch. 6, p. 191. Elsevier/North Holland, Biomedical Press.

Karlin, A., Prives, J., Deal, W. & Winnik, M. (1971) J. Mol. Biol., 61, p. 668

Katz, B. & Miledi, R. (1972) J. Physiol. (London), 244, p. 665.

Katz, B. & Thesleff, S. (1957) J. Physiol. (London), 138, p. 63.

Kemp, G., Morley, B., Dwyer, D. & Bradley, R.J. (1980) Memb. Biochem., 3, p. 229.

Kerkut, G.A., Shapira, A. & Walker, R.J. (1965a) Comp. Biochem. Physiol., 16, p. 37.

Kerkut, G.A., Leake, L.D., Shapira, A., Cowan, S. & Walker, R.J. (1965b) Comp. Biochem. Physiol., 15, p. 485.

Kolomytkin, O.V., Kuznetsov, V.I. & Akov, I.G. (1979a) Zh. Biol. Khim. 1979 Abstr. 5 F 46.

Kolomytkin, O.V., Kuznetsov, V.I. & Ermishkin, L.N. (1979b) Deposited Doc VINITI p. 1672.

Krogsgaard-Larsen, P., Honore, T. & Hansen, J.J. (1980) Nature, 284, p. 64.

Kuznetsov, V.I. & Mogilyanskii, D.N. (1975) Stud. Biophys., 49, p. 61.

Langcake, P.R. & Clements, A.N. (1974) Insect Biochem., 4, p. 225.

Langley, J.N. (1905) J. Physiol. (London), 33, p. 374.

Lea, T.J. & Usherwood, P.N.R. (1973) Comp. Gen. Pharmacol., 4, p. 333.

Leake, L.D. & Walker, R.J. (1980) In: "Invertebrate Neuropharmacology" Blackie, Glasgow and London.

Lee, C.Y. & Chang, C.C. (1966) Mem. Inst. Butantan. Internac., 33, p. 555.

Lewis, S.E. (1953) Nature (London), 172, p. 1004.

Lindstrom, J. Cooper, J. & Tzartos, S. (1980a) Biochemistry, 19, p. 1454.

Lindstrom, J., Walter, B. & Einarson, B. (1979) Biochemistry, 18, p. 4470.

Lindstrom, J., Gullick, W., Conti-Tronconi, B. & Ellisman, M. (1980b) Biochemistry, 19, p. 4791.

Lindstrom, J., Anholt, R., Einarson, B., Engel, A., Osame, M. & Montal, M. (1980c) J. Biol. Chem., 255, (17) p. 8340.

Lodish, H.F. & Rotham, J.E. (1979) Scientific American, 240, p. 35-53.

Lowry, J., McGregor, J., Rosenstone, J. & Schmidt, J. (1976) Biochemistry, 15, p. 1522.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951) J. Biol. Chem., 193, p. 265.

Lunt, G.G. (1973) Comp. Gen. Pharmacol., 4, p. 75.

McDonald, T.J. (1972) Comp. Gen. Pharmacol., 3, p. 319.

McDonald, T.J., Farley, R.D. & March, R.B. (1974) J. Insect Physiol., 20, p. 1761.

Mandelstom, Y.E. (1967) In: "Symposium on Neurobiology of Invertebrates" (Ed. Salanki, J.) Plenum Press, New York. p. 49.

Mansour, N.A., Eldefrawi, M.E. & Eldefrawi, A.T. (1977) Biochem., 16, p. 1426.

Mansour, N.A., Pessah, I.N. & Eldefrawi, A.T. (1980) In: "Insect Neurobiology and Pesticide Action Neurotox 79" p. 201. London: Society of Chemical Industry.

March, C.S., Cattell, K.J. & Donnellan, J.F. (1980) Biochem. Soc. Trans., 591, p. 742.

March, S. C., Parikh, I. & Cuatrecasas, P. (1974) Analyt. Biochem., 60, p. 145.

Maruyama, K. (1965) In: "The Physiology of Insecta" (Ed. Rockstein, M.) 2, Academic Press, New York and London p. 451.

Mathers, D.A. & Usherwood, P.N.R. (1978) Comp. Biochem. Physiol., 60C, p. 7.

May, T.E. & Piek, T. (1979) J. Insect Physiol., 25, p. 685

Meunier, J.C., Huchet, M., Boquet, P. & Changeux, J-P. (1971) Comp. Rend. Acad. Sci. Paris, 272, p. 117.

Meunier, J.C., Olsen, R.W., Menez, A., Fromageat, P., Boquet, P. & Changeux, J-P. (1972) Biochem., 11, p. 1200.

Michaelis, E.K. (1975) Biochem. Biophys. Res. Comm., 65, p. 1004.

Michaelis, E.K. (1979) Biochem. Biophys. Res. Comm., 87, p. 106.

Michaelis, E.K., Grubbs, R.D., Belieu, R.M. & Michaelis, M.L. (1980) Proc. Natl. Acad. Sci. USA (in press).

Michaelis, E.K., Michaelis, M.L. & Boyarsky, L.L. (1974) Biochim. Biophys. Acta, 367, p. 338.

Michaelis, E.K., Mulvaney, M.J. & Freed, W.J. (1978) Biochem. Pharmacol., 27, p. 1685.

Miledi, R. (1960) J. Physiol. (London), 151, p. 1.

Miledi, R. & Potter, L.T. (1971) Nature, 233, p. 599.

Miller, T.A. (1980) In: "Insect Neurobiology and Pesticide Action Neurotox 79" P. 145. London: Society of Chemical Industry.

Miller, R., Leaf, G. & Usherwood, P.N.R. (1973) Comp. Biochem. Physiol., A44, p. 991.

Nathanson, N.M. & Hall, Z.W. (1979) Biochem., 18, p. 3392.

Olsen, R.W., Van Ness, P.C., Napias, C., Bergman, M. & Tourtelotte, W.W. (1980) In: "Advances in Biochemical Psychopharmacology" Vol. 21: "Receptors for Neurotransmitters and Peptide Hormones" (Ed. Pepeu, G., Kuhar, M.J. & Enna, S.J.) p. 451. Raven Press, New York.

Osborne, M.P. (1970) In: "Insect Ultrastructure", Symp. Roy. Ent. Soc. (Ed. Neville, A.C.), 5, p.77. Blackwell, Oxford.

Oswald, R.E. (1979) Ph.D. Thesis, Vanderbilt University.

Oswald, R. E. & Freeman, J.A. (1979) J. Biol. Chem., 254, p. 3419.

Oswald, R.E. & Freeman, J.A. (1981) Neurosci., 6, p. 1.

Peachey, L.D. (1968) A. Rev. Physiol., 13, p. 1471.

Pichon, Y. (1974) In: "The Physiology of Insecta" (Ed. Rockstein, M.) 2nd Edn., p. 101. Academic Press, New York.

Piek, T. & Djie Nijo, K. (1979) Advs. Insect. Physiol., 14, p. 185.

Piek, T., Mantel, P. & Engels, E. (1971) Comp. Gen. Pharmac., 2, p. 317.

Piek, T. & Spanjer, J. (1978) In: "Pesticide and Venom Neurotoxicity" (Ed. Shankland, D.L., Hollingworth, R.M. & Smith, T.) Plenum, New York.

Pipa, R.L. (1973) In: "Developmental Neurobiology of Arthropods" (Ed. Young, D.) Cambridge University Press p. 105.

Prescott, D.J., Hildebrand, J.G., Sanes, J.R. & Servett, S. (1977) Comp. Biochem. Physiol., 56C, p. 77.

Pringle, J.W.S. (1967) Prog. Biophys. Molec. Biol., 17, p. 1.

Pringle, J.W.S. (1972) In: "The Structure and Function of Muscle" (Ed. Bourne, G.H.) 2nd Edn. Vol. 1. Pt. 1., p. 491. Academic Press, New York and London.

Pumphrey, R.J. & Rawdon-Smith, A.F. (1937) Proc. Roy. Soc. B., 122, p. 106.

Raftery, M.A., Schmidt, J., Clark, D.B. & Wolcott, R.G. (1971) Biochem. Biophys. Res. Comm., 45, p. 1622.

Raftery, M., Blanchard, S., Elliott, J., Hartig, P., Moor, H-P., Quast, U., Schimerlik, M., Witzemann, V. & Wu, W. (1979) Adv. Cytopharmac., 3, (Ed. Ceccarelli, B. & Clementi, F.) Raven Press, New York, p. 159.

Rang, H.P. (1975) Q. Rev. Biophys., 7, p. 283.

Rathmeyer, W. (1962) Z. Vergl. Physiol., 45, p. 413.

Rees, D. & Usherwood, P.N.R. (1972) Comp. Biochem. Physiol., 43A, p. 83.

Reynolds, J.A. & Tauford, C. (1976) Proc. Natl. Acad. Sci. USA., 73, p. 4467.

Robbins, J. (1959) J. Physiol. (London), 148, p. 39.

Roberts, P.J. (1974) Nature (London), 252, p. 399.

Roberts, P.J. (1981) In: "Glutamate:Transmitter in the Central Nervous System". (Ed. Roberts, P.J., Storm-Mathisen, J. & Johnston, G.A.R.), John Wiley & Sons. London.

Roeder, K.D. (1948) John Hople. Hosp. Bull., 83, p. 587.

Roeder, K.D., Kennedy, N.K. & Samson, E.A. (1947) J. Neurophysiol., 10, p. 1.

Rubsamen, H., Eldefrawi, A.T., Eldefrawi, M.E. & Hess, G.P. (1978) Biochem., 17, p. 3818.

Rudloff, E. (1978) Exp. Cell. Res., 111, p. 185.

Rudloff, E., Jimenez, F. & Bartels, J. (1980) In: "Receptors for Neurotransmitters, Hormones and Pheromones in Insects". (Ed. Sattelle, D.B., Hall, L.M. & Hildebrand, J.G.) p. 85, Elsevier/North Holland, Amsterdam.

Rudnick, G. (1977) J. Biol. Chem., 252, p. 2170.

Salvaterra, P.M. & Mahler, H.R. (1976) J. Biol. Chem., 251, (20), p. 6327.

Sattelle, D.B. (1978) In: "Pesticide and Venom Neurotoxicity" (Ed. Shankland, D.L., Hollingworth, R.M. & Smith, T. Jr.) p. 7 Plenum Press, New York.

Sattelle, D.B. (1980a) Adv. Insect Physiol., 15, p. 215.

Sattelle, D.B. (1980b) In: "Neurotransmitters of Invertebrates". (Ed. S-Rozsa, K.) Hungarian Academy of Sciences.

Sattelle, D.B., David, J.A., Harrow, I.D. & Hue, B. (1980) In: "Receptors for Neurotransmitters, Hormones and Pheromones in Insects" (Ed. Sattelle, D.B., Hall, L.M. & Hildebrand, J.G.) p. 125. Elsevier/North Holland, Amsterdam.

Sattelle, D.B., Harrow, I.D., Hue, B., Gepner, J.I. & Hall, L.M. (1981) J. Exp. Biol. (in press)

Sattelle, D.B., McClay, A.S., Dowson, R.J. & Callec, J-J. (1976) J. Exp. Biol., 64, p. 13.

Scatchard, G. (1949) Ann. N.Y. Acad. Sci. USA, 51, p. 660.

Schachman, H.K. (1959) In: "Ultracentrifugation in Biochemistry", Academic Press, London and New York.

Schmidt, J., Hunt, S. & Polz-Tejera, G. (1980) In: "Neurotransmitters, Receptors and Drug Action". (Ed. Essman, W.B.) Spectrum Publications, Inc.

Schmidt, J. & Raftery, M.A. (1973) Analyt. Biochem., 52, p. 349.

Schmidt-Nielsen, B.K., Gepner, J.I., Teng, N.N.H. & Hall, L.M. (1977) J. Neurochem., 29, p. 1013.

Seto, A., Arimatsu, Y. & Amand, T. (1977) Neuroscience Lett., 4, p. 115.

Shafiq , S.A. (1964) Quart. J. Micr. Sci., 105, (1) p. 1.

Shankland, D.L., Rose, J.A. & Donniger, C. (1971) J. Neurobiol., 2, p. 247.

Sharif, N.A. & Roberts, P.J. (1980a) J. Neurochem., 34, p. 779.

Sharif, N.A. & Roberts, P.J. (1980b) Brain Res., 194, p. 594.

Sharif, N.A. & Roberts, P.J. (1980c) Eur. J. Pharmacol., 61, p. 213.

Sherrington, C.S. (1925) Proc. Roy. Soc. B., 97, p. 519.

Shorr, R.G., Lyddiatt, A., Lo, M.M.S., Dolly, J.O. & Barnard, E.A. (1981) Eur. J. Biochem., 116, p. 143.

Simon, J.R., Contrera, J.F. & Kuhar, M.J. (1976) J. Neurochem., 26, p. 141.

Smallman, B.N. (1956) J. Physiol. (London), 132, p. 343.

Smallman, B.N. (1975) Pestic. Biochem. Physiol., 5, p. 170.

Smith, D.S. (1960) J. Biophys. Biochem. Cytol., 3, p. 447.

Smith, D.S. (1965) J. Biophys. Biochem. Cytol., 11, p. 119.

Smith, D.S. & Sacktor, B. (1970) Tissue and Cell, (21) 2, p. 355.

Smith, D.S. & Treherne, J.E. (1963) In: "Advances in Insect Physiology" 1, (Ed. Beament, J.W.L., Treherne, J.E. & Wigglesworth, V.B.) Academic Press, London and New York. p. 401.

Smith, D.S. & Treherne, J.E. (1965) J. Cell Biol., 26, p. 445.

Snodgrass, S.R. (1979) Soc. Neurosci. Abst., 5, p. 572.

Snyder, S.H., Chang, K.J., Kuhar, M.J. & Yamamura, H.I. (1975) Fedn. Proc., 34, p. 1915.

Sobel, A., Heidmann, T., Cartaud, J. & Changeux, J-P. (1980) Eur. J. Biochem., 110, p. 13.

Sobel, A., Weber, M. & Changeux, J-P. (1977) Eur. J. Biochem., 80, p. 215.

Sohal, R.S., Sharma, S.P. & Couch, E.F. (1972) Z. Zellforsch, 135 p. 449.

Stephenson, F.A. (1980) Ph. D. Thesis, University of Bath, Bath.

Stephenson, F.A., Harrison, R. & Lunt, G.G. (1981) Eur. J. Biochem., 115, p. 91.

Sugiyama, H. & Changeux, J-P. (1975) Eur. J. Biochem., 55, p. 505.

Sumikawa, K., Houghton, M., Eintage, J.S., Richards, B.M. & Barnard, E.A. (1981) Nature, 292, p. 862.

Takeuchi, A., Onodera, K. & Kawagoe, R. (1980) Proc. of the Japan Acad. 56, (4), p. 246.

Tauford, C., Nazaki, Y., Reynolds, J.A. & Makino, S. (1974) Biochem., 13, p. 2369.

Tobias, J.M., Kollross, J.J. & Savit, J. (1946) J. Cell Comp. Physiol., 28, p. 159.

Tripathi, R.K., Tripathi, H.L. & O'Brien, R.D. (1979) Biochim. Biophys. Acta., 586, p. 624.

Turner, P.D. (1980) Ph.D. Thesis, University of Bath, Bath.

Twarog, B.M. & Roeder, K.D. (1956) Biol. Bull., 111, p. 278.

Twarog, B.M. & Roeder, K.D. (1957) Ann. Ent. Soc. Amer., 50, p. 231.

Urbaniak, S.J., Penhale, W.J. & Irvine, W.J. (1973) Clin. Exp. Immunol., 15, p. 345.

Usherwood, P.N.R. (1963) J. Insect Physiol., 9, p. 811.

Usherwood, P.N.R. (1969) Nature (London), 223, (5204), p. 411.

Usherwood, P.N.R. (1973) J. Exp. Biol., 59, p. 1.

Usherwood, P.N.R. (1978a) Adv. Comp. Physiol. & Biochem., 7, p. 227.

Usherwood, P.N.R. (1978b) Adv. Pharmacol. Therap., 1, p. 107. Pergamon Press, Oxford and New York.

Usherwood, P.N.R. & Cull-Candy, S.G. (1975) In: "Insect Muscle" (Ed. Usherwood, P.N.R.) Ch. 4 p. 207, Academic Press, London and New York.

Usherwood, P.N.R. & Grundfest, H. (1965) J. Neurophysiol., 28, p. 487.

Usherwood, P.N.R. & Machili, P. (1968) J. Exp. Biol., 49, p. 341.

Usherwood, P.N.R., Machili, P. & Leaf, G. (1968) Nature (London), 219, p. 1169.

Van Harreveld, A. & Mendelson, M. (1959) J. Cell Comp. Physiol., 54, p. 85.

Veratti, E. (1902, 1961) Mem. Inst. Lamb. Classe Sci. Mat. Nat., 19, 87-113 (1902). Translated by C. Bruini, H.S. Bennett & D. de Koven and reprinted, J. Biophys. Biochem. Cytol., 10, Suppl. 1 (1961) p. 1.

Vincent, S.R. & McGeer, E.G. (1980) Brain Res., 184, p. 99.

Weber, K. & Osborn, M. (1969) J. Biol. Chem., 244, p. 4406.

Weill, C.L., McNamee, M.G. & Karlin, A. (1974) Biochem. Biophys. Res. Comm., 61, p. 997.

Werman, R. (1966) Comp. Biochem. Physiol., 18, p. 745.

Whittaker, V.P. (1965) Progr. Biophys. Mol. Biol., 15, p. 39.

Whittaker, V.P. (1969) In: "The Structure & Function of Nervous Tissue" (Ed. Bourne, G.H.) Vol. 3, p. 1. Academic Press, New York.

Whittaker, V.P. & Barker, L.A. (1972) In: "Methods of Neurochemistry" Vol. 2 (Ed. Freid, R.) Dekker, New York, Ch. 1, p.1.

Wigglesworth, V.B. (1959a) Quart. J. Micr. Sci., 100, p. 285.

Wigglesworth, V.B. (1959b) Quart. J. Micr. Sci., 100 p. 299.

Wonnacott, S., Harrison, R. & Lunt, G.G. (1980a) Life Sci., 27, p. 1769.

Wonnacott, S., Harrison, R., Lunt, G.G. & Barkas, T. (1980b) Eur. J. Biochem., 108, p. 621.

Wu, W.C.S., Moore, H.P.H. & Raftery, M.A. (1981) Proc. Natl. Acad. Sci. USA, 78, (2), p. 775.

Wu, W.C.S. & Raftery, M.A. (1981) Biochem., 20, (4), p. 694.

Yamamura, H.I., Kuhar, M.J., Greenberg, D. & Snyder, S.H. (1974a) Brain Res., 66, p. 541.

Yamamura, H.I., Kuhar, M.J. & Snyder, S.H. (1974b) Brain Res., 80, p. 170.

Yamamura, H.I. & Snyder, S.H. (1974a) Proc. Natl. Acad. Sci. USA., 71, p. 1725.

Yamamura, H.I. & Snyder, S.H. (1974b) Mol. Pharmacol., 10, p. 861.

Yamamura, H.I. & Snyder, S.H. (1974c) Brain Res., 78, p. 320.