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A BIOCHEMICAL INVESTIGATION INTO ASPECTS OF THE NEURO ACTIVE AMINO ACID RECEPTOR COMPLEXES IN THE INSECT NERVOUS SYSTEM

by

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A thesis submitted to the Council for National Academic Awards in partial fulfilment for the Degree of Doctor of Philosophy

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Publications

Baines P., Wilson R.G. and Wright R.A. (1983) : The mechanism of amidine resistance in a strain of the cattle tick, Boophilus microplus.
10th Int. Conf. W.A.A.V.P., Perth, W. Australia.

Nicholson R.A., Baines P. and Robinson P.S. (1986) : Insect synaptosomes in superfusion: a technique to investigate the actions of ion channel directed neurotoxicants by monitoring their effect on transmitter release.
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"Of many books there is no end, and much study is a weariness to the flesh."

Ecclesiastes 12:12.

Abbreviations

AMPA	γ-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (XVI)
APB	aminophosphonobutyric acid (VI)
2APV	2-amino-5-phosphonovaleric acid (VII)
B_{max}	maximum receptor density
BTX	α-bungarotoxin
CNS	central nervous system
cpm	counts per minute
DABA	di-aminobutyric acid (XX)
DAPA	2-dimethylaminophenylacetonitrile (XXXVIII)
dpm	disintegrations per minute
GABA	γ-aminobutyric acid (XVII)
GABA-T	γ-aminobutyric acid transaminase E.C.2.6.1.19
GAD	glutamate decarboxylase E.C.4.1.1.15
K_a	equilibrium association constant
K_d	equilibrium dissociation constant
kdr	knock down resistance
K_m	Michaelis constant for enzymes
L-glu	L-glutamic acid (I)
mineps	miniature endplate potentials
NMDA	N-methyl-D-aspartic acid (IV)
nmj	neuromuscular junction
PTX	picrotoxinin (XLII)
skdr	super knock down resistance
SSD-D	succinic semialdehyde dehydrogenase E.C.1.2.1.16
TBOB	1-phenyl-4-t-butyl-2,6,7-trioxabicyclo-[2,2,2]-octane (XLVII)
TBPS	t-butylbicyclophosphorothionate (XLVI)
THIP	4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol (XXIII)
V_{max}	maximum rate of enzyme conversion
μCi	micro Curie:2.22×10^6 dpm

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Abstract

A biochemical investigation into aspects of the neuro active amino acid receptor complexes in the insect nervous system.

Peter Baines

Biochemical investigations were undertaken to study the glutamate (L-glu) and γ-aminobutyric acid (GABA) receptors in the central nervous system (CNS) of the American cockroach, Periplaneta americana. The aim was to provide a simple, quick and cost effective method of screening new chemical entities as potential insecticides acting on these systems, and to provide structure activity information once a series had been suggested as active in these systems.

Using radioligand binding techniques, specific binding sites were revealed for both these amino acids in a P_2 preparation from whole CNS material. The receptor for L-glu showed similar receptor binding properties to excitatory amino acid receptors in the mammalian CNS, having a K_d of 1.80×10^{-7}M and a B_{max} of 10.6fmoles/μg. Despite the similarities to the mammalian receptor, there were subtle differences in the pharmacological profile which may be exploitable in the design of safe insecticides. This work represents the first piece of evidence for the presence of L-glu as a transmitter in the CNS of insects, as opposed to the peripheral nervous system, where its role as a transmitter is now well accepted.

The receptor for GABA was studied in more detail. Specific binding of this ligand was found, but the equilibrium constants obtained (K_d 1.79×10^{-6}M, B_{max} 16.1fmoles/μg) showed the receptor to be less avid than that normally found with a GABA neuro-receptor site. Reasons why this may be so are discussed. However, the pharmacological profile obtained using the potent GABA-mimetic, muscimol, suggest that this site is pertinent to the role of GABA as a neurotransmitter in the insect CNS, and that this receptor resembles the $GABA_A$ receptor described in mammals. However, differences in the exact profile of the mammalian and insect receptors again suggest that this system may provide a site of action for a new and safe series of insecticides.

Other areas of the biochemistry of the GABA receptor were investigated, and the results are discussed. Such positive results as were obtained confirm the identification of this receptor as relevant to the role of GABA as a neurotransmitter.

Although relevant receptors for both L-glu and GABA were demonstrated, it was not possible to use these as routine screens for the testing of potential new insecticides because of the large number of replicates needed to overcome the variations inherent in the technique.

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N.B. The Roman numerals in the text refer to the 'Compound structures' section from p.121 onwards.

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CHAPTER 1

Introduction

1.1 The insect nervous system.

The insect nervous system provides a communications network through which sensory information generated by the activation of peripheral sensory receptor cells can be rapidly passed to the central nervous system (CNS), where it is processed and relayed to the appropriate effector cells which will react accordingly. The afferent sensory neurons with their associated sensory receptor cells and the efferent motor and effector cells make up the insect peripheral nervous system. Communication between the afferent and the efferent pathways is mediated by the interneurons in the ganglia of the CNS.

The insect nervous system is bilaterally symmetrical and segmented. A schematic diagram of the structure of the CNS of the American cockroach, Periplaneta americana, is given in Figure 1.1. The CNS consists of a dorsal anterior brain and a central nerve cord. The segmentation of the cerebral ganglia is no longer apparent, as the segmental boundaries have become lost in the evolutionary process. Within the cerebral nerve mass there is a supraoesophageal ganglion (the brain proper) connected to the suboesophageal ganglion and the ganglia of the stomatogastric nervous system. This is then connected to the central nerve cord, which consists of three thoracic and six abdominal ganglia. These ganglia are double laterally fused structures joined to the others by pairs of interganglionic connectives. In higher orders of insects, such as the dipteran flies, these ganglia have become fused into one nerve mass located in the thorax. The peripheral nervous system arises from the CNS and consists of the neuromuscular, the sensory and the stomatogastric systems.

The basic element of the nervous system is the neuron. This specialised cell consists of the following elements (Figure 1.2):

a) the cell body or soma containing the nucleus;
b) the dendritic tree, a structure which is often highly branched and through which is received the afferent nervous input;
c) the axon, which acts as the efferent component of the neuron through which the nervous signal is propagated for transmission to other neurons or effector cells;

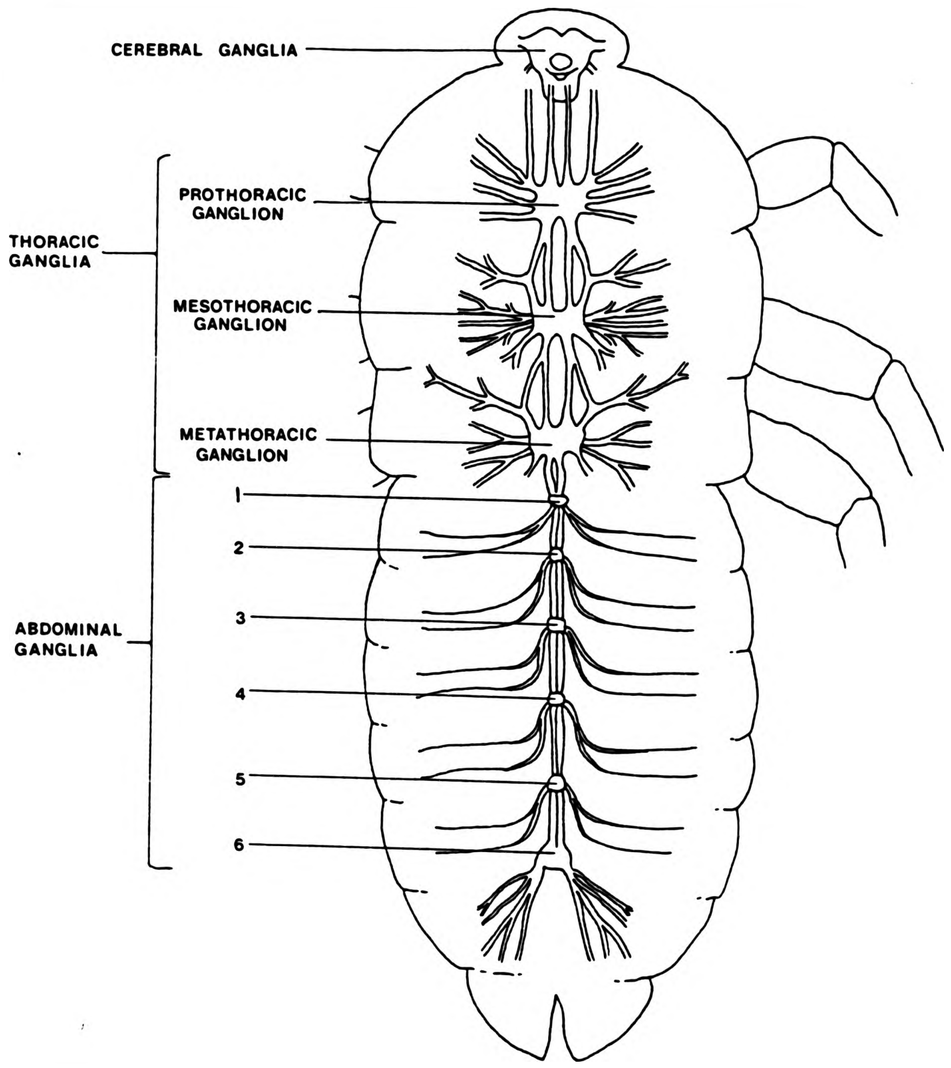

Figure 1.1

The nervous system of the American cockroach, P.americana.

This diagram shows the segmental nature of the cockroach CNS. The cerebral ganglia are a fusion of what were once separate ganglia. However, in the cockroach, the ganglia of the central nerve cord remain separate, consisting of three thoracic and six abdominal ganglia. In species such as the dipteran flies, these ganglia have also become fused into a second nerve mass.

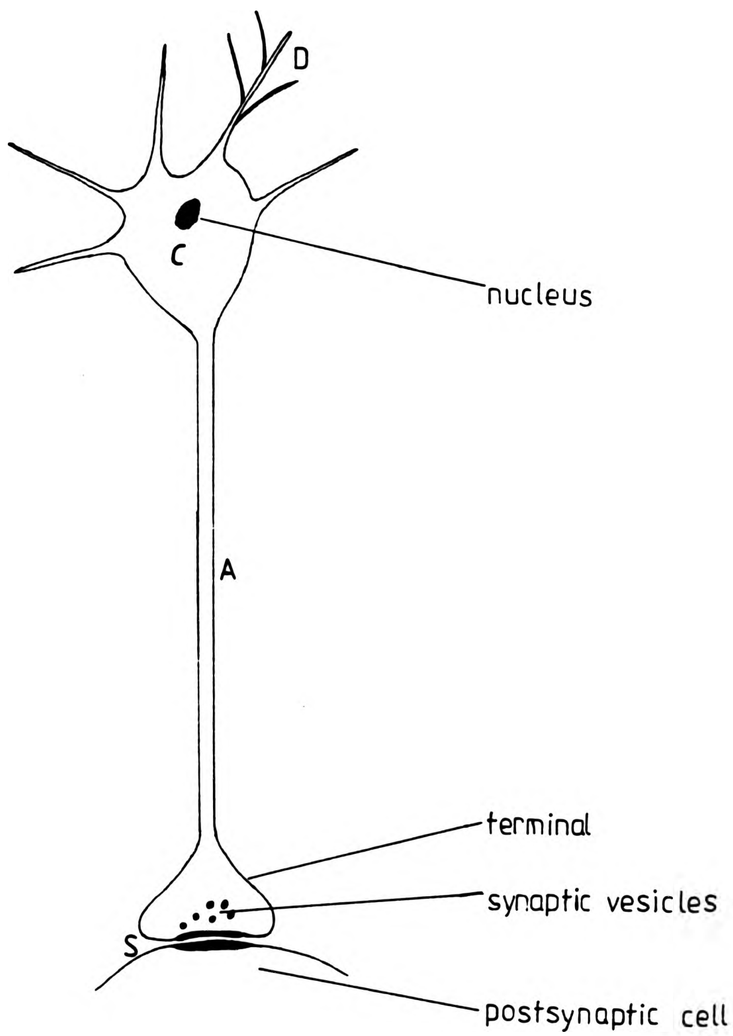

Figure 1.2

A schematic diagram of a simple neuron.

This diagram shows a simple neuron, as described in the text. The following features are labelled:

C - cell body

D - dendritic tree

A - axon

S - synapse

d) the synapse, which is the area where contact is made between the various elements of the nervous system, which consists of a specialised area of closely opposed presynaptic and postsynaptic membranes.

Contact between neurons within the insect nervous system may be axo-axonic, axo-dendritic, dendro-axonic or dendro-dendritic. Unlike vertebrates, all insect neuronal cell bodies are located on the periphery of the ganglia.

1.1.1 The chemical synapse.

The chemically mediated synapse in the insect has a synaptic cleft of between 5 and 25nm separating the pre- and postsynaptic elements (Osborne:1970). Within the CNS these elements are characterised by electron-dense thickening of the opposed membranes (Osborne:1966). The electron dense material on the cytoplasmic surface of the postsynaptic membrane is of a uniform nature, whilst that on the cytoplasmic surface of the presynaptic membrane is formed into projections or rod like structures. Within the presynaptic terminal are vesicles which are believed to contain the neurotransmitter. These synaptic vesicles are often attached to the presynaptic specialisations by filamentous material which may facilitate transmitter release (Osborne:1975). The presynaptic terminals also contain large numbers of mitochondria, microtubles and endoplasmic reticulum, indicative of a high metabolic turnover. Many of these structures can be seen in the sub-cellular entities known as synaptosomes. These 'pinched -off' nerve terminals are obtained by tissue fractionation and are often used in biochemical studies of the nervous system. As well as the characteristic elements of the pre-synaptic terminal, they often contain the membranes of the post-synaptic specialisation (Figure 1.3). The structure of the nerve-muscle synapse is very similar.

When the neuron receives the appropriate stimulus, an action potential is sent along the axon to the terminal. This consists of a wave of depolarisation mediated by transient changes in the axonic membrane's permeability to sodium and potassium ions. When this arrives at the nerve terminal, large numbers of the pre-synaptic

Figure 1.3

An electronmicrograph of a synaptosome from tissue from the CNS of the American cockroach.

This electronmicrograph is taken from material prepared from tissue from the CNS of the American cockroach as described in the text in the section on Materials and Methods. In the micrograph can be seen:

M - mitochondrion

P - synaptic cleft and post-synaptic membrane

V - synaptic vesicles.

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This electronmicrograph is taken from material prepared from tissue from the CNS of the American cockroach as described in the text in the section on Materials and Methods. In the micrograph can be seen:

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vesicles collide and merge with the pre-synaptic membrane in a calcium dependent manner, causing the release of their contents into the synaptic cleft. The post-synaptic response is produced when the transmitter substance binds to receptors on the post-synaptic membrane. This in turn activates ionophores causing transient changes in the postsynaptic membrane's ion permeability. The response may be excitatory, in which case the postsynaptic cell is depolarized, usually by the influx of sodium ions, or inhibitory. This latter response is caused by the influx of chloride ions, which may or may not cause a hyperpolarisation of the cell, as the resting potential in insects is usually close to the chloride reversal potential (Piek:1975).

Even in the absence of a depolarising current small numbers of neurotransmitter vesicles are thought to interact with the pre-synaptic membrane, the release of the transmitter contained within them producing small, quantum responses in the post-synaptic cell. Such responses are termed miniature endplate potentials (mineps) and are the main evidence for the quantal nature of neurotransmitter release (Fatt and Katz:1952).

1.1.2 Neural control of the insect muscle.

The organisation and control of the insect skeletal muscle has been described by Hoyle (1975). Insect muscles are small and consist of only a few muscle units. For this reason, control of the degree of contraction cannot be achieved in the same manner as is found in vertebrates. In vertebrates, control is achieved by the "all-or-nothing" contraction of a small number of motor units, each being only a small part of a whole muscle. To produce a more powerful response, extra muscle units are recruited. This is in contrast to the situation in the insect, where a graded mechanical response is achieved by multi-terminal innervation of each unit by excitatory and inhibitory axons, the tension produced in these muscles depending upon the balance of these axons' activities.

Excitatory axons may be termed "fast" or "slow" according to the response they induce in the post-synaptic muscle cell (Hoyle:1955). Fast axons produce a large post-synaptic potential upon which an electrically excited component is superimposed, giving a brief but powerful twitch of the muscle which decreases in strength with the distance from the endplate. Increasing the rate of stimulation causes fusion of the contractions giving a smooth, sustained muscle contraction at about 20 stimuli per second. Slow axons produce a small depolarisation of the muscle cell membrane, producing very little mechanical response. Repetitive stimulation causes facilitation of this small postsynaptic potential, increasing the velocity and extent of the muscle contraction. It has been proposed that these two types of excitatory response are mediated by two different transmitters, but this is far from proven (Irving and Miller:1980).

1.2 The insect nervous system as a site for pesticide action.

The insect nervous system is not as well shielded as that of vertebrates. The vertebrate CNS is well screened from the environment. Not only is part of it segregated by the so-called 'blood-brain barrier', but it is bathed by a specialised fluid (the cerebro spinal fluid) which is not accessible to environmentally introduced contaminants. The insect system is not as well protected. Although it has a partial 'blood -brain barrier' (Lane:1974; Treherne:1974), it is not as effective and lacks the support of a separate bathing fluid. This ready accessibility from the outside makes the insect nervous system a prime target for insecticides.

Many groups of insecticides already in use act upon this system. Three major examples are given below:

a) Organophosphates - Acetyl choline acts as an excitatory transmitter at both the central and peripheral levels in insects. It is normally removed from the synaptic cleft by the action of the enzyme acetyl choline esterase. Compounds such as Malathion (LIV) and Diazinon (LV) are now known to act by inhibiting this enzyme. The enzyme forms a suicide complex with this group of insecticides, causing increased

levels of acetyl choline in the synapase, leading to hyperactivity and death in the insect poisoned with these compounds.

b) Pyrethroids and DDT - Pyrethroids, such as permethrin (LVI), and DDT (LVII) are known to act by opening the sodium channel, causing an influx of sodium ions and thus depolarization of the nerve. This will lead to excitability in the insect and in turn to death.

c) Cyclodienes and Lindane - Whilst the two groups of compounds given above lead to death through overstimulation of excitatory responses, the group of compounds which include cyclodienes, such as dieldrin (XLIV), and γ-BHC (or Lindane:XLV) cause death by perturbing the effects of the inhibitory control of the nervous system. These compounds act by blocking the chloride channel, once more causing hyperexcitability and death.

Through prolonged use in the field, insects have developed resistance mechanisms to all of these groups of compounds. That is to say that the amount of insecticide required to achieve a desired level of control has increased. In extreme cases, the resistance may be such that control can no longer be achieved. Even if this is not the case, the increased level of insecticide required to obtain the desired level of control may be such that that it is no longer cost-effective. Such resistance can broadly be of two sorts. In one, the insecticide is prevented from reaching the target site. This form of resistance is commonly caused by an increase in the ability of the insect to metabolise the toxic compound. This form of resistance can often be overcome quite simply by the use of so-called synergistic compounds. These are compounds of low intrinsic activity which prevent the degradation of the main insecticide by the inhibition of the metabolising enzymes. Such a compound is piperonyl butoxide (LVIII), frequently used to enhance the activity of the pyrethroid insecticides. The second type of resistance involves a change in the target site itself. This is more difficult to overcome and can have additional problems in that it can render other classes of insecticides working through the same mechanism ineffective. When

this occurs, the classes of insecticides are said to be cross-resistant.

Such target site resistance has developed for both the pyrethroid and the cyclodiene insecticides. The mode of action and the mechanism of resistance for the cyclodiene insecticides has been reviewed by Matsumura (1985), who has shown in previous work mentioned in this review that the resistance is caused by an alteration at the site on the chloride channel which binds picrotoxinin (PTX:XLII). This alteration seems to confer resistance to many of the compounds which bind at this site, thus dieldrin, lindane and PTX all have lower toxicity towards those insect strains which exhibit this type of resistance. The resistance mechanisms to the pyrethroids have been studied for many years (Tsukamoto et al.:1965). They are known as kdr (knock down resistance) and skdr (super knock down resistance), which confere levels of resistance of some 10 and 100 fold to permethrin respectively. These are caused by genetic modifications to the sodium channel and were initially brought about through the use of DDT, an insecticide which acts at the same site as the pyrethroids (Osborne and Hart:1979).

With such problems developing in the current series of insecticides and the danger of cross-resistance occuring in new series which use the same modes of action as pesticides currently in use, it would seem prudent to search for new insecticides which act on an area of the nervous system not yet exploited as a site of insecticide activity. Such areas may be provided by the receptor sites for the closely related amino acids L-glumatic acid (L-glu:I) and γ-aminobutyric acid (GABA:XVII).

1.3 Criteria for the identification of neurotransmitters.

Werman (1966) laid down six criteria for the identification of a compound as a neurotransmitter. These were:

i) that the compound should be present in the neurotransmitter pool.

ii) that the compound be released by neural stimulation from the pre-synaptic terminal in sufficient amounts.

iii) that the compound have an identical action at the post-synaptic membrane to the natural transmitter.

iv) that the compound interacts with the post-synaptic membrane in the same way as the natural transmitter.

v) that synthetic and degradative enzymes be present pre-synaptically.

vi) that there be a mechanism for the removal of the compound from the synaptic cleft in order to terminate the action of the compound.

Ideally all these criteria should be fulfilled before a compound can be classed as a neurotransmitter.

1.3.1 General introduction to the use of ligand binding experiments.

Many of the criteria outlined above can only be studied adequately by the use of electrophysiological techniques. However, biochemical studies do have a significant role to play as well and the use of radiolabelled ligands to study the biochemistry of receptors of pharmacological and physiological interest is now well established. Care must be taken to ensure that the ligand binding that is being studied is as expected; that is to say that it is not just an experimental artefact caused either by the equipment used, or by an interaction with parts of the biological material other than that supposed to be under investigation. Even after many years experience with this type of experiment the problems of equipment artefacts are still with us. Thus, although Cuatrecasas and Hollenberg (1976) reviewed the problems they had encountered with the 'specific' non-receptor binding of various peptide hormones to glassware and membrane filters ten years ago, such problems still bedevil biochemists today (eg. the interesting H_1-receptor in glass filters reported by Bielkiewicz and Cook:1985). The field of receptor studies is also well strewn with examples of non-pharmacological receptors (eg. the example of Guth:1982), or receptors that were not what they at first appeared (eg. the supposed binding of [^{3}H]-kainic acid to the central receptors for L-glu). Various sets of criteria have been laid down to try to avoid such mistakes. In his 1985 review, Burt laid down the following basic criteria:

a) Saturability - although this is sometimes seen as a sufficient criterion, it is only the beginning.

b) Kinetics - the binding of the neurotransmitters themselves should be readily reversible, as should that of any other ligands that are known to be pharmacologically reversible. To help to confirm this, the association and dissociation rates should be studied and their ratio should be found to be close to the experimental equilibrium constant.

c) Distribution - subcellular, tissue, regional and species distribution should follow the known pharmacological distribution of the receptor under study.

d) Pharmacology - the binding of ligands to the site under study should follow the known pharamacological profile in the areas of stereospecificity, agonist and antagonist compounds. With many compounds it is crucial to choose the correct parameters as they bind to more than one receptor. Thus lysergic acid diethylamide binds to both dopamine and serotonin receptors in a stereospecific manner; when this is used as a radioligand, the two sites can be distinguished by the careful selection of the displacing agent.

It is rare that any study will match all of these criteria. Even if that were possible, there would still be room for doubt until the receptor had been totally solublised and reconstructed, as has been achieved with the acetylcholine receptor from the electric fish, Torpedo marmorata. However, such criteria should be borne in mind when using radioligands to try to study pharamacologically interesting receptors.

In studying the biochemistry of receptors using ligand binding techniques, mention is frequently made of three classes of binding and three basic types of plot. The types of binding are the specific, saturable or displaceable binding; the non-specific or non-displaceable binding and the total binding. These are defined as follows:

a) The specific binding is considered as that component of the radioligand binding which is actually bound to the receptor under consideration. As there are only a finite number of these receptors this binding can easily be diluted out by adding excess cold ligand or specific displacer - a compound that is a potent agonist or antagonist of the in vivo pharmacological action. The choice of the compound used to define the specific binding must be made with care. Thus PTX (XLII) is often considered to be a specific antagonist of the action of GABA (XVII), which is an opener of chloride channels. However, receptor binding studies soon showed that these two compounds act through different receptors - the binding of GABA to its receptor promotes the opening of the chloride channel, which action is antagonised by PTX because this compound blocks the chloride ionophore itself.

b) The non-specific binding is that component of the binding that cannot be diluted out by an excess of the appropriate displacer. This component is considered to be caused by such non-receptor phenomena as the partitioning of the radioligand into the lipid environment of the membrane preparation, the association of the ligand with non-specific proteins and the interaction of the ligand with the equipment used in the experimental protocol.

c) The total binding is the sum of these two.

In practice, the specific binding is arrived at by the difference between the total binding - measured when no displacer is present, and the non-specific binding - measured in the presence of excess displacer.

The three plots used are the semi-log, the Scatchard and the Hill plots. They are as follows:

i) The semi-log plot shows the percentage of the specific sites occupied as a function of the log of the displacer concentration.

ii) The Scatchard plot is the graph of the ratio of the concentration of the amount of ligand specifically bound to the receptor over the concentration of the free ligand against the concentration of the free ligand. As such, it is analogous to the Eadie plot used in enzyme studies. A straight line is indicative of a single population of non-cooperative receptors. In such a case it is easy to derive the equilibrium dissociation constant (K_d) and the number of receptors present (B_{max}) from the slope of the line and its intercept on the x-axis. It can also be used to study the potency and class of inhibitors of the interaction between the receptor and the radioligand.

iii) The Hill plot is the graph of the log of the ratio of the concentration of the number of receptor sites bound to the ligand over the total concentration of receptor sites less the concentration of those bound against the log of the concentration of the free ligand. A linear relationship for this plot will yield both the number of ligand molecules bound to each receptor site (from the slope) and the K_d of the receptor-ligand complex (from the intercept on the x-axis). If this plot gives a non-linear relationship, then the limiting slope of the curve yields the number of ligand molecules bound to each receptor molecule.

The K_d referred to in the descriptions above is an inverse measure of the affinity of the ligand for its receptor. Thus a low value for the K_d indicates that the ligand has a high affinity for the receptor (ie. it is a good ligand for that receptor), whilst a high value indicates that the ligand has a low affinity for the receptor (ie. that it is a poor ligand for the receptor). The curves described above are treated in more detail in the Results section, where the mathematical proof of the relationships is also derived from the law of mass action (See Sections 3.2.1 and 3.2.2).

1.4 The role of L-glu as a neurotransmitter in the insect

Neurotransmitter roles for both L-glu (I) and GABA (XVII) had been proposed in the 1950's because of their action on arthropod (Van Harreveld and Mendelson: 1959) and mammalian (Florey: 1954; Hayashi: 1954) nervous systems. However, this role for L-glu was not accepted for many years because of its ubiquitous nature and central position in intermediate metabolism. Thus, in homogenates of the muscle from the locust Schistocerca gregaria, the content of L-glu was found to be 2277μg/g tissue (Briley et al.:1982), whilst its level in the haemolymph of various insects lay between 0.1mM and 2.0mM (Wright: 1984). Such levels were higher than those required to activate the appropriate channels on the insect muscle, whilst the metabolic role of this amino-acid can be seen from Figure 1.4, which schematically outlines the involvement of the two amino acids in this area. As can be seen, L-glu not only takes part in the synthesis of proteins, but is also the precursor for other amino-acids and has a vital role to play in the metabolism of ammonia. However, the evidence for the transmitter role of L-glu soon became overwhelming.

1.4.1 The electrophysiological evidence for the role of L-glu as a neurotransmitter at the arthropod neuromuscular junction.

L-glu was shown to be excitatory in both mammals (Hayashi: 1954; Curtis and Watkins: 1960a) and crustacea (Van Harreveld and Mendelson: 1959) many years ago, but was not initially accepted as a putative neurotransmitter. However, the evidence continued to mount, until L-glu is now accepted as the major excitatory transmitter at the arthropod neuromuscular junction (nmj). There have now been many reviews on the subject (e.g. Lunt: 1975; Usherwood: 1981). In his 1981 review, Usherwood could make the following statement concerning the evidence for L-glu as the transmitter at the arthropod nmj:

> "1. Identity of action: The postsynaptic receptors gate action of selective ionophores, the reversal potential of the synaptic current and the L-glu current having the same value. The mean conductance and lifetime of these ionophores is also identical whether they are gated through the action of L-glu or the natural transmitter.....

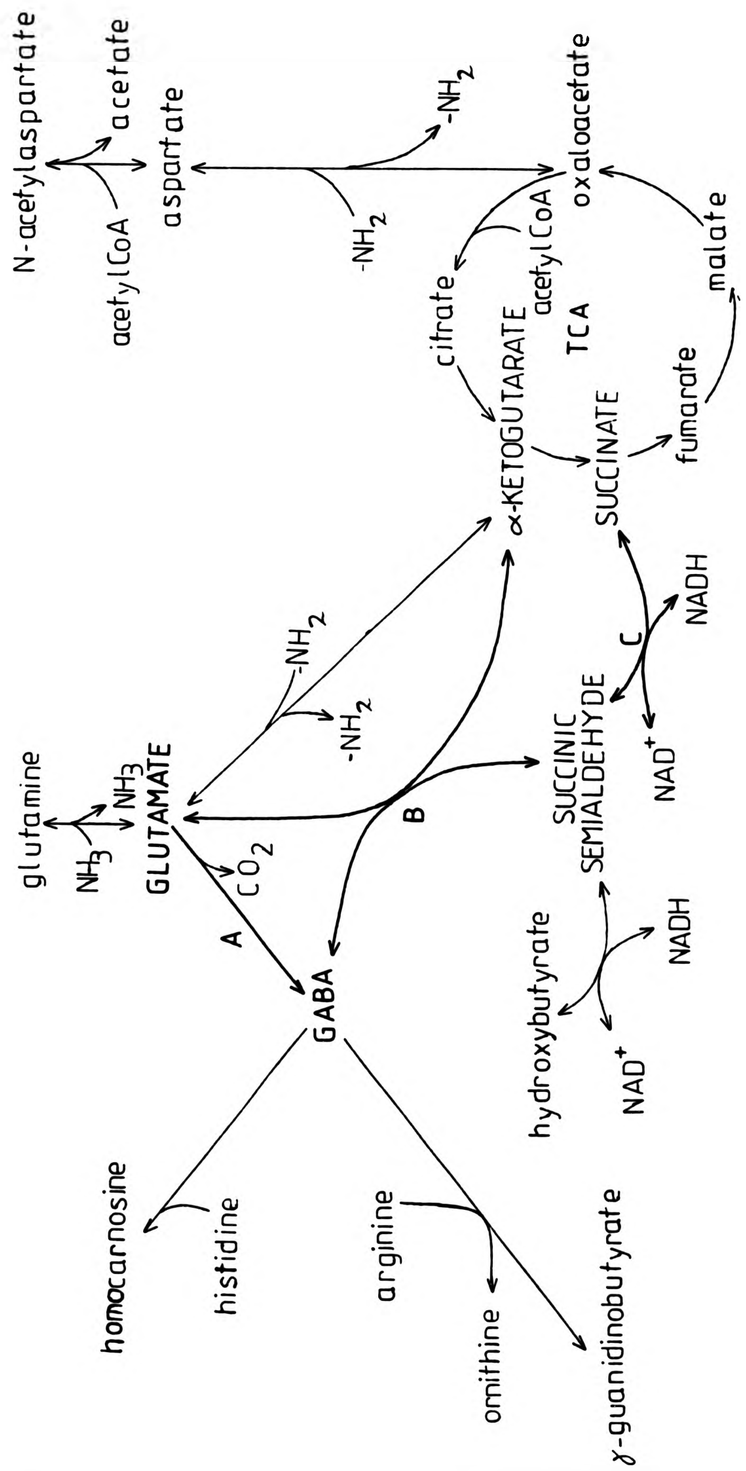

Figure 1.4

The metabolic roles of L-glu and GABA

This diagram shows schematically the central importance of L-glu (I) and GABA (XVII) in intermediate metabolism. The diagram is based upon that given in Carolei et al. (1978).

The enzymes mentioned in this thesis are:

	E.C. NUMBER	I.U.B. NAME	TRIVIAL NAME
A	4.1.1.15	L-glutamate 1-carboxy-lyase	glutamate decarboxylase
B	2.6.1.19	4-aminobutyrate: 2-oxoglutarate aminotransferase	γ-aminobutyric acid transaminase
C	1.2.1.16	succinic semialdehyde-NAD^+ oxidoreductase	succinic semialdehyde dehydrogenase

2. Release from the nerve terminal: L-glu is released from resting locust nerve-muscle preparations in small quantities and in larger quantities... from stimulated preparations...
3. Inactivation of transmitter: High-affinity uptake of L-glu into nerve terminals has been demonstrated at synapses on some insect muscles, but available physiological data suggest that removal of transmitter from the cleft is diffusional.....
4. Precursors and synthesizing enzymes: Although enzymes and precursors for the syntheses of L-glu have not been demonstrated in insect motor nerve terminals, given the ubiquitous distribution of this amino acid their presence at these sites seems highly likely.
5. Presynaptic pharmacology: It could be argued that the presence of presynaptic receptors for L-glu at locust excitatory nerve-muscle junctions supports the contention that this compound is the transmitter at these sites...
6. Extrajunctional receptors:..... Extrajunctional receptors on locust leg muscle comprise topographically heterogeneous populations of two receptor types; D-receptors which are similar pharmacologically to the junctional receptors found postsynaptically at excitatory junctions and H-receptors which have distinctive pharmacology and gate Cl^- ionophores.
7. Denervation supersensitivity: When locust muscle is denervated there is a significant increase in the sensitivity of the extrajunctional membrane to L-glu due to an increase in the population density of D-receptors (not H-receptors).....

On the basis of the evidence presented in 1-7 above, one is compelled to accept the conclusion that L-glu is the transmitter at the insect excitatory nerve-muscle junction".

However, there are still some observations that cast doubt upon this hypothesis. These were pointed out by Florey and Rathmayer (1981) and include the following:

i) In low Ca^{2+} medium the effect of applied L-glu is reduced; not so the effect of the natural transmitter.

ii) Diltiazem (LIX) increases the action of the natural transmitter but depresses the effect of applied L-glu.

iii) Concanavaline A prevents the desensitization to L-glu but enhances the depressant effect of L-glu on the amplitude of the excitatory post synaptic potential (epsp).

iv) bath-applied L-glu often causes a decrease of membrane conductance, in contrast to the natural transmitter which increases membrane conductance.

Some of these contradictions may be explained by the presence in the arthropod of multiple types of L-glu receptor, as detailed below.

1.4.2 Multiple receptor types from L-glu in the insect nerve-muscle systems and the mammalian CNS.

Electrophysiological investigation of the insect nerve-muscle system has suggested the existence of five receptors for L-glu. This work has been reviewed by Usherwood (1980) and Walker et al. (1980). Three of these receptors are found at the nmj. Using the extensor tibia muscle of the locust S.gregaria, these can be classed by their preferred conformation for the L-glu molecule (Gration et al.: 1979). They all gate Na^+,K^+ and Ca^{2+}, and are as follows:

a) This receptor prefers L-glu in its fully folded conformation, a conformation which leaves that compound similar in shape to L-aspartic acid (II), hence this receptor is classed as L-aspartic acid preferring.

b) This receptor prefers L-glu in its fully extended conformation and thus it responds to ibotenic acid (XLV) and is classed as ibotenic acid preferring.

c) The final receptor prefers L-glu in a partially folded conformation, being activated by neither L-aspartic acid nor ibotenic acid.

In studies on the extensor tibia preparation it is the final type of receptor that accounts for the majority of those present. Types (a) and (b) account for only some 15-20% each and their exact function in the in vivo role of L-glu as the transmitter at the insect nmj is unknown.

There are also two types of extrajuctional receptors. The D-receptors (for depolarising) seem to gate the same ions as the junctional receptors described above. The exact pharmacology of these receptors is unknown and they may turn out to be heterogeneous in the same way as the junctional receptors. The second class of extrajunctional receptors is quite distinct. These are the H-receptors (for hyperpolarising) and they are found to gate Cl^- ions and are sensitive to ibotenic acid. Thus on different areas in the same preparation, ibotenic acid elicits both depolarising and hyperpolarising activity.

Although there are no reports of the electrophysiological demonstration of L-glu receptors in the CNS of insects, it has been found to play a role in other arthropods. Thus Walker et al. (1981) failed to find any response to L-glu in the CNS of the cockroach, P.americana whilst finding an excitatory response in crab, Limulus polyphemus.

The presence of three types of excitatory amino acid receptor at the nmj parellels the situation in the mammalian CNS. Here three receptors are also found for the excitatory amino acids, although they have a different pharmacology to those in the insect. The electrophysiological work on the mammalian system has been mainly undertaken using the cat spinal neurons. This work has been reviewed by Watkins et at. (1981) and Fagg (1985).

The three classes of mammalian receptors are differentiated on their response to the antagonists L-glutamic acid diethyl ester (V) and 2-amino-5-phosphonovaleric acid (2APV:VII). The latter compound is an antagonist of the first class of such receptors. 2APV antagonises the action of the neuroexcitatory compound N-methyl-D-aspartic acid (NMDA:IV). These responses are also inhibited by raised (20mM) levels of the divalent ion Mg^{2+}. The actions of the two isomers of

2,4-piperidine dicarboxylic acid (IX) suggest that L-glu interacts with this receptor in a partially folded or aspartic acid-like manner. Thus the trans isomer is quite active, in contrast to the cis isomer which shows little or no activity. Work on the dorsal root evoked synaptic excitation of frog motorneurons suggests that these receptors play the major part in L-glu mediated neurotransmission.

In the mammal, the second class of excitatory amino acid receptors are those activated by the extended analogue of L-glu, quisqualic acid (XII). These receptors are antagonised by L-glutamic acid diethyl ester, but not by 2APV. They are also insensitive to the presence of Mg^{2+} ions.

The final class of mammalian excitatory amino acid receptors are activated by kainic acid (XIV). These receptors are not antagonised by either of the two compounds mentioned above and may not be activated by L-glu at all.

Thus there would appear to be differences in the major receptors responsible for the excitatory action of L-glu in insects and mammals, a fact which may be able to be exploited for the development of specific insecticides.

1.4.3 The radioligand receptor binding evidence for the identification of L-glu as an excitatory neurotransmitter.

The amount of material obtainable for radioligand binding studies in the insect is limited. Because of the small size of test subjects, harvesting sufficient quantities of material can take considerable time, and even with care the product will be of questionable quality. Thus the favoured method of obtaining CNS material from flies is to sieve the insects with solid CO_2 and use the heads as a crude source. However, this will not only contain CNS material, but also optic pigments, muscles and other contaminants. Obtaining purer preparations in sufficient quantities takes much longer than this method. Because of these difficulties much of our knowledge of the biochemistry of the L-glu receptor comes from studies on preparations from mammalian tissue-usually the rat brain.

Early work on the binding of radiolabelled L-glu to its putative neuroreceptor was carried out using L-[^{14}C]-glu. With this ligand, Roberts (1974) and Michaelis et al. (1974) showed the presence of the specific binding of L-glu in membranes derived from the rat brain, whilst De Robertis and Fiszer de Plazas (1976) showed similar results for a chloroform/methanol proteolipid extract from the same source. Further study was hampered by the high concentration of the radioligand needed because of the low specific activity of the ^{14}C label. This was overcome with the arrival of tritium labelled L-glu, a label capable of giving a much higher specific activity. Thus in their 1981 review, Roberts and Sharif were able to list a wide range of compounds with their ability to displace the specific component of the L-glu membrane binding.

In this early work there were differences between the receptor types demonstrated biochemically and electrophysiologically. Thus the radioligand studies suggested that there was only one class of L-glu neuroreceptor, as opposed to the three types demonstrated by the use of electrophysiological techniques. The L-glu specifically bound at this site could be readily displaced by quisqualic acid, but neither NMDA nor kainic acid showed any such ability (Roberts and Sharif: 1981).

However, with improved techniques, it is now possible to demonstrate up to four types of L-glu receptor in membranes derived from rat brains. Three of these are similar to those described in the electrophysiological studies. Radiolabelled L-glu proved to be a non-specific ligand, being displaced both by 2APV (the NMDA antagonist) and ɣ-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA:XVI; a new and specific agonist at the quisqualic acid site; Honore et al.: 1981b) from membrane preparations. When these two compounds are used to displace radiolabelled L-glu from autoradiographs of thin tissue slices, they are found to do so from different areas of the brain - further indicating that they represent different receptor types. As expected, kainic acid is found to be a poor displacer of bound L-glu. This area of work has been reviewed by Fagg in his 1985 paper.

The fourth type of receptor is one not described above. These receptors are revealed by the addition of 2.5mM $CaCl_2$. The addition of this compound increases the number of L-glu binding sites by some three fold. This increase is antagonised by 2-amino-4-phosphonobutyric acid (APB:VI). The exact purpose of these receptors is uncertain (Fagg et al.:1982).

1.4.4 Radioligand binding studies using labelled L-glu with tissue derived from the insect muscle.

Despite the paucity of material, some studies into the binding of L-glu to insect tissue have been undertaken. Early on in the study of radioligand binding of L-glu to its postsynaptic receptor in mammalian CNS there was a claim to have discovered a proteolipid receptor for the amino acid using the locust muscle as a starting point (Lunt:1973). However, this receptor preparation did not prove reliably reproducable and was dropped in favour of a membrane bound receptor preparation (Briley et al.:1979). This system initially gave a K_d of 30 μM for L-glu, but further refinement finally showed two populations of L-glu binding sites, having K_d values of 12.5nM and 1.3mM (Briley et al.:1982). A putative L-glu receptor has also been shown on thoracic muscle membranes of the housefly Musca domestica (Filbin et al.:1985). This receptor shows a K_d of 0.5μM, and a displacement profile similar - but by no means identical - to that found early on in the work on the mammalian CNS receptor for L-glu. It would thus appear that the insect work is at a stage similar to that attained in the mammalian work in the early 1980's.

There would appear to be no reports of L-glu radioligand binding in the CNS of insects.

1.5 The role of GABA as a neurotransmitter in the insect

A neurotransmitter role for GABA (XVII) was accepted much more readily than for L-glu (I) because of the much less important role this amino acid plays in intermediary metabolism. Most of Werman's criteria for the indentification of a compound as a neurotransmitter (Werman:1966; see above) have been fulfilled by GABA in mammalian systems.

a) Presence in the neurotransmitter pool: GABA was shown to be one of the amino acids present in the extracts made from mouse brains by Roberts and Frankel (1950)

b) Release: With the small quanities released on nerve stimulation, this can be difficult to show in vivo. However, Obata and Takeda (1969) have shown that calcium ions induce the release of endogenous GABA into the fourth ventricle of the cat and the release of preloaded radiolabelled GABA by the use of depolarising agents on cortical slices (Srinivasen et al.: 1969) and synaptosomes (De Belleroche and Bradford:1972) is also calcium dependent.

c) Identity of action: GABA has been shown to have an inhibitory action similar to the natural transmitter in central neurons (Curtis and Watkins:1960b)

d) Synthesis and degradation: GABA is synthesised by glutamate decarboxylase (GAD). Levels of this enzyme in various areas of the brain correlate well with the GABA content of that area (Fonnum et al.:1970). In fact GAD is now used as a major cytochemical marker for the identification of GABA-ergic neurons (Wood et al.:1976). GABA is degraded via the enzymes γ-amino-butyric acid transaminase (GABA-T) and succinic semialdehyde dehydrogenase (SSA-D). Both these enzymes are found in regions similar to those containing GAD (Salvador and Alberts:1959)

e) Removal: Removal of GABA from the synaptic cleft is believed to be by uptake into the glial cells. High affinity, sodium-dependent uptake into these cells has been shown (Henn and Hamberger:1971).

GABA is now also accepted as the major inhibitory transmitter in insects.

1.5.1 The electrophysiological evidence for GABA as a neurotransmitter at the insect nmj and the insect CNS.

GABA is accepted as the major inhibitory transmitter at the insect nmj. Experiments carried out in vertebrate models such as the cat cortical neurones (Krnjević and Schwartz:1967) and cat spinal motorneurones (Curtis et al.:1968) paved the way for this acceptance, as did similar work on the crustacean nmj (Takeuchi and Takeuchi:1965). Using the extensor tibia muscle of the metathoracic leg of the locust S.gregaria Usherwood and Grundfest showed in 1964 that GABA could mimic inhibitory post synaptic potentials and in 1965 that these could be blocked by PTX (XLII). In his 1980 review, Usherwood could say that the role of GABA as the inhibitory transmitter at the insect nmj was readily accepted.

In 1980, Brookes and Werman studied the effect of ion concentrations upon the reponsiveness of the flexor tibialis muscle of the locust Locusta migratoria. From the Hill plots obtained in their experiments they were able to show that the number of GABA molecules needed to activate the receptor/ionophore complex varied between 2 and 4, depending upon the ionic conditions used in the test bath. Using a saline that mimics physiological conditions they found that the number of GABA molecules needed for activation was 3. This suggests that the receptor can adopt at least three states dependent upon the ionic environment.

As with the nmj, so with the CNS of the insect; GABA is readily accepted as a neurotransmitter. As long ago as 1969 Kerkut et al. showed that the abdominal ganglion of the cockroach P.americana was inhibited by very small amounts of GABA (10^{-13}moles), Roberts et al. (1981) have since shown that this inhibitory effect is mediated by a receptor which accepts GABA in the partially extended form. They showed that the GABA agonist dihydromuscimol (XXII) was the most potent of those compounds which they tested on both P.americana and L.polyphemus central neurones.

1.5.2 The evidence of receptor binding studies for GABA as a neurotransmitter.

As with L-glu, most of the work into the radioligand binding properties of the GABA receptors have been carried out on membrane fractions derived from the rat brain. Such studies show some of the dangers in the interpretation of receptor binding assays, for it is possible to define up to 36 different sub-types of the GABA receptor (Beart:1982). These have been resolved into two major groups - the $GABA_A$ and the $GABA_B$ receptors. The $GABA_A$ site is postulated as the classical receptor site for the action of GABA as an inhibitory neurotransmitter, the action of GABA at this site being classically antagonised by bicuculline (XLI). This site will be dealt with in more detail below.

The uncovering of the $GABA_B$ site arose from the work of Bein (1972) and Kerberle and Faigle (1972), which led to the production of Baclofen (XXXVI). This compound caused the inhibition of cat spinal reflexes in a bicuculline-insensitive manner. Radioligand binding studies at first showed that this compound did not displace specifically bound GABA from the normal membrane preparations. However, when $CaCl_2$ was added to slightly modified buffers (containing Tris-HCl as opposed to Tris-citrate to avoid the chelation of the Ca^{2+}), the amount of GABA bound was found to increase in a manner that was sensitive to the addition of Baclofen. These conditions also reveal specific binding of radiolabelled Baclofen (Hill and Bowery: 1981). The exact role of the $GABA_B$ sites is uncertain, but they may be involved in the presynaptic modulation of release of neurotransmitters. The area is reviewed by Bowery (1982).

The $GABA_A$ receptor in the mammal is part of a supramolecular complex which includes the Cl^- ionophore and binding sites for benzodiazepines and barbiturates. The three major binding sites on this complex will be considered below. Useful reviews of this area are provided by Enna (1981) and Olsen (1981).

1.5.2.1 The $GABA_A$ recognition site.

Studies on the $GABA_A$ recognition site are usually carried out using freeze-thawed and Triton X-100 treated membranes in sodium free Tris-citrate buffer (Enna and Snyder: 1977). For a long time the major displacer used at this site was the GABA-mimetic, muscimol (XXI). However, this compound is known to have other actions besides those mediated through the $GABA_A$ receptor, being also a GABA uptake blocker and a substrate for GABA-T. In order to better study the role of the $GABA_A$ receptor, more specific GABA agonists were developed. The three main ones were
4,5,6,7-tetrahydroisoxzolo[5,4-c]pyridin-3-ol (THIP:XXIII), isoguvacine (XXIV) and piperidine-4-sulphonic acid (XXV). Although all of these compounds are less potent than muscimol at displacing GABA from its recognition site (isoguvacine and piperidine-4-sulphonic acid are about as potent as GABA, whilst THIP has only one fifth the activity), they all show greater specificity for the receptor site. All of these were reported by Krogsgaard-Larsen and Falch (1981).

1.5.2.2 The benzodiazepine recognition site.

Benzodiazepines are a group of compounds possessing relaxant, hypnotic, anti-convulsant and anxiolytic properties. Many of these actions seem to be mediated through receptors allosterically linked to the $GABA_A$ receptor site, binding to this site causing a facilitation of GABA function. This site binds the benzodiazepine representative [^{3}H]-flunitrazepam (LI) with high affinity (a K_d value of 1.54nM). In the presence of GABA or agonists such as muscimol the amount of the radioligand bound is increased due to an increase in the binding affinity (the K_d is reduced to 0.90nM). The GABA antagonist bicuculline was found to reduce the amount of flunitrazepam bound (Wong et al.:1980).

Rigid GABA agonists such as THIP and piperidine-4-sulphonic acid are unable to produce this increase in the binding of benzodiazepines at 0°C (Krogsgaard-Larsen and Falch:1981). However, the addition of Cl^- ions or the elevation of the incubation temperature to 30°C enabled these agonists to mimic GABA in the increase of the binding affinity

of the flunitrazepam (Supavilai and Karobath: 1980).

Various endogenous ligands have been proposed for these receptor sites, but none have gained general acceptance. Thus in a paper in 1981, Mohler suggested the following possibilities; inosine, hypoxanthine, nicotinamide, ethyl-β- carboline-3-carboxylate, thromboxane A_2 and GABA-modulin. The search for an endogenous ligand still continues.

1.5.2.3 The chloride ion ionophore.

The chloride ion ionophore is believed to be the site of action of compounds such as PTX and the other cage convulsants. Much work has been carried out using PTX reduced with tritium as a ligand for this site (eg. Leeb-Lundburg et al.: 1981). However, dihydropicrotoxinin (DPTX:XLIII) has proved to be a poor ligand because of the high level of non-specific binding associated with its use. Since then, two cage convulsants have provided more suitable ligands for this site. They are t-butyl-bicyclophosphorothionate (TBPS:XLVI), labelled with ^{35}S and t-butyl-bicylcoorthobenzoate (TBOB:XLVII), labelled with ^{3}H. Both these compounds act on the same receptors as PTX (Casida et al.: 1985; Lawrence et al.:1985). All of these compounds act by blocking the ionophore and as such act as non-competitive GABA antagonists in electrophysiological studies, having no effect in radioreceptor studies on the binding of GABA.

Other compounds which displace these ligands from this site are the convulsant benzodiazepine RO5-3663 (LII) and the barbiturates (Olsen and Leeb-Lundberg: 1981). Thus this unusual benzodiazepine would seem to work by blockage of the Cl^- channel, whilst the barbiturates work by prolonging the opening of it. There is also an allosteric interaction between the barbiturates and the other two sites as they can enhance the binding of both the high affinity GABA sites in certain circumstances (using fresh membranes as opposed to the normally employed freeze-thawed and detergent washed membranes; Johnson and Willow: 1981) and can also enhance the binding of the benzodiazepines (Stephenson and Olsen: 1982).

A schematic diagram of the $GABA_A/Cl^-$ channel complex is given in Figure 1.5.

1.5.3 The binding of the various classes of $GABA/Cl^-$ channel complex ligands to membranes derived from insect tissue.

Radioligand binding studies with GABA have proved harder to perform in the insect than those using L-glu. This is presumably due to a higher level of non-specific binding to be expected using a ligand with a lower charge and thus more lipophilic. Despite this, more groups have investigated the insect GABA receptor than is the case for the L-glu receptor and investigations have been carried out on all of the three sites involved in the complex.

The initial work on the binding of compounds to the GABA receptor site in arthropods was carried out by Olsen's team (Olsen et al.: 1975; Meiners et al.: 1979). In 1982, Briley et al. made a preliminary report on the discovery of muscimol binding to a membrane fraction from the muscle of S.gregaria. This was to the same fraction as that used to show L-glu binding in the locust muscle. However, it was only recently that the presence of a specific GABA receptor in insect CNS was reported. In 1985 a number of groups showed such activity in a number of insect species:

i) Breer and Heilgenberg reported the presence of one class of Na^+- independent sites for muscimol with a K_d of 0.1mM in the CNS of L.migratoria.

ii) Lummis and Sattelle, working on the cockroach P.americana, found a receptor site which bound GABA with a K_d of 0.38μM. This site showed properties that were characteristic of neither the $GABA_A$ nor the $GABA_B$ sites in mammals.

iii) Lunt et al. studied both S.gregaria and M.domestica and found specific sites in the CNS of each of these species. the K_d were 10 and 40nM respectively.

All these investigators found the Hill coefficient to be close to unity, indicating that there was no cooperativity between the receptors.

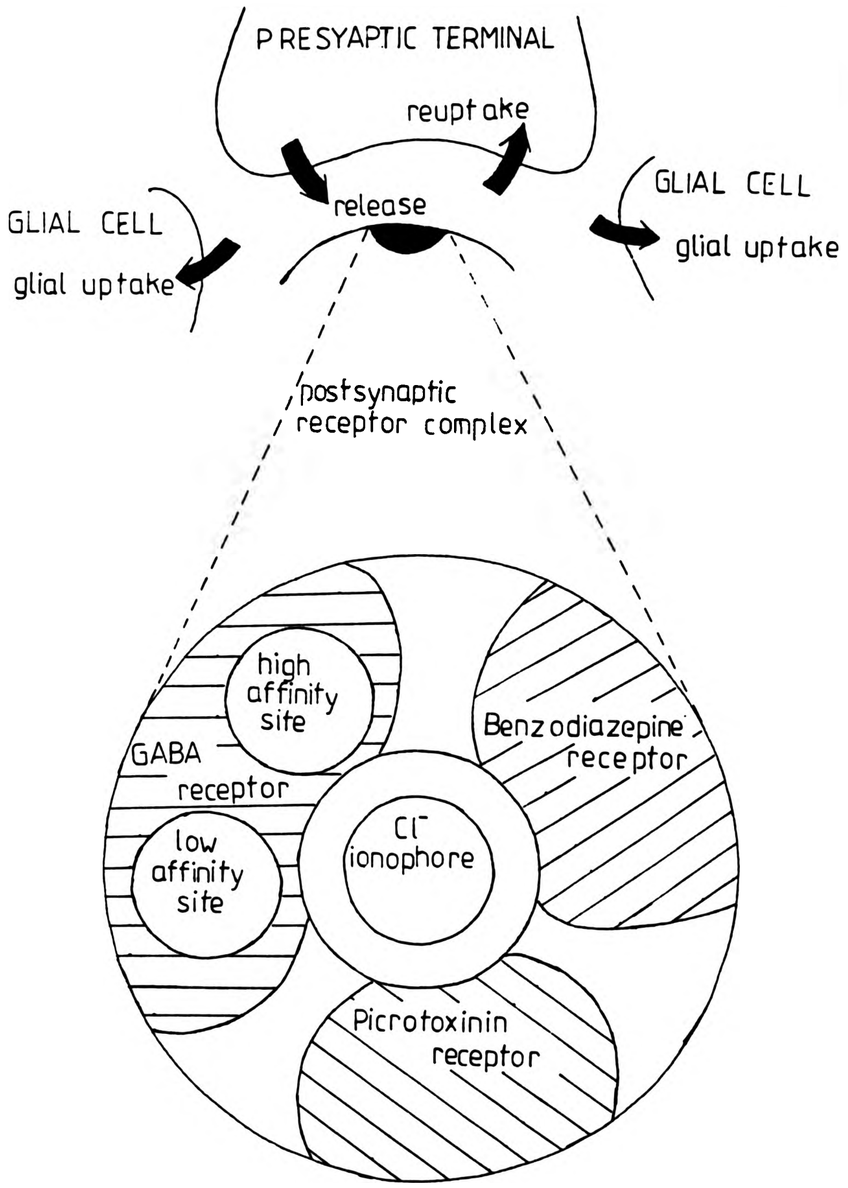

Figure 1.5

A schematic representation of the GABA/Cl^- channel complex.

This diagram is taken from Krogsgaard-Larsen and Falch (1981). It shows the typical GABA-ergic terminal with the various receptor sites to be found on the post-synaptic GABA/Cl^- channel complex receptor.

The Cl^- channel in arthropods was also first investigated using ligand binding techniques by Olsen and his team on crayfish muscle, where they claimed to have found a site that specifically bound DPTX (Olsen et al.: 1978). The presence of a similar site in P.americana CNS was reported by Tanaka et al. in 1984. This had a K_d value of 0.8μM. More recently, work on the ionophore site has been undertaken using the bicyclophosphate ligand [^{35}S]-TBPS. Cohen and Casida (1985;1986) have shown the presence of specific TBPS binding in housefly muscle preparations, whilst Lunt et al. (1985) give preliminary reports of such binding in a preparation from the head of the same species. The K_d values recorded varied between 10 and 210nM, but in each case the reported Hill coefficient was close to unity.

Finally benzodiazepine receptors have also been sought in insect tissue. Preliminary results suggested that these receptors did not exist in insects. Thus both Nielsen et al. (1978) and Maggi et al. (1980) undertook work which indicated that benzodiazepines receptors were of a late evolutionary appearance. However, in 1983 Abalis et al. discovered two sites that specifically bound flunitrazepam in the thorax muscle of the housefly (K_d values of 24 and 994 nM), whilst Robinson et al. (1985) found a single site in the CNS of both locust and housefly (K_d values around 50nM). The work of the latter group suggested that the reason for the failure of earlier groups to find these sites was the lack of Ca^{2+} in the medium, as this ion was found to be essential to reveal the binding sites with which they were dealing.

The nature of the interactions between these three sites have not been fully investigated as yet, but it would appear that the insect CNS does contain a GABA/Cl^- channel complex that bears many similarities to the one found in mammalian systems.

1.6 The scope of the present study

In the search for new insecticides there is a need to evaluate the intrinsic activity of candidate compounds on their putative target site as well as to determine the overall toxicity of these entities to the target species. This need arises because many compounds do

not reach the target site, being prevented from doing so by such factors as metabolism and difficulties in penetration. To reject such compounds on the basis of toxicity data alone is to run a grave risk of ignoring potentially fruitful chemical leads. And once a lead has been established, radioligand binding studies can be used to establish quantitative stucture activity relationships and so aid the chemists in the production of the most potent compound in the shortest time. Receptor binding studies offer the scope for a high throughput, low cost screening system for such compounds. These systems have been used in screening new psychoactive drugs (Creese: 1985), why should they not be used in the development of pesticides? One major aim of this study was to provide such a system to aid in the development of novel series of insecticides acting through the neuroreceptors for the amino acids L-glu (I) or GABA (XVII).

A secondary aim was to fill some of the gaps in the knowledge of the biochemistry of the insect receptors for the amino acids L-glu and GABA. For although there has now been some considerable work undertaken to extend the biochemical investigation of the amino-acid transmitter receptors into the insect field, this area of study is still far behind the biochemical studies in mammals and the field of biochemistry is far behind that of electrophysiology in this whole area.

The CNS of the American cockroach was chosen as the major target tissue for the following reasons:

a) The American cockroach is a major pest. It is frequently found in hospitals and eating areas, as well as in many homes. It may be a carrier of human pathogens and is the cause of much food spoilage (Roth: 1981). In the U.S.A. alone, the market for entities which control the roach pest is £400M; clearly a method of control of this pest would be of economic interest.

b) The species breeds rapidly and has a comparatively large CNS (60mg wet weight), thus plenty of material is readily available for study.

c) The CNS is readily amenable to the techniques of electrophysiology, so that it will be easy to make comparisons with work done in the future by others in the laboratory.

The GABA/Cl^- channel complex provided the major area of study because the radioligands for the study of this area are more effective, although some work on the L-glu area was also undertaken.

CHAPTER 2

Materials and Methods

2.1 Sources of Materials

2.1.1 Chemicals and Solvents

Unless stated to the contrary, the chemicals used were from the following sources:-

General chemicals and solvents - British Drug Houses, Broom Road, Poole. BH12 4NN (Analar Grade where available).

Biochemicals - Sigma, Fancy Road, Poole. BH17 7NH

Radiochemicals - Amersham International, P.O. Box 16, Amersham. HP7 9LL (AI)
or - New England Nuclear/Du Pont, Wedgewood Way, Stevenage. SG1 4QN (NEN).

2.1.2 Insects

Cockroaches : Adult male Periplaneta americana were reared in the Applied Biology Section at Wellcome Research Laboratories, Berkhamsted Hill. Their diet consisted of calf-nuts and water ad lib, and they were maintained at 26°C.

Green Bottle Flies : Adult Lucilia sericata were supplied by the Veterinary Entomology Section, Coopers Animal Health, Berkhamsted Hill. They were maintained on a diet of sugar and water ad lib until required. The temperature of the holding room was maintained at 26°C and a 12 hour light/dark cycle was used.

Locusts : Locusta migratoria were reared in house in Griffin and George locust cages. The cages were kept in a holding room maintained at 28° and 20% relative humidity, with a 12 hour light/dark cycle. The locusts were fed fresh cut grass and water. Fully developed adults were used for the experiments.

Schistocerca gregaria were purchased from Bioserv Ltd.

Unless otherwise stated, all procedures described were carried out at 4°C.

2.2 Measurement of radioactivity.

Radioactivity was measured by liquid scintillation counting using an Intertechnique SL4000 scintillation counter.

For β-particle emitters (^{3}H, ^{14}C, ^{35}S), samples were added to the Triton/xylene based scintillation medium Instagel (Packard Instruments Ltd) and counted using the appropriate factory set gates. The counting efficiency for each series of experiments was determined by replicate determinations of the cpm from a known amount of the appropriate radioactive standard (usually [^{3}H] - or [^{14}C]-n-hexadecane, ex AI).

For the γ-emitter ^{125}I, samples were placed in polythene inserts and counted on the SL4000 in γ-vials (Koch-Light Ltd) using the factory settings for ^{14}C. The counting efficiency was determined by counting replicate samples containing a known amount of γ-emitting substance.

2.3 The binding of ligands to a potential glutamic acid receptor in membranes derived from P. americana central nervous tissue.

2.3.1. The binding of [^{3}H]-L-glutamic acid.

The procedures used to prepare membranes to study the binding of L-[^{3}H]-glu (I) are schematically laid out in Figure 2.1.

2.3.1.1 Gray and Whittaker (1962) published a method for the preparation of synaptosomes from whole rat brains. This method was followed to produce synaptosome-like material from P. americana central nervous tissue. This material was then osmotically and sonically shocked to give membranes suitable for binding assays.

The method used was as follows:

Total central nervous tissue (cerebral, thoracic and abdominal ganglia) were dissected and homogenised in 0.32M sucrose (pH 7.2 with Tris base) using 12 strokes of a Jencons 'Uniform' 15ml glass in glass homogeniser. The homogenate was centrifuged at 1000 x g(max) for 10 minutes, and the supernatant re-centrifuged at 20000 x g(max) for 45 minutes. The resulting pellet (P_2) was resuspended in 3mls 0.32M sucrose, and carefully layered on top of a sucrose step-gradient consisting of 6mls of each of 1.2M and 0.8M sucrose (pH 7.2 with Tris base). The step gradient was centrifuged at 150000 x g(max) for 60 minutes. The synaptosomal material was removed from the boundary between the 0.8M and 1.2M sucrose using a 10ml syringe with a wide-bore (19 gauge) 90° needle. This was added to a 100ml measuring cylinder and slowly diluted with an equal amount of distilled water. This solution was centrifuged at 38000 x g(max) for 45 minutes. The resulting pellet was osmotically shocked by resuspension in 2mls distilled water, and then sonicated in a Kerry 'Sonibath' for a total of 3 minutes. This was finally centrifuged 38000 x g (max) for 45 minutes, and the pellet used for the binding experiments as described below (2.3.1.3.).

2.3.1.2. A method closely following that used by Sharif and Roberts (1980) to produce synaptic membranes from rat brains was used. The method used differed slightly in the production of the final pellet following the sonication and washing phase.

Total central nervous tissue was dissected and homogenised in 0.32M sucrose (pH 7.2 with Tris base) using 10 strokes of a 15ml Jencons 'Uniform' glass in glass homogeniser. The homogenate was centrifuged at 1000 x g(max) for 20 minutes and the supernatant carefully removed. This was re-centrifuged at 17000 x g(max) for 20 minutes. The resulting pellet (P_2) was resuspended in distilled water, and sonicated for 3 minutes using a Kerry 'Sonibath'. The homogenate was pelleted and washed twice by resuspension in distilled water, and a final pellet produced by centrifuging at 38000 x g(max) for 15 minutes. This washed P_2 was then used for binding experiments (2.3.1.3).

NB : Unless otherwise stated, all experiments used an homogenisation rate of 1CNS per 40µl of homogenisation buffer. Where washes were performed, these were all carried out in 5ml distilled water.

The method used was as follows:

Total central nervous tissue (cerebral, thoracic and abdominal ganglia) were dissected and homogenised in 0.32M sucrose (pH 7.2 with Tris base) using 12 strokes of a Jencons 'Uniform' 15ml glass in glass homogeniser. The homogenate was centrifuged at 1000 x g(max) for 10 minutes, and the supernatant re-centrifuged at 38000 x g(max) for 45 minutes. The resulting pellet (P_2) was resuspended in 3mls 0.32M sucrose, and carefully layered on top of a sucrose step-gradient consisting of 6mls of each of 1.2M and 0.8M sucrose (pH 7.2 with Tris base). The step gradient was centrifuged at 150000 x g(max) for 60 minutes. The synaptosomal material was removed from the boundary between the 0.8M and 1.2M sucrose using a 10ml syringe with a wide-bore (19 gauge) 90° needle. This was added to a 100ml measuring cylinder and slowly diluted with an equal amount of distilled water. This solution was centrifuged at 38000 x g(max) for 45 minutes. The resulting pellet was osmotically shocked by resuspension in 2mls distilled water, and then sonicated in a Kerry 'Sonibath' for a total of 3 minutes. This was finally centrifuged 38000 x g (max) for 45 minutes, and the pellet used for the binding experiments as described below (2.3.1.3.).

2.3.1.2. A method closely following that used by Sharif and Roberts (1980) to produce synaptic membranes from rat brains was used. The method used differed slightly in the production of the final pellet following the sonication and washing phase.

Total central nervous tissue was dissected and homogenised in 0.32M sucrose (pH 7.2 with Tris base) using 10 strokes of a 15ml Jencons 'Uniform' glass in glass homogeniser. The homogenate was centrifuged at 1000 x g(max) for 20 minutes and the supernatant carefully removed. This was re-centrifuged at 17000 x g(max) for 20 minutes. The resulting pellet (P_2) was resuspended in distilled water, and sonicated for 3 minutes using a Kerry 'Sonibath'. The homogenate was pelleted and washed twice by resuspension in distilled water, and a final pellet produced by centrifuging at 38000 x g(max) for 15 minutes. This washed P_2 was then used for binding experiments (2.3.1.3).

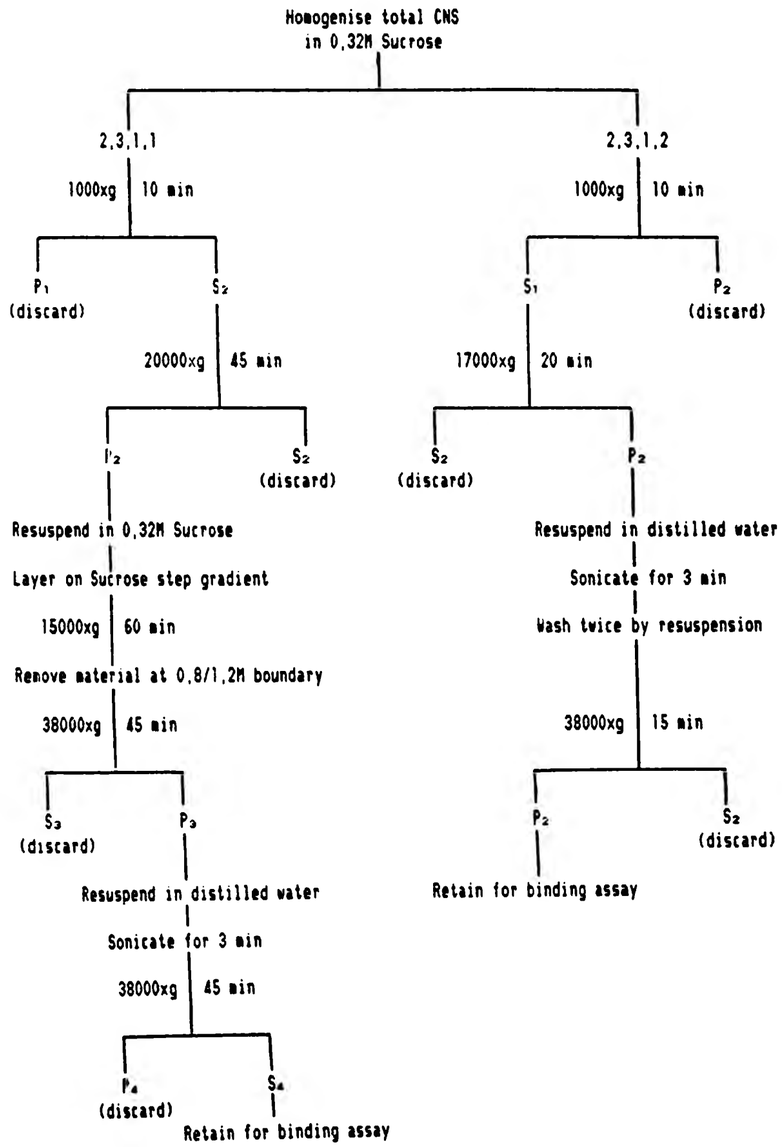

Figure 2.1.

Methods of membrane preparation to study glutamate binding.

Schematic outline for the preparation of membranes from P. americana central nervous tissue to study the binding of L-[^{3}H]-glu (I). The methods are based upon those of Gray and Whittaker (1962) and Sharif and Roberts (1980).

NB : Unless otherwise stated, all experiments used an homogenisation rate of 1CNS per 40µl of homogenisation buffer. Where washes were performed, these were all carried out in 5ml distilled water.

2.3.1.3 The final pellet was resuspended in 0.05M Tris/citrate buffer (pH 7.1) to give a final protein content of between 0.01 mg/ml and 0.1mg/ml. The binding assay was initiated by adding 360µl of the resuspended membrane preparation to 1.5ml conical test tubes (Wm. Sarstedt type 72,690) containing 20µl displacer and 20µl L-[G-^{3}H]-glutamic acid. The [^{3}H]-glu was diluted 1:35 from the stock solution (1µCi/µl; 30Ci/m mole; ex AI) to give a final ligand concentration of 50nM. Experiments were also carried out in which the binding saline was modified by the addition of various cations.

The membranes were incubated at 4°C for 20 minutes, and the assay was terminated by centrifugation. This involved transferring the assay solution to 400µl fine pointed tubes (Wm. Sarstedt type 72,702) which were placed in pre-formed holders made from Rapid Araldite (R.S. Components Ltd). These were centrifuged at 100000 g(max) for 5 minutes in a 'swing-out' rotor. The tubes were removed from their holders and the bulk of the supernatant aspirated and discarded. The tip of the tube containing the membrane pellet was removed and placed in a glass scintillation vial (F.G.B. Trident Ltd) containing 500µl Soluene (Packard Instruments Ltd). This was left over night before liquid scintillation counting.

2.3.2. The binding of D,L-[^{3}H]-2-amino-4-phosphonobutyric acid (APB).

Membranes were prepared as described in Section 2.3.1.2. The pellet was resuspended in 0.05M Tris/citrate buffer (pH 7.1) for use in binding assays. The assay was carried out as described in Section 2.3.1.3 with the following modifications. The ligand used was D,L-[3,4-^{3}H]-2-amino-4-phosphonobutyric acid diluted 1:30 from stock (iµCi/µl; 26.1Ci/m mole;ex NEN) giving a final ligand concentration of 30nM. The binding saline was also modified by the additon of $CaCl_2$, as suggested by Butcher et al (1983) after their studies of the binding of D,L-[^{3}H]-APB (VI) to rat brain synaptic membranes.

2.3.3. The binding of [^{3}H]-kainic acid

Three methods of membrane preparation were used to try to show the binding of [^{3}H]-kainic acid (XIV). These are described below.

2.3.3.1. Because it has been shown to produce membranes containing glutamic acid receptors which show displaceable binding of L-[^{3}H]-glu, the method of Sharif and Roberts (1980) was used (Section 2.3.1.2).

2.3.3.2. Membranes were also prepared using the method of Staatz et al. (1983). This method was used to study the effects of pyrethroids on the binding of [^{3}H]-kainic acid to membranes from the mouse brain.

Total central nervous tissue was dissected and homogenised in glass distilled water using 20 strokes of a 15ml Jencons 'Uniform' glass in glass homogeniser. The homogenate was centrifuged at 100000 x g(max) for 15 minutes. The supernatant was discarded and the pellet resuspended in glass distilled water and left to stand for 30 minutes at 37°C. The suspension was re-centrifuged and the pellet resuspended in 0.05M Tris/citrate buffer (pH 7.1) the solution was finally centrifuged at 100000 x g(max) for 15 minutes, and the pellet used for binding assays as described below (2.3.3.4)

2.3.3.3. Finally, membranes were prepared using the method of Ruck et al. (1980) This method was used to study the binding of [^{3}H]-kainic acid to rat cerebellum membranes.

Total central nervous tissue was dissected and homogenised in 0.32M sucrose (pH 7.1 with Tris base). The homogenate was centrifuged at 1000 x g(max) for 10 minutes. The supernatant was re-centrifuged at 20000 x g(max) for 20 minutes. The resulting pellet was resuspended in distilled water and sonicated intermittantly for 20 seconds using a Dawe Soniprobe on maximum setting. The shocked membranes were centrifuged at 8000 x g(max) for 20 minutes. The supernatant was removed, and used to carefully rinse the pellet to remove its top (lighter) layer to produce an augmented supernatant (S_3^+). This supernatant was centrifuged at 48000 x g(max) for 20 minutes. The pellet was washed by resuspension, and the final pellet stored at -25°C for 24-72 hours.

On the day of use, the pellet was thawed and resuspended in 0.05M Tris/citrate buffer (pH 7.1) using the Dawe Soniprobe. This was centrifuged at 48000 x g(max) for 10 minutes and this final pellet used in the binding assay.

2.3.3.4. For the methods described in Sections 2.3.3.1. and 2.3.3.2. the membranes were resuspended in 0.05M Tris/citrate buffer (pH 7.1) at a concentration of 1 CNS equivalent per 400µl. 380µl of this homogenate were added to 20µl of displacer and 2µl of [^{3}H]-kainic acid (1µCi/µl; 60Ci/m mole; ex NEN) giving a final ligand concentration of 80nM. This was incubated at 4°C for 20 minutes, and the incubation stopped and counted as described in Section 2.3.1.3.

For the experiment described in Section 2.3.3.3. the procedure was similar, the only modification being that the membranes were resuspended at the equivalent of 1 CNS per 500µl, and 480µl of this homogenate were used per assay. This gave a final ligand concentration of 65nM.

2.4. The binding of ligands to a potential GABA receptor and related sites on the GABA/Cl^- channel complex in membranes derived from P. americana central nervous tissue.

2.4.1 The binding of γ-[^{3}H]-amino butyric acid and [^{3}H]-muscimol.

The experimental procedure follows that used by Enna and Synder (1977) to study the binding of [^{3}H]-GABA (XVII) to rat brain synaptic membranes. The procedure followed is schematically described in Figure 2.2.

Total central nervous tissue was dissected and homogenised in 0.32M sucrose (pH 7.1 with Tris base) using 10 strokes of a Jencons 'Uniform' 15 ml glass in glass homogeniser. The homgenate was centrifuged at 1000 x g(max) for 10 minutes. The pellet (P_1) was discarded and the supernatant re-centrifuged at 20000 x g(max) for 10 minutes. The resulting pellet (P_2) was resuspended in distilled water and disrupted further by using a 2.5l Kerry 'Sonibath' for 10

minutes. The shocked membranes were centrifuged at 38000 x g(max) for 40 minutes. The pellet (P_3) was retained and stored at -26°C for at least 18 hours.

On the day of use, the pellet was thawed and resuspended in 0.05M Tris/citrate buffer (pH 7.1) containing 0.05%(w/v) Triton X-100 (Koch Light, Colnbrook). The homogenate was incubated at 37°C for 30 minutes and the detergent washed membranes harvested by centrifuging at 38000 x g(max) for 40 minutes. This pellet was finally resuspended in 0.05M Tris/citrate buffer (pH 7.1, no Triton X-100) at a protein concentration of 0.1mg/ml to 0.5 mg/ml. 360µl of this suspension were added to 20µl displacer and 20µl ^{3}H-ligand. The ligands used were γ-[2,3-^{3}H]-amino butyric acid (1µCi/µl; 60Ci/m mole; AI) diluted to give a final ligand concentration of 25nM; and [methyl-^{3}H]-muscimol (1µCi/µl; 20Ci/m mole; ex AI) diluted to give a final concentration of 75 nM. After incubation at 4°C for 30 minutes the assay was stopped as described in Section 2.3.1.3.

2.4.2. The binding of ligands directed at the Cl^- channel.

Various Cl^- channel ligands are available for use, and three of these were tried out under various conditions.

2.4.2.1. The binding of [^{3}H]-dihydropicrotoxinin (DPTX).

The method used to study the binding of [^{3}H]-DPTX (XLIII) was based upon the method used by Olsen et al. (1978) to study the binding of this ligand to membranes derived from crayfish muscle. The procedure used here differed from the original in missing out an initial high speed centrifugation and in adding the multi-function oxidase inhibitor piperonyl-butoxide to the binding medium. These modifications were used by Kadous et al. (1983) in their study of the binding of [^{3}H]-DPTX to membranes derived from the central nervous tissue of the German cockroach, Blattella germanica

Central nervous tissue from the cerebral and three thoracic ganglia was dissected and homogenised in van Harreveld's saline (NaCl:205mM; KCl: 5.4mM; $CaCl_2$: 13.6mM; $MgCl_2$: 2.6mM; Tris HCl: 5mM; pH 7.9 by the NaOH) using ten strokes of a Jencons 15 ml 'Uniform' glass in glass

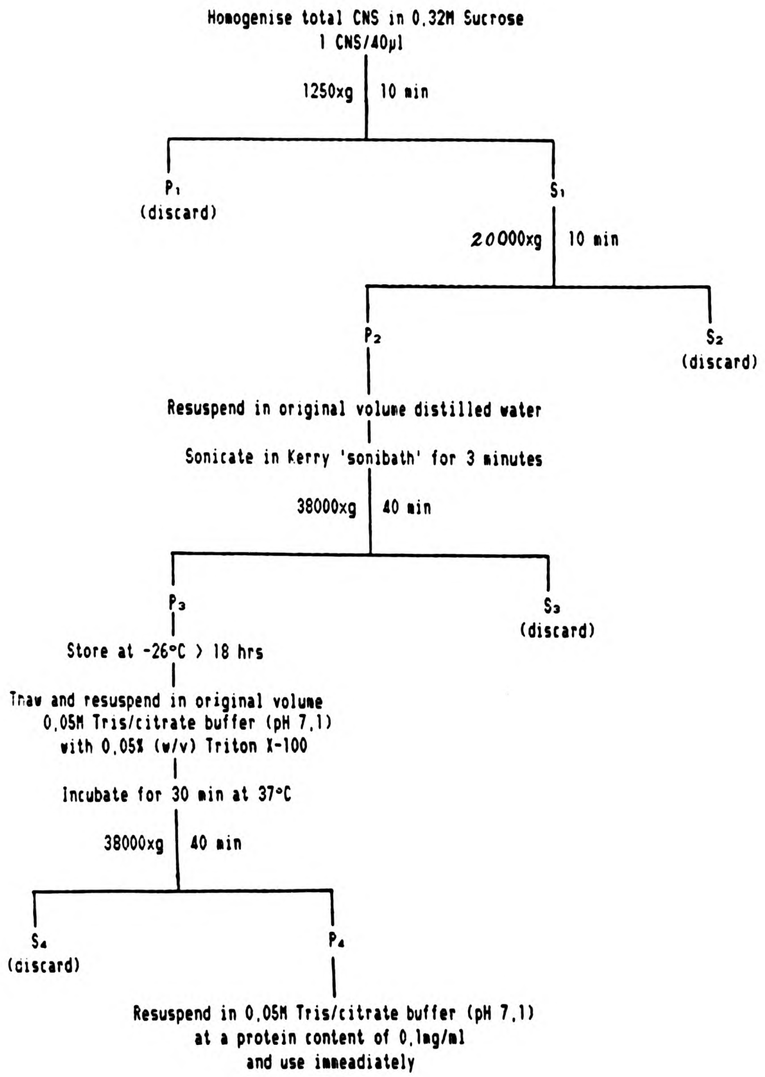

Figure 2.2.

Method used to prepare membranes to study GABA binding.

The schematic outline for the preparation of membranes from P. americana CNS which were used to study the displacable binding of [^{3}H]-GABA (XVII) and [methyl-^{3}H]-muscimol (XXI). This method follows the one published by Enna and Synder (1977).

homogeniser. The homogenate was centrifuged at 1000 x g (max) for 10 minutes, and the resulting supernatant re-centrifuged at 140000 x g (max) for 35 minutes. The pellet (P_2) was retained.

The P_2 was resuspended at a protein concentration of 1-3 mg/ml in a binding saline consisting of 200mM NaCl; 5mM Na_3PO_4 at pH 7.0 (by HCl). To 380µl of this were added 2µl piperonyl butoxide solution (0.676mg/ml, giving a final concentration of 10^{-5}M). Five minutes later, 2µl [^{3}H]-DPTX (1µCi/µl; 30Ci/m mole; a final concentration of 20nM - ex NEN) and 20µl displacer were added. The tubes were incubated at 4°C for 15 minutes before the assay was terminated and the membrane bound radioactivity estimated in the normal way.

2.4.2.2. The binding of t-[^{35}S]-butylbicyclophosphorothionate (TBPS)

2.4.2.2.1. One method used was based upon that published by Ramanjaneyulu and Ticku (1984) to reveal displaceable binding of [^{35}S]-TBPS (XLVI) to membranes derived from rat brains. Cerebral and thoracic ganglia were dissected and homogenised in 1mM EDTA, pH 7.0, using a Jencons 15ml 'Uniform' glass in glass homogeniser. The homogenate was centrifuged at 1000 x g (max) for 10 minutes, and the pellet discarded. The retained supernatant was re-centrifuged at 140000 x g (max) for 20 minutes. This time the supernatant was discarded and the pellet (P_2) retained. The P_2 was then resuspended in 1mM EDTA. This was then either dialysed overnight against 2l distilled water, or washed twice by centrifugation and resuspension. The membrane suspension was finally centrifuged at 140000 x g (max) for 20 minutes, and the washed membranes resuspended in a buffer containing 200 mM KBr; 5 mM Tris HCl; pH 7.5(by KOH) and finally pelleted for use in binding assays.

2.4.2.2.2. The method of Nicholson et al (1986) was followed. This method has been shown to give synaptosomes with functional Cl^- channels when using central nervous tissue from a wide variety of insects (L. sericata, S. gregaria, P. americana) as the starting material (See Figure 2.4).

Cerebral and thoracic ganglia were dissected and homogenised in 0.25M sucrose buffered with 0.1M Tris to pH 7.2 using 20 strokes of a 30mm bore Whittaker type homogeniser. The homogenate was centrifuged at 1000 x g (max) for 10 minutes. The supernatant was removed and stored on ice, whilst the pellet was washed twice by rehomogenisation. The combined supernatants were then centrifuged at 25000 x g(max) for 45 minutes to produce a crude synaptosomal pellet (P_2). The P_2 was then either used for the binding assay (normal) or osmotically shocked and washed to remove any endogenous Cl^- channel ligand which may have been present.

To shock the P_2 it was resuspended in 1mM EDTA, centrifuged at 38000 x g (max) for 40 minutes and washed by a second resuspension centrifugation cycle. This gave the 'shocked' P_2.

2.4.2.2.3. The binding assay was similar in all the above cases. The membranes from Section 2.4.2.2.1. were resuspended in saline containing 200mM KBr; 5mM Tris HCl; pH 7.5 to give a membrane concentration equivalent to 1 CNS per 400µl. The membranes from Section 2.4.2.2.2. were initially carefully resuspended in 400µl 0.25M sucrose buffered with 0.1M Tris, using a Jencons 1ml 'Uniform' glass in glass homogeniser. These membranes were then carefully introduced into various binding salines (200mM KCl/5mM Tris HCl; 200mM KBr/5mM Tris HCl; 200mM KI/5mM Tris HCl; Nicholson et al. (1986) Synaptosome saline - NaCl : 203mM; KCl : 3.1mM; $CaCl_2$: 1.9mM; $MgCl_2$: 6.6mM. Na_2HPO_4 : 2.4mM; NaH_2PO_4 : 2mM; Glucose: 8.8mM; pH 7.2) to give a final membrane concentration equivalent to 1 CNS per 400µl.

400µl of these homogenates were then added to 4µl displacer (either PTX or cold TBPS) and 2µl [^{35}S]-TBPS (diluted 1:10 from a stock containing 1µCi/µl; 99Ci/m mole; a final concentration of 6nM; this ex NEN). The experiments were incubated for 30 minutes at 25°C, and the incubation terminated by filtration : 2.5cm Whatman GF/B glass fibre filters were used with a Millipore filtration unit and pump and the membranes were washed with 2.5ml ice-cold binding saline. The filters, with the attached membranes and associated radioactivity, were estimated in the normal way.

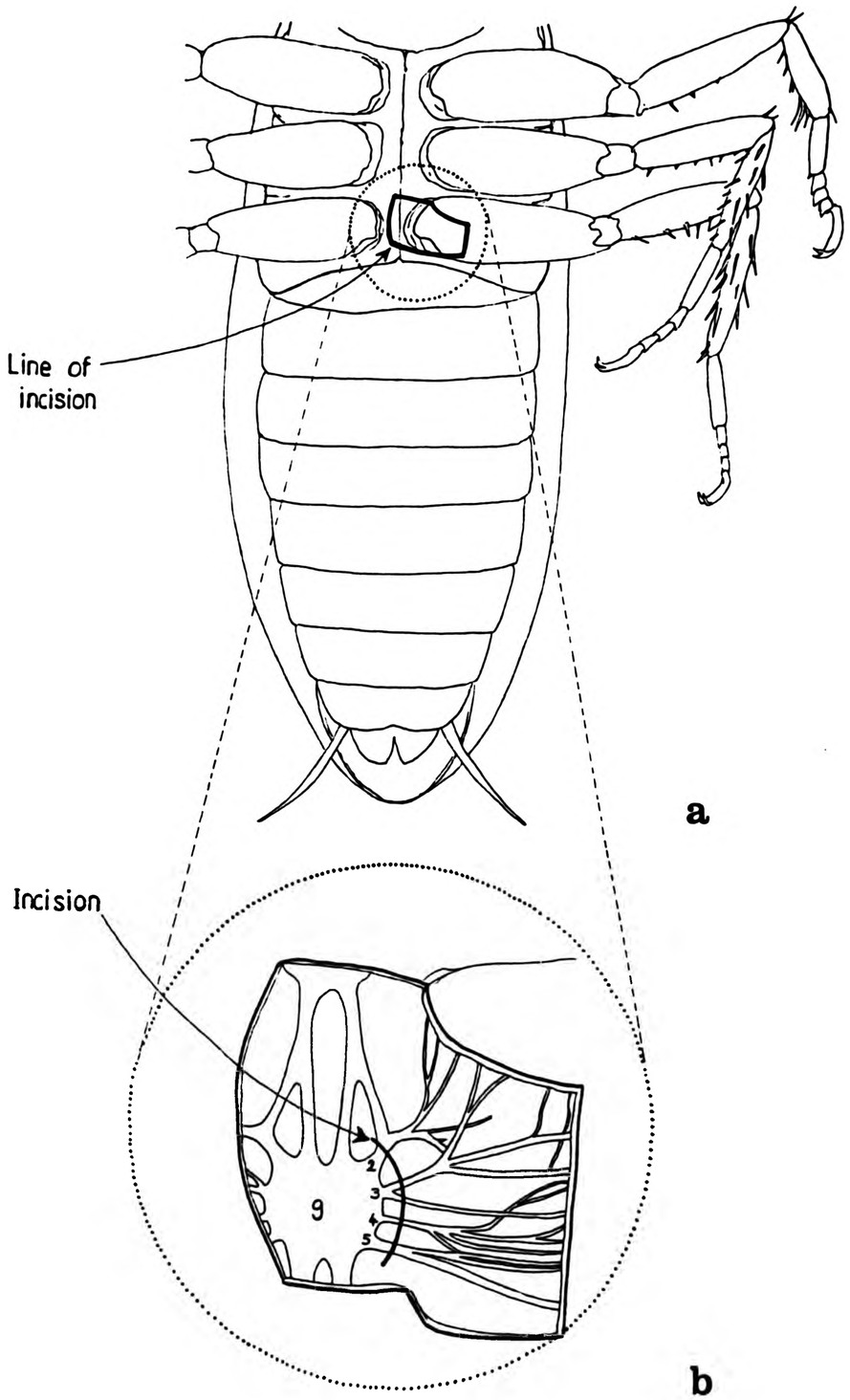

Figure 2.3

A schematic view of the denervation of P. americana coxa.

a. A ventral view of P. americana showing the area of cuticle to be removed.

b. A close up of the area exposed once the cuticle has been cut. This shows the meta thoracic ganglion (g), with the nerve trunks (2-5) to be severed. It is important to ensure nerves 3 and 5 are completely severed.

2.4.2.3. The binding of 1-[^{3}H]-phenyl-4-t-butyl-2,6,7-trioxabicyclo-[2,2,2]-octane (TBOB).

[^{3}H]-TBOB (XLVII) is available in a solution of 1μCi/μl at a specific activity of 55Ci/m mole (NEN). It was used diluted 1:5 from stock for the following experiments.

2.4.2.3.1. Once more, the method Ramanjaneyulu and Ticku (1984) was closely adhered to (Section 2.4.2.2.1.). Briefly, total central nervous tissue was dissected and the P_2 obtained using extensive washing, as previously described. The P_2 was then resuspended in 0.2M KBr; 5mM Tris HCl; pH 7.5 so as to yield a membrane concentration equivalent to 3 CNS per 400μl.

400μl of this homogenate was incubated with 4μl displacer and 1μl [^{3}H]-TBOB (giving a final TBOB concentration of 1nM) for 40 minutes. The incubation was terminated by filtration; the filters being washed rapidly with 2 x 2ml ice-cold saline. The filters were then counted in the normal manner.

2.4.2.3.2. The method used by Lawrence et al. (1985) to study the binding of [^{3}H]-TBOB to rat brain synaptic membranes was slightly modified. Total central nervous tissue was dissected and homogenised in 1mM EDTA (pH 7.0) using 30 strokes of a 30mm bore Whittaker type homogeniser. The P_2 was produced by centrifuging the homogenate at 1000 x g (max) for 10 minutes, and re-centrifuging the resultant supernatant at 10000 x g (max) for 20 minutes. The pellet was washed by resuspension and centrifugation. This was done twice in 1mM EDTA and once in 500mM NaCl; 50mM PO_4^{2-}; pH 7.5 (binding saline). The washed membranes were centrifuged at 25000 x g (max) for 20 minutes.

The pellet was suspended in binding saline at a membrane equivalent of 12 CNS per ml. The incubation was initiated by adding 250μl of these resuspended membranes to 250μl binding saline containing 4μl displacer and 1μl[^{3}H]-TBOB - a final concentration of 0.8nM. The assay was incubated for 30 minutes at 30°C and the incubation stopped by adding 2ml ice-cold binding saline, followed by rapid filtration: for this assay, Whatman GF/C filters were used in place of the GF/B. The filters were washed with two lots of 2ml of ice-cold binding

saline. The radioactivity associated with the membranes was estimated in the normal way.

2.5. **The binding of ligands associated with amino-acid coupled ion channels to membranes derived from other insect tissues.**

2.5.1. The binding of ligands to membranes derived from P. americana coxal muscle.

2.5.1.1. Various electrophysiological results suggest that TBPS (XLVI) acts at peripheral rather than central sites in the insect (G. Lees and S. Irving, personal communications). To test the binding of [^{35}S]-TBPS to peripheral sites in the American cockroach, the coxal muscles of the metathoracic leg were carefully squeezed out using the flat of a scapel blade, and membranes prepared from these using the method based on that of Ramanjaneyulu and Ticku (1984) as described in Section 2.4.2.2.1. The binding assay was carried out as described in Section 2.4.2.2.3. with the membranes being resuspended in the KBr/Tris binding saline at the equivalent of four coxa per 400µl.

2.5.1.2. Denervation of muscles has been shown to increase the neurotransmitter receptor density on the denervated muscles (e.g. Usherwood 1969). The left metathoracic leg was denervated by carefully removing a flap of cuticle near to the top of the leg and cutting nerve trunks 3 and 5 from the metathoracic ganglion using a Wick microsurgical scalpel (supplied by J. Weiss and Son Ltd). (Figure 2.3.). The flap of cuticle was replaced and the P. americana maintained for 21 days before using the coxa for binding experiments.

2.5.1.2.1. Sarcoplasmic membranes were prepared from the coxal muscles of right (normal) and left (denervated) metathoracic legs of P. americana treated as described in Section 2.5.1.2. These were prepared as described by Olsen et al. (1978) and used to study the binding of [^{3}H]-glu (I) to such membranes.

The metathoracic legs were removed and the coxa carefully squeezed out. These were homogenised in van Harreveld's saline using a Jencons 50ml 'Uniform' glass in glass homogeniser. The homogenate was centrifuged at 140000 x g (max) for 30 minutes. The supernatant was discarded, and the pellet resuspended in the original volume of saline. This was centrifuged at 1000 x g (max) for 10 minutes. The supernatant was stored on ice whilst the pellet was washed once. The combined supernatant was centrifuged at 140000 x g (max) for 30 minutes to produce the microsomal pellet (P_3).

The P_3 was resuspended in 3ml van Harreveld's saline and carefully placed on the top of a sucrose step gradient consisting of 6ml of each of 1.0M, 0.75M and 0.32M sucrose in 100mM NaCl; 5mM Tris, pH 7.7. This step gradient was centrifuged at 50000 x g (max) for 120 minutes and the sarcoplasmic membranes harvested by collecting the material at the 0.75M/1.0M sucrose boundary. The membranes were centrifuged to give a final pellet which was used in binding studies with [^{3}H]-glu, as described in Section 2.3.1.3.

2.5.1.2.2. Membranes were also prepared from the normal and denervated coxa to study the binding of [^{3}H]-muscimol (XXI). The coxa from the right (normal) and left (denervated) metathoracic legs were removed and membranes prepared and used in the binding assay as described in Section 2.4.1.

2.5.2. The binding of radio-ligands to amino-acid related receptors in membranes derived from insects other than P. americana.

2.5.2.1. The binding of [^{3}H]-GABA to membranes derived from the heads of L. sericata

L. sericata were chilled and then sieved with solid CO_2 using a coarse 'Endicott' sieve. With vigorous shaking, the heads and thoraces separated, and the heads then fell through the sieve, leaving the thoraces behind. Membranes were then prepared from the separated heads and used for a binding assay as described in Section 2.4.1.

2.5.2.2. The binding of [^{3}H]-amino acids to membranes derived from the cerebral and thoracic ganglia of the locust L. migratoria.

The method used by Breer and Jeserisch (1980) to produce synaptosomes from locust central nervous tissue was used to prepare membranes to study the binding of [^{3}H]-glu and [^{3}H]-GABA (XVII). The cerebral and three thoracic ganglia were dissected and homogenised in 0.25M sucrose; 0.1M Tris; pH 7.3 using 20 strokes of a 30mm bore Whittaker type homogeniser. The homogenate was centrifuged at 1000 x g (max) for 10 minutes. The supernatant was kept on ice, whilst the pellet was washed twice in half the original volume of sucrose. The combined supernatants were centrifuged at 25000 x g (max) for 20 minutes. The resulting supernatant was discarded and the pellet (P_2) retained.

The P_2 was carefully resuspended in 150µl sucrose containing 12%(w/v) Ficoll 400 (Pharmacia) and added to 750µl of the same. This was thoroughly mixed and centrifuged in 400µl tubes at 10000 x g(max) for 75 minutes. This produced a supernatant with a small floating 'pellicle'. The supernatant, with the 'pellicle' was mixed with 3.6ml of sucrose and centrifuged at 25000 x g (max) for 20 minutes to produce the synaptosomal pellet (P_3).

The membranes so produced were used to study the binding of [^{3}H] - glutamic acid (as described in Section 2.3.1.3) and [^{3}H]-GABA (as described in Section 2.3.3.4.)

2.6. A study of the enzymes involved in the immediate production and degradation of GABA in P. americana central nervous tissue.

2.6.1. Glutamic acid decarboxylase (GAD)

This method of obtaining and estimating the activity of GAD is based upon methods used to obtain the bacterial enzyme, as modified for screening purposes by Helen White of the Burroughs Wellcome Co. in America (White: 1981). Whole central nervous tissue was dissected and homogenised in a buffer consisting of : 0.1M potassium phosphate; 0.5% (w/v) Triton X-100; 1mM pyridoxal phosphate, 1mM dithiothreitol; pH 6.9. The homogenate was centrifuged at 16000 x g (max) for 30

minutes and the pellet discarded. The supernatant was brought to 25% saturation with ammonium sulphate and stirred gently for 60 minutes. The solution was centrifuged at 16000 x g (max) for 30 minutes and the pellet discarded once more. The supernatant was brought to 45% saturation with ammonium sulphate and stirred gently for 60 minutes before once more centrifuging at 16000 x g(max) for 30 minutes. This time the pellet was retained, and resuspended in a buffer containing 0.1M potassium phosphate; 1mM pyridoxal phosphate; 1mM dithiothreitol; pH 6.9. The resuspended membranes were dialysed against at least 1000 volumes of the resuspension buffer for 6 hours, and the resulting suspension stored at -20°C for up to three months, and slowly thawed before use.

A Pierce-Wariner 3.5ml 'Reactivial' was used as the reaction vessel. 100µl of reaction buffer (0.1M potassium phosphate; 1mM pyridoxal phosphate; 0.1mM dithiothreitol; pH 6.9) and 25µl 0.03M glutamic acid containing 1.25µCi [U-^{14}C]-glutamic acid (ex AI) were carefully added to the bottom of this. The reaction was initiated by adding 25µl of the enzyme preparation. A wick (1cm x 1cm Whatman No.1 filter paper) soaked with 25µl of 10% hyamine 10-X hydroxide in methanol was carefully wrapped around the septum, inserted into the reaction vessel and the cap screwed on well. The vessel was then incubated for 30 minutes at 37°C.

The incubation was terminated by carefully introducing 100µl of 10% trichloracetic acid into the reaction vessel using a gas chromatography syringe (S.G.E. Limited) introduced through the septum. The vial was allowed to stand for 60 minutes to allow the evolution of the $^{14}CO_2$ to be completed. The $^{14}CO_2$ was produced by the decarboxylation of the [^{14}C]-glu, and was trapped by the hyamine wick. This was counted in the normal way.

2.6.2. γ-Aminobutyric acid transaminase (GABA-T)

This method is a shortened version of that used by Watts and Atkins (1983) to study GABA-T in the nematode Nippostrongylus braziliensis. Total central nervous tissue was dissected and rinsed in 10mM potassium phosphate buffer, pH 7.2 The tissue was then homogenised in a buffer consisting of 50mM potassium phosphate; 0.1mM reduced

glutathione; 0.03mM pyridoxal phosphate; 0.2% (v/v) Triton X-100; pH 8.0; using 10 strokes of a Jencons 5ml 'Uniform' glass in glass homogeniser. The homogenate was centrifuged at 20000 x g (max) for 30 minutes and the supernatant retained. This was brought to 45% saturation with ammonium sulphate and stirred gently for 60 minutes. The solution was centrifuged at 4000 x g (max) for 30 minutes, and the supernatant (S_2) retained.

For the standard assay, the S_2 was dialysed overnight against a buffer containing : 100mM potassium phosphate; 5mM dithiothreitol; 0.1mM EDTA; 0.04mM pyridoxal phosphate; pH 8.0. After dialysis, the enzyme preparation was stored until required at -26°C.

For the preliminary investigations, the S_2 was brought to 65% saturation with ammonium sulphate and stirred gently for 60 minutes. This was then centrifuged at 44000 x g (max) for 30 minutes. The pellet (P_3) was stored at -26°C until required, and the supernatant was dialysed as described above before storage.

To assay for GABA-T activity, 50µl of thawed enzyme preparation were placed in a 1.5ml conical test tube (Wm Sarstedt type 72,690) to which were added 25µl of assay mixture (dialysis buffer containing 0.8mM 2-oxoglutarate and 0.8mM 4-amino-n-[U-^{14}C]-butyric acid at a specific activity of 2.2Ci/mole-ex AI) and 25µl of buffer, with or without amino-oxyacetic acid. The reaction mixture was incubated at 37°C for 30 minutes, and the reaction was stopped by adding 10µl 2M HCl.

The reaction products were separated from the unchanged GABA (XVII) by adding 1ml of a suspension of thoroughly washed Dowex 50W-X8 (H^+form) in distilled water (0.4 g/ml). The tubes were briefly centrifuged in a Beckman TJ-6 bench-top centrifuge to consolidate the ion-exchange resin. 0.4ml of the supernatant was carefully removed and added to 0.8 ml distilled water so that the amount of [^{14}C] - radioactivity could be estimated in the normal manner.

2.6.3 The effect of GAD and GABA-T inhibitors upon the levels of GABA in the CNS of P.americana.

An experiment was carried out to study the effect of various compounds upon the levels of GABA found in the CNS of P. americana. 120µg of each compound (4µl of aqueous solution containing 30mg/ml) were injected into the haemocoel using an SGE 10µl gas-tight syringe, introducing the needle through the intersegmental membrane between the 7th and 8th abdominal sternites. After 24 hrs in the normal culture conditions; the roaches were chilled and the total central nervous tissue dissected. Each CNS was individually homogenised in 20µl absolute ethanol, using a Jencons 0.1 ml 'Uniform' glass in glass homogeniser. The homogenate was transferred to a Sarstedt 1.5 ml concial test-tube and centrifuged briefly to precipitate the protein. 2µl aliquots were then removed for GABA estimation.

The GABA content was estimated by micro-dansylation. 1µl[^{14}C]-GABA (224mCi/mmole, ex AI) was added to the supernantant as an internal standard and the sample carefully freeze-dried in a Sarstedt 0.75ml concial test-tube (cat no. 72,680). To this were added: 1µl 0.2M $NaHCO_3$; 3µl 2:1 acetone:water; 4µl [^{3}H]-dansyl chloride solution (containing 1.56mg/ml and 1mCi/ml, ex AI) This was incubated at 37°C for 20 minutes, the reaction being stopped by freezing. 1µl of the reaction product was then spotted onto a 7.5cm x 7.5cm polyamide thin layer chromatography plate (Schleicher and Schull) and developed in two dimensions - once in 3% aqueous formic acid and subsequently in 3:3:1 heptane:butanol:acetic acid.

The developed plate was visualised by U.V irradiation (254 nm) and the spot corresponding to dansyl-GABA outlined. This spot was then carefully cut out and counted, using gates set up for dual label counting of ^{3}H and ^{14}C. The recovery of GABA was estimated using the [^{14}C]-GABA as an internal standard (the recoveries varied from 45% to 75%) and the original concentration of GABA in the sample estimated from the amount of [^{3}H]-dansyl-GABA present on the piece of excised polyamide plate.

Figure 2.4

The method used to prepare insect synaptosomes for superfusion.

a. Flow chart showing the preparation and loading of insect synaptosomes ready for use in the superfusion system.

b. A diagramatic representation of the superfusion system.

This method is as described by Nicholson et al. (1986).

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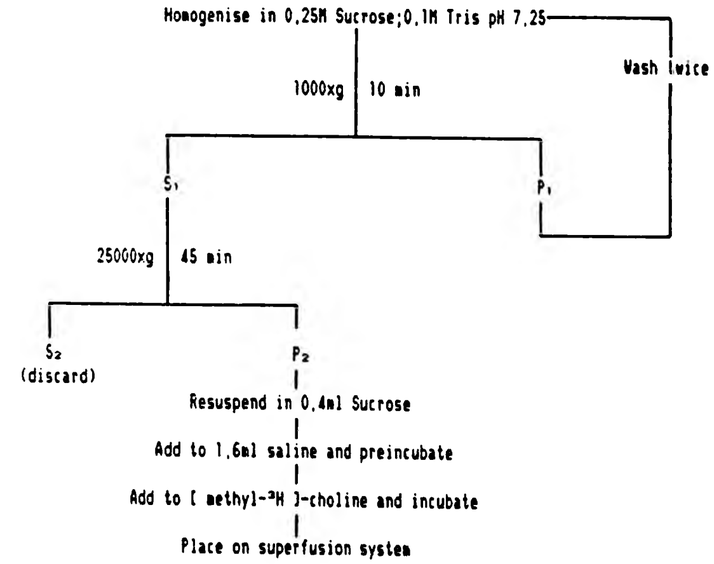
a)



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b)

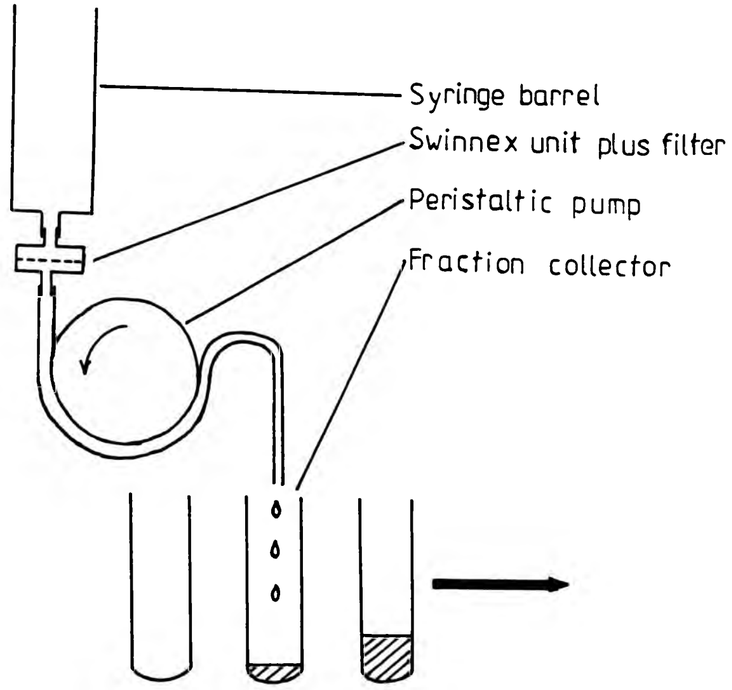


Figure 2.4

The method used to prepare insect synaptosomes for superfusion.

a. Flow chart showing the preparation and loading of insect synaptosomes ready for use in the superfusion system.

b. A diagramatic representation of the superfusion system.

This method is as described by Nicholson et al. (1986).

2.7 The production of synaptosomes from the central nervous tissue of P. americana and their use in transmitter release studies.

This method, a modified version of the one developed by Breer and Jeserisch (1980), was described by Nicholson et al. (1986). Head and thoracic ganglia were dissected and homogenised in 0.25M sucrose buffered with 0.1M Tris at pH 7.2, using 20 stokes of a 30mm bore Whittaker type homogeniser. The homogenate was centrifuged at 1000 x g(max) for 10 minutes. The supernatant was removed and stored on ice, and the pellet was washed twice by resuspension and re-centrifugation, the supernatants from each wash being combined with the original supernatant. The combined supernatants were centrifuged at 25000 x g (max) for 45 minutes, giving a pellet (P_2) which was retained. The P_2 has been shown to contain considerable numbers of intact synaptosomal profiles when viewed under the electron microscope (Nicholson et al. 1985).

The P_2 was carefully resuspended in 0.4ml Tris buffered 0.25M sucrose using a Jencons 1ml 'Uniform' glass in glass homogeniser. The homogenate was then slowly introduced into 1.6 ml oxygenated saline consisting of: NaCl:203mM; KCl:3.1mM; $CaCl_2$:1.9mM; $MgCl_2$:6.6mM; Na_2HPO_4: 2.4mM; NaH_2PO_4: 2mM; Glucose : 8.8mM; pH 7.25. This was preincubated at 30°C for 5 minutes. The synaptosomal solution was then added to 100µl saline containing 50µCi [methyl-^{3}H]-choline (1µCi/µl; 80Ci/m mole; ex AI) and incubated for 15 minutes at 30°C.

100µl of the [methyl-^{3}H]-choline loaded synaptosomes were carefully placed onto Whatman GF/B filters held in Millipore Swinnex filter holders, and then washed onto the filters using 3 x 200µl saline. The synaptosomes were then superfused with 30ml oxygenated saline followed by 20 ml saline containing the various compounds to be tested. The flow rate was roughly 0.8ml/minute maintained by a Watson Marlow 502U peristaltic pump, and fractions were collected every 3 minutes using an LKB Ultrorac II fraction collection. The amount of ^{3}H present in each fraction was estimated in the normal way.

Figure 2.4 shows a flow chart of the preparation and a diagramatic representation of the superfusion system.

2.8 Miscellaneous experiments

2.8.1. The binding of D,L-[pyrrolidinyl-^{3}H(N)]-nicotine to membranes derived from the central nervous tissue of P. americana.

In this experiement, the binding of [^{3}H]-nicotine (LX) and [^{3}H]-glu (I) to the same synaptosomal membrane fraction was compared. The method used was based upon Breer (1981) in which he did some of the initial work on characterising his locust central nervous tissue derived synaptosomes. The major modification involved replacing sucrose by mannitol. Total central nervous tissue was dissected and homogenised in 0.25M mannitol; 1M EDTA; pH 7.4 using 10 stokes of a Schutt Teflon in glass homongeniser. The homogenate was centrifuged at 1250 x g (max) for 10 minutes and the supernatant re-centrifuged at 45000 x g(max) for 10 minutes. The pellet (P_2) was retained.

The P_2 was carefully resuspended and transfered to the Schutt homogeniser using 2 x 150µl mannitol buffer. The tube was then rinsed with 1ml mannitol buffer supplemented with 12%(w/v) Ficoll 400. The rinsings were added to the homogeniser and the P_2 evenly dispersed using three strokes of the plunger. The homogenate was transferred to a centrifuge tube, and the homogeniser rinsed well with a further 0.7ml of the mannitol/Ficoll 400 buffer. The rinsings were added to the centrifuge tube, and the contents thoroughly but gently mixed. The resuspended P_2 was centrifuged at 20000 x g (max) for 75 minutes. The supernatant was diluted with 2-3 volumes of mannitol buffer and re-centrifuged at 75000 x g (max) for 20 minutes to produce the synaptosomal pellet (P_4).

P_4 was resuspended in 5ml of 50mM Tris/citrate buffer (pH 7.1) and 360µl used for binding assays. For [^{3}H]-glu the procedure described in Section 3.2.1.2. was followed. For [^{3}H]-nicotine 20µl of a diluted radioligand solution (diluted 1:150 from stock, which was 5µCi/µl, 35Ci/mmole; ex NEN) was used in place of [^{3}H]-glutamate (giving a [^{3}H]-nicotine content of 50nM), otherwise the same procedure was used.

2.8.2 The binding of [^{125}I]-bungarotoxin to membranes derived from the central nervous tissue of S. gregaria

A crude P_2 was produced and was then further fractionated. α-[^{125}I]-Bungarotoxin was used to identify those fractions containing acetyl choline receptors, which are in turn a marker for synaptosomal membranes.

The total central nervous tissue was dissected from S. gregaria and was homogenised in 0.25M sucrose containing 0.1M Tris and 1mM EDTA at pH 7.4 using 10 stokes of a small motor driven Whittaker type homogeniser. The homogenate was centrifuged at 1000 g (max) for 10 minutes, and the supernatant re-centrifuged at 20000 x g (max) for 20 minutes. The supernatant was discarded, but the pellet (P_2) was retained.

The P_2 fraction was carefully resuspended in 2ml of the homogenisation buffer and carefully placed upon a discontinuous Ficoll step gradient. This consisted of 1ml of each 11%, 9%, 7%, 5% (w/v) Ficoll 400 in homogenisation medium. The gradient was spun at 70000 x g (max) for 90 minutes.

The binding assay followed that used by Fertuck and Salpeter (1976) in their studies on the localization of acetylcholine receptors on mouse motor end plates. Each fraction from the step gradient was diluted to a protein content of between 0.25 and 2mg/ml using α-bungarotoxin binding medium (NaCl:50mM; sucrose: 250mM; Tris: 10mM; BSA: 5mg/ml; pH 7.3). 360µl of each membrane solution was added to 20µl diluted [^{125}I]-bungarotoxin (1:100 from stock which was 0.5mCi/ml; 200Ci/mmole; (AI) - a final concentration of 0.6nM). This was incubated for 90 minutes at 20°C, before stopping the incubation by transferring the assay medium to the 400µl fine pointed tubes and harvesting the membranes by centrifugation. The tips of the tubes containing the harvested pellets were removed and the associated radioactivity counted.

2.8.3. A check for metabolism of [^{3}H]-TBOB by P. americana homogenates.

Central nervous tissue membranes were produced as described in Section 2.4.2.3.2. The P_2 was resuspended in the binding saline at a membrane concentration equivalent to 6 CNS per ml. To this was added 1µCi [^{3}H]-TBOB (XLVII), and this was incubated for 30 minutes at 4°C, 21°C and 37°C. The membrane solutions were then filtered through Millipore Swinnex disposable filters, and 15µl of the filtrate assayed by HPLC under conditions which would separate TBOB from its major metabolites. This involved a 10cm C-18 reverse phase column (Chrompack) with an eluting solvent of 70% methanol/30% water at a flow rate of 0.4ml/min and maintained at a temperature of 20°C. The eluate was monitored at 210nm, and fractions for liquid scintillation counting were collected at one minute intervals.

2.9 Protein estimations

The two methods of protein estimation described below were used unsystematically, the method chosen being largely dependent upon the amount and range of protein contents expected in the samples. For small amounts, the Coomassie Blue method tended to be used, for large amounts, or a large range, the modified Lowry was used.

2.9.1. The method using Coomassie Brillant Blue G250.

This method was described by Sedmark and Grossberg (1977) and was a modification of the assay by Bradford (1976). Coomassie Blue G250 was prepared as a 0.06% solution in perchloric acid, and filtered through a Whatman No. 1 paper. Protein solutions were prepared in 0.5ml unbuffered saline and added to 0.5ml of the Coomassie Blue solution. The two were mixed, and the absorbance at 620nm read. A standard curve was prepared for each batch of protein estimations and the protein content calculated accordingly.

2.9.2 The modified Lowry method.

This method was described by Peterson (1977) and was a modification of the original method of Lowry et al. (1951). The following stock reagents were made up:

1. Copper-tartrate-carbonate: A solution of about 20% sodium carbonate was added slowly with stirring to a copper sulphate/sodium-potassium tartrate solution to give final concentrations of 0.1% copper-sulphate; 0.2% potassium tartrate, 10% sodium carbonate. This solution was stable for at least 2 months.

2. 10% sodium dodecyl sulphate.

3. 0.8M sodium hydroxide.

4. 2M Folins - Ciocaltau phenol reagent.

From these the following working solutions were made:

A. Mix equal parts of 1, 2 and 3 and distilled water. This solution will keep at least two weeks.

B. Solution 4 was diluted 1:5 with distilled water, and kept in an amber bottle.

The protein was brought to between 5µg/ml and 100µg/ml with distilled water, and 1ml of this added to 1ml Reagent A. This was allowed to stand for 10 minutes. 0.5ml of Reagent B was then added and rapidly mixed. This was allowed to stand for at least 30 minutes, and the absorbance of 750nm read between then and 2 hours after Reagent B had been added. Once more a standard protein curve was run with each set of samples.

All the procedures for protein estimation were carried out at 20°C.

CHAPTER 3

Results

3.1 Statistics

Throughout this chapter, the methods outlined below were used to obtain the various statistical parameters.

3.1.1 Means and standard errors.

The group means and the standard errors of those means, were calculated using a Hewlett-Packard HP25 programable calculator.

3.1.2 Statistically significant differences between groups.

Where two or more groups have been treated for statistically significant differences, a package written in APL for the mainframe IBM370/148 computer was used. The program used Bartlett's test for homogeneity of group variances, (Bartlett 1937), followed by Duncan's multiple range test (Duncan 1955), or Shuster and Boyett's non-parametric method (Shuster and Boyett 1979) in cases where the normal restrictions for parametric statistical tests were not met. (Burch and Macpherson 1980).

3.1.3 Straight lines.

Data were fitted to straight lines by calculating the least squares fit using a Texas Instruments TI-66 programable calculator.

3.2 Radioligand binding assays.

The results have been calculated by dividing the total binding into its component parts of 'specific binding' and 'non-specific binding'. The 'total binding' was taken as the total amount of radioactivity associated with the membranes and counted in the scintillation counter. The 'non-specific' component was taken as that portion of the radioactivity that associates with the membranes due to non-specific interactions; eg. lipophilicity, ionic interactions, van der Waal's forces, solution entrapped in the pellet, radioacitivity associated with the filter. This component was not saturable within the limits of experimentation. As the amount of radioligand was constant for each series of experiments, the 'non-specific' binding

was independent of the amount of displacing compound added. It was estimated by adding a large excess of displacing compound, so that the amount of radioligand associated with the membranes due to receptor-ligand interactions was less than 0.1% of the total counts present. The 'specific binding' was obtained by subtracting the 'non-specific binding' from the 'total binding' and represents the amount of radioactivity associated with the membrane due to receptor-ligand interactions. It is on the 'specific binding' component that all the various transformations were carried out.

3.2.1 The Scatchard plot.

The plot of [bound ligand (B)]/[free ligand (F)] vs [bound ligand] was first used for receptor-ligand interactions by Scatchard (1949) and is analogous to the Eadie plot of the Michaelis-Menten equation for enzyme kinetics (Eadie: 1942). It assumes one type of receptor with a 1:1 interaction between the ligand and the receptor and is derived from the equilibrium equation for the interaction as below:

$$R + L \rightleftharpoons RL$$

where R represents the receptor, L the ligand and RL the receptor-ligand complex. Thus:

$$K_a = \frac{[RL]}{[R][L]} = \frac{1}{K_d}$$

But:

$$[R_T] = [RL] + [R]$$

where R_T represent the total number of receptors present,

and:

$$[RL] = [B] \; ; \; [L] = [F]$$

Thence:

$$K_d = \frac{([R_T] - [B])[F]}{[B]}$$

or:

$$\frac{[B]}{[F]} = \frac{1}{K_d} ([R_T] - [B])$$

Thus a plot of [B]/[F] vs [B] gives a straight line with a slope of $-1/K_d$ and an intercept on the abscissa of $[R_T]$. Should the initial assumptions be incorrect and more than one type of receptor exist, or the interaction between the receptor and the ligand prove not to be 1:1, then the plot gives a curve.

This analysis was used for both the glutamate and the GABA receptors in those cases where the tritiated ligand was being displaced by an excess amount of the same cold material.

3.2.2. The Hill plot.

The Hill or logit plot is explained by Enna (1985). This is derived from the law of mass action for the following reaction equation:

$$R + nL \rightleftharpoons RL_n$$

Thus:

$$K_d = \frac{[R]\,[L]^n}{[RL_n]}$$

Replacing as before:

$$K_d = \frac{([R_T] - [B])[L]^n}{[B]}$$

or:

$$\frac{[B]}{([R_T]-[B])} = \frac{1}{K_d}[F]^n$$

Taking logarithms:

$$\lg \frac{[B]}{([R_T]-[B])} = -\lg K_d + n \lg[F]$$

Thus a plot of $\lg([B]/[R_T]-[B])$ against $\lg[F]$ will give a straight line for which the slope will give the number of ligand molecules binding with each receptor molecule and the intercept on the ordinate will give $-\lg K_d$. When the slope of the line is unity, this will show that the ligand and receptor molecules bind 1:1 and that there is no cooperativity in binding.

The value for $[R_T]$ obtained from the Scatchard plots constructed as in Section 3.2.1. was used to construct Hill plots for these experiments.

3.3 The binding of ligands to the glutamate receptor in membranes derived from the central nervous tissue of P. americana.

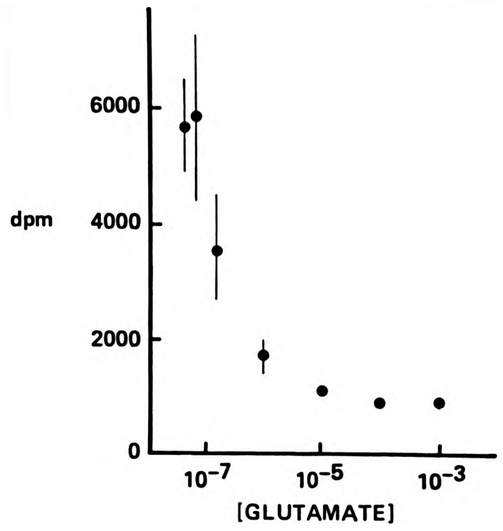
The results of the various experiments undertaken to investigate the glutamate receptor are given below.

3.3.1 The binding of L-[^{3}H]-glutamic acid.

For these experiments 'non-specific binding' was taken as that level of radioactivity remaining associated with the membranes in the presence of 10^{-3}M cold L-glu (I). In the experiments using the method of Sharif and Roberts (Section 2.3.1.2) the non-specific binding varied between 20% and 50% of the total radioactivity bound at 5×10^{-8}M glu. The standard error about the mean for groups of replicate samples was normally about 6% of the mean, but it was occasionally as high as 20% of the mean. The amount of membrane protein used for each assay was about 30µg.

Figure 3.1 gives graphical representations of results obtained from an experiment to show the displacement of the [^{3}H]-glu from the membrane preparation by the addition of unlabelled glu. The Scatchard plot gave a value for the K_d of glu and its membrane receptor of 1.84×10^{-7}M, with a receptor density of 10.6fmoles/µg of

a.



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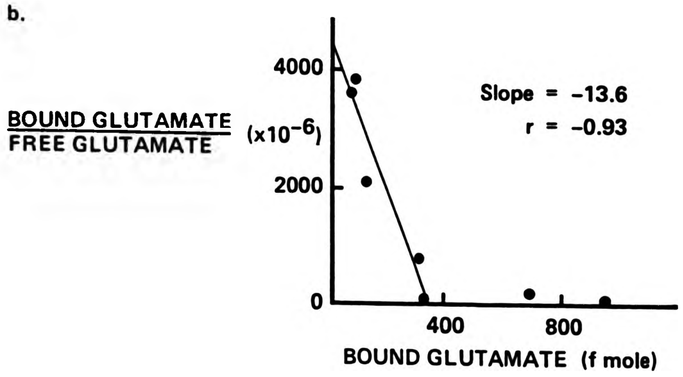

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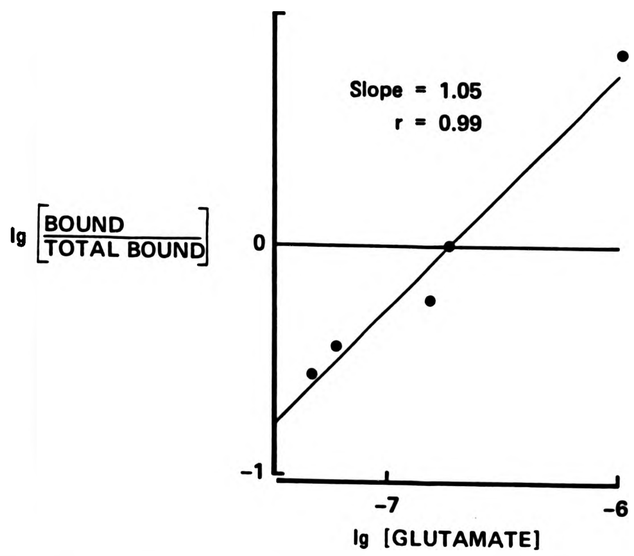


Figure 3.1

The displacement of L-[^{3}H]-glutamic acid by excess cold L-glutamic acid

a) A semi-logarithmic plot showing the amount of [^{3}H]-glu (I) bound to the membranes (as disintegrations/minute) against the total concentration of glu added. Each point is the mean of 3-6 samples and the error bars give the standard error about this mean.

b) A Scatchard plot using data derived from the results graphically displayed in figure 3.1(a). The non-specific binding was taken as that amount of [^{3}H]-glu bound in the presence of 10^{-3}M cold glu. This was 911dpm for this experiment. 32µg protein were used per assay, thus the number of glu receptors present was 10.6fmoles/µg of protein and the K_d for glu was 1.84×10^{-7}M.

c) A Hill plot using data derived from the results of the Scatchard plot (Figure 3.1(b)). The slope of this line was 1.04 and the intercept on the x-axis -6.75 (giving a K_d of 1.78×10^{-7}M).

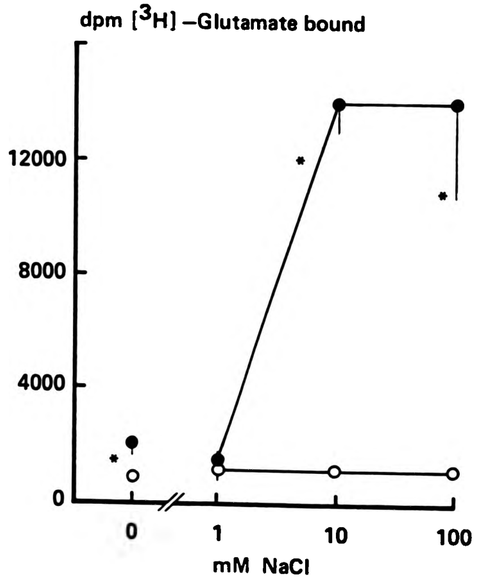


Figure 3.2

The effect of Na^+ ion concentration upon the binding of glutamate to its receptor in membranes derived from P.americana CNS.

The points show the mean and the standard error about that mean, each point being assayed at least in triplicate. Each sample contained 10µg of protein.

o-o Non-specific binding : radioactivity bound in the presence of 10^{-3}M cold glu.

●-● Total binding : radioactivity bound in the presence of the diluted ligand only (47.5nM glu).

* Significant difference.

Table 3.1

The effect of various cations on the binding of glutamic acid to its receptors in membrane fragments derived from P. americana central nervous tissue.

Each cation was added at a final concentration of 100mM and the mean and standard error for the amount of radioactivity bound calculated. Each value is the result of at least triplicate samples. The terms 'non-specific' and 'total' binding are as defined in the text.

* Significant difference between the 'non-specific' and the 'total' amounts of radioactivity bound.

CATIONS PRESENT	NON-SPECIFIC BINDING dpm	SPECIFIC BINDING dpm
None(standard)	1737+/-107	2774+/-443*
Mg^{++}	1814+/-191	2802+/-191*
Ca^{++}	1563+/-151	2419+/- 21*
K^{+}	1714+/-151	2223+/-251

membrane protein. The two points at high ligand concentrations were not used in calculating these values. Such points have been reported to give aberant results because the amount of 'specific binding' is too small to be statistically significant when compared with the amount of 'non-specific binding' or because of the presence of saturable non-specific sites of very low ligand affinity (Cuatrecasas and Hollenberg 1976). The slope of the Hill plot of 1.04 confirmed that the binding of L-glu to the membrane receptors was non-cooperative and that the K_d was around 1.8×10^{-7}M, the plot giving a value of 1.78×10^{-7}M.

The ion dependency of the binding of L-glu to its membrane receptors was studied, the various ions being added as concentrated solutions in place of the 20µl displacer. Figure 3.2 shows the effect of Na^+ (added as NaCl). The system was highly sensitive to Na^+, the amount of L-glu associated with the membranes increased some 8-10 fold with the addition of only 10mM NaCl. K^+ (as KCl), Mg^{2+}(as $MgSO_4$) and Ca^{2+}(as $CaCl_2$) were all added at 100mM and the results are given in Table 3.1 It is clear that none of these ions had any effect on the binding of L-glu to the membrane receptors.

Finally, the ability of various glu agonists and antagonists to displace the 'specific' component of the membrane bound glu was studied. The displacing compounds were added at a final concentration of 10^{-3}M and the percentage of the 'specific binding' displaced calculated. The results are given in Table 3.2. Of the compounds tested, D- and L-aspartic acid (II) and L-amino-adipic acid (III) are all potent displacers of the specifically bound component of the glu membrane binding, whilst 1-amino-1,2-dicarboxy-cyclobutane (IX) and dihydrokainic acid (XV) show no such ability. The other compounds listed fell into two groups:

a) those having some appreciable tendancy to displace the specifically bound L-glu (N-methyl-L-aspartic acid (IV); 2,6-dicarboxypiperidine (VIII); DL-APB (VI); 1-amino-1,3-dicarboxy -cyclopentane (X). These all displace some 60% of the specifically bound glutamic acid).

Table 3.2

The effect of various compounds upon the amount of L-[^{3}H]-glutamic acid specifically bound to P.americana central nervous tissue membranes.

Various putative glutamic acid agonists and antagonists were added to the membrane preparation at a final concentration of 10^{-3}M. Their ability to displace the specifically bound L-glu (I) was measured by the percentage of that component displaced. The figures give the mean and standard error of at least triplicate samples.

x Significantly different to 0% inhibition (no added cold glu)

+ Significantly different to 100% inhibition (10^{-3}M added cold glu).

COMPOUND	NUMBER	% SPECIFIC BINDING DISPLACED BY 10^{-3}M
L-aspartic acid	II	123+/- 4*
L-aminoadipic acid	III	87+/-14*
D-aspartic acid	II	71+/-38*
N-methyl-L-aspartic acid	IV	61+/-20*
piperidine-2,6-di-carboxylic acid	VIII	59+/-12*+
DL-aminophospho-butyric acid	VI	58+/- 5*+
1-amino-1,3-di-carboxy-cyclopentane	X	56+/-15*+
D-aminoadipic acid	III	46+/-22*
DL-2-aminophospho-valeric acid	VII	44+/-22*
D-glutamic acid	I	39+/- 5*
1-amino-1,2-di-carboxy-cyclobutane	XI	6+/-51*
dihydrokainic acid	XV	1+/-51*

Table 3.3

The binding of other putative glutamic acid receptor ligands.

METHOD (SECTION)	RADIOLIGAND	DISPLACER	NON-SPECIFIC BINDING(dpm)	TOTAL BINDING(dpm)
SHARIF AND ROBERTS (2.3.2)	DL-[^{3}H]-APB	2mM DL-APB	567+/- 63	596+/- 12
• +2.5mM$CaCl_2$	•	•	505+/- 165	581+/- 47
• +$CaCl_2$/20°C	•	•	593+/- 56	565+/- 7
• +$CaCl_2$/20°C/ 40 min	•	•	623+/- 63	565+/- 7
SHARIF AND ROBERTS (2.3.3.1)	[^{3}H]-kainic acid	1mM kainic acid	3044+/- 224	3340+/- 833
•	•	1mM L-glu	2953+/- 113	•
•	•	1mM DL-APB	2979+/- 306	•
STATZ *et al* (2.3.3.2)	[^{3}H]-kainic acid	1mM kainic acid	20807+/- 194	21137+/-3163
•	•	1mM L-glu	13479+/-3157	•
•	•	1mM DL-APB	18247+/-1972	•
ROCK *et al* (2.3.3.3)	[^{3}H]-kainic acid	1mM kainic acid	23470+/- 423	21577+/-3836
•	•	1mM L-glu	23574+/- 748	•

The binding of [^{3}H]-labelled APB (VI) and [^{3}H]-labelled kainic acid (XI) were both used to try to reveal specific, displaceable binding of the glu receptor. Neither ligand proved useful, despite the use of various methods.

b) those for which the glu displacing ability was equivocal (D-2-amino-adipic acid (III); 2-amino phosphovaleric acid (VII); D-glu (I); all displacing some 40% of the specifically bound glu, but having standard arrors large enough for this not to be signicantly different to the 'total binding' control).

A brief study was also made on the binding of L-[^{3}H]-glu to membrane fragments produced using Gray and Whittaker's sucrose step gradient (Section 2.3.1.1). This method gave more protein in the final pellet (75μg) and specific, displaceable glu binding was clearly present (with no added cold glutamic acid the membrane fragments bound 15323 ±767 dpm); with 10^{-3}M added cold glu they bound 1963 ± 767 dpm). However, this was not a significant improvement on the shorter method of Sharif and Roberts and so it was not taken any further.

3.3.2 The binding of other putative glutamic acid receptor ligands.

Various other glu receptor ligands were tried on various membrane preparations (described in Sections 2.3.2 and 2.3.3) and the results are given in Table 3.3. Neither D,L-[^{3}H]-APB (VI) acid nor [^{3}H]-kainic acid (XIV) were able to reveal specific, displaceable membrane binding, despite various modifications to the method (for kainic acid) or to the binding saline (e.g. by the addition of $CaCl_2$ in the case of APB).

3.4 The binding of ligands to the GABA/Cl^- channel complex in membranes derived from P. americana central nervous tissue.

The results of these experiments are given below.

3.4.1 The binding of ligands to the GABA (XVII) recognition site (Section 2.4.1)

For the experiments investigating the binding of [^{3}H]-GABA to Triton treated membranes, the samples contained between 30 and 40μg protein each. They showed that the 'non-specific binding' was between 30% and 50% of the total radioactivity bound in the presence of 2.5×10^{-8}M GABA. With the standard error of the mean for each group of

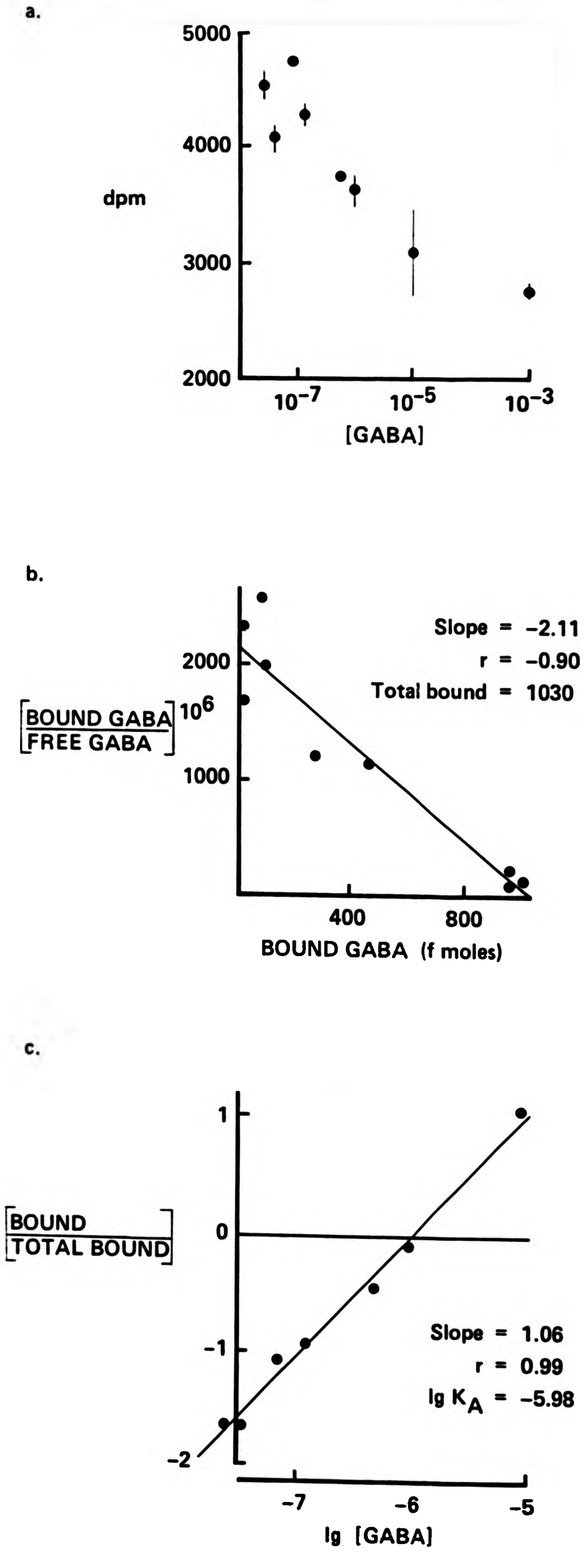


Figure 3.3

The graphical representation of the displacement of [^{3}H]-GABA by added cold GABA from membranes derived from P. americana central nervous tissue.

a) A semi-log plot showing the amount of [^{3}H]-GABA (XVII) bound to the membranes (as disintegrations per minute) against the total concentration of GABA added. Each point is the mean of triplicate samples, the error bars giving the standard error.

b) A Scatchard plot using data derived from the results graphically displayed in Figure 3.3(a). The 'non-specific binding' was taken as the amount of [^{3}H]-GABA bound in the presence of 10^{-3}M cold GABA - a value of 2779 dpm. For this experiment, 45µg of membrane protein were used per assay. Thus the number of receptors present was 22.9fmoles/µg protein and the K_d was $1.18.10^{-6}$M.

c) A Hill plot using the value for the number of receptors present derived from the Scatchard plot given in Figure 3.3.(b). The slope of this line was 1.06 and the intercept on the abscissa -5.98 (giving a K_d value of 1.06×10^{-6}M).

replicate samples being between 10% and 30% of the mean, this meant that many of the experiments performed showed no statistically significant displaceable binding of the [^{3}H]-labelled GABA probe. However, it was possible to obtain Scatchard and Hill plots from some of these experiments, and Figure 3.3 shows one such example. These experiments showed the membranes to contain 16.1 $\pm$ 6.8fmoles GABA receptor/μg membrane protein. The K_d values were 1.79 $\pm$ 0.61μM by the use of Scatchard plots and 2.01 $\pm$ 0.96μM by use of Hill plots. The Hill plots gave slopes between 0.95 and 1.06, confirming that the assumption on non-cooperativity made for the Scatchard plots was valid.

The use of [^{3}H]-muscimol (XXI) in place of [^{3}H]-GABA did not improve the low level of 'specific binding' obtainable, however, it did seem to reduce the variations within groups of replicate samples - the standard error being only 5% to 10% of the mean when using [^{3}H]-muscimol. A typical curve for the displacement of [^{3}H]-muscimol by the addition of unlabelled GABA is given in Figure 3.4. This shows an apparent K_i of 1.9×10^{-7}M.

To test the efficacy of various compounds as GABA agonists and antagonists at the P. americana central nervous tissue GABA recognition site, [^{3}H]-muscimol was used as the radioligand and the compounds added at a final concentration of 10^{-3}M. Of the 18 compounds listed in Table 3.4, only five (piperidine-4-sulphonic acid (XXV); N(pyrimidin-2,4-dione-6-yl)-hydrazine (XXVII); aminopropane sulphonic acid (XVIII); N-(dodecyl)-2-hydroxy-benzylamine (XXVIII); 2,6-dicarboxypiperidine (VIII)) showed the ability significantly to reduce the amount of [^{3}H]-muscimol bound. The other 13 compounds gave results that did not differ significantly from the amount of [^{3}H]-muscimol bound with no cold displacer present.

3.4.2 The binding of radioligands directed at the Cl^- channel.

[^{3}H]-DPTX (XLIII), [^{35}S]-TBPS (XLVI) and [^{3}H]-TBOB (XLVII) were all tried to no avail, as in no case was specific displaceable binding revealed. The results are given in Table 3.5.

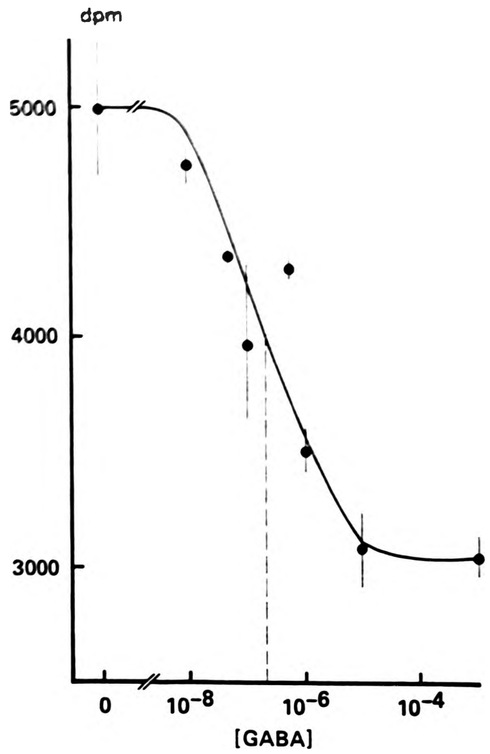


Figure 3.4

The ability of cold GABA to displace membrane bound [^{3}H]-muscimol.

This diagram shows the amount of [^{3}H]-muscimol (XXI) bound to membranes derived from P. americana CNS against the concentration of cold GABA added to the system. Each point represents the mean of triplicate samples and the error bars show the standard error about that mean. 40µg of protein were used for each sample. This displacement curve gives an apparent K_i of 1.9×10^{-7}M.

Table 3.4

The ability of various compounds to displace [^{3}H]-muscimol.

The results obtained by adding various compounds to a preparation of membranes displaying specific [^{3}H]-muscimol (XXI) binding. The compounds were added at 10^{-3}M and the percentage of the specific [^{3}H]-muscimol binding displaced calculated. The table gives the results as the (mean) ± (standard error) of at least triplicate samples.

x Significantly different from 0% inhibition (no added cold GABA (XVII)).

\+ Significantly different from 100% inhibition (10^{-3}M cold GABA added)

o Also tested at 10^{-5}M, when it gave the result of 5 ± 21% which is not statistically different from the value with no added compound.

COMPOUND	NUMBER	%SPECIFIC BINDING DISPLACED BY 10^{-3}M
piperidine-4-sulphonic acid	XXV	102+/-11°o
N-(pyrimidin-2,4-dione-6-yl)-hydrazine	XXVII	85+/-24*
Y-aminopropanesulphonic acid	XVIII	83+/- 7*
N'-(dodecyl)-2-hydroxy-benzylamine	XXVIII	64+/-16*
piperidine-2,6-dicarboxylic acid	VIII	60+/-42*
aminophosphonovaleric acid	VII	30+/-36*
N-methyl-L-aspartic acid	IV	29+/-18*
L-aminoadipic acid	III	26+/- 7*
4-(aminopropyl)-3-hydroxy-2-methyl-pyrazine	XXIX	19+/-41*
5-(aminomethyl)-3-hydroxy-pyrazine	XXX	18+/-36*
3-(piperidin-6-yl)-propionic acid,HCl	XXXI	12+/-12*
3-aminoethyl-pyridine,HCl	XXXIII	11+/-26*
3-phenyl-4-aminobutyric acid	XXXVII	6+/-14*
ethyl-2-(p-chlorobenzyl)-3-(piperidin-6-yl)-propionate	XXXII	1+/-19*
2-(piperidin-6-yl)-ethyl-sulphonic acid	XXXIV	- 3+/- 7*
nipecotic acid	XXVI	-14+/-36*
2-phenyl-4-hydroxy-6-aminomethyl piperidine,HCl	XXXV	-15+/-27*
3-hydroxy-GABA	XIX	-24+/-18*

Table 3.5

The displacement of various Cl^- channel ligands from binding sites on membranes derived from the central nervous tissue of P. americana.

Various methods were tried to reveal specific, displaceable binding of the Cl^- channel ligands [^{3}H]-DPTX (XLIII), [^{35}S]-TBPS (XLVI) and [^{3}H]-TBOB (XLVII). These are listed in this table, with the results given as the mean and standard error of at least triplicate samples.

METHOD (SECTION)	RADIOLIGAND	DISPLACER	NON-SPECIFIC BINDING(dpm)	TOTAL BINDING (dpm)
OLSEN *et al* (2,4,2,1)	[^{3}H]-DPTX	10^{-3}M PTX	14088+/-1177	10758+/- 772
RAMANJANEYULU AND TICKU (2,4,2,2,1)	[^{35}S]-TBPS	10^{-3}M PTX		
-dialysis	"	"	2057+/- 260	2352+/- 213
-washings	"	"	6369+/- 364	6577+/- 218
NICHOLSON *et al* (2,4,2,2,2)	[^{35}S]-TBPS	10^{-3}M PTX		
unshocked				
-200mM KBr	"	"	586+/- 129	703+/- 37
-200mM NaCl	"	"	"	500+/- 75
-200mM KCl	"	"	"	504+/- 2
-200mM KI	"	"	"	514+/- 15
shocked				
-200mM KBr	"	"	739+/- 13	671+/- 43
-200mM NaCl	"	"	"	849+/- 107
-200mM KCl	"	"	"	667+/- 44
-200mM KI	"	"	"	818+/- 82
RAMANJANEYULU AND TICKU (2,4,2,3,1)	[^{3}H]-TBOB	10^{-4}M PTX	1615+/- 60	1596+/- 217
LAWRENCE *et al* (2,4,2,3,2)	[^{3}H]-TBOB	$5,10^{-5}$M TBOB	4356+/- 202	5467+/- 484

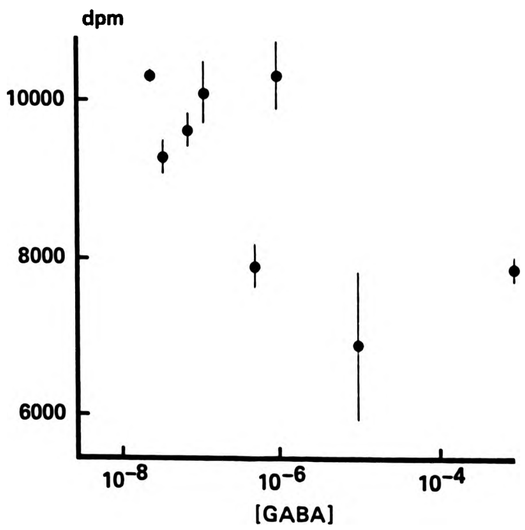


Figure 3.5

The binding of [^{3}H]-GABA to membranes derived from L.sericata.

This diagram shows the relationship between the amount of [^{3}H]-GABA (XVII) bound and the total concentration of GABA present in experiments using the fly-head preparation described in 2.3.2.1. Each point gives the mean and standard error about that mean for triplicate samples, each sample containing 0.1mg of membrane protein and 1.25×10^6dpm of [^{3}H]-GABA. The correlation coefficient for the derived Scatchard plot is only 0.31, this plot actually having a positive slope of 0.53.

The method of Ramanjaneyulu and Ticku (Section 2.4.2.2.1) was also used to check the time course for the binding of [^{35}S]-TBPS to both mammalian and insect central nervous tissue derived membranes. The membranes were resuspended in binding saline containing 200mM KBr and incubated for up to 70 minutes, the displacing agent used being 10^{-4}M 1-parabromophenyl-4-t-butyl-2,6,7-trioxabicyclo-[2,2,2]-octane (XLVIII). Whilst the mammalian (rat) membranes were showing maximal displaceable binding within 10 minutes (1142 ± 18 dpm with and 4035 ± 546 dpm without added displacer), the insect (P. americana) tissue showed no displaceable binding at any time up to 70 minutes (when the values with and without displacer were 1444 ±19 dpm and 1856 ±204 dpm respectively).

3.5 The binding of ligands associated with amino-acid coupled ion channels to membranes derived from other insect tissue.

3.5.1. The binding of ligands to membranes derived from P. americana coxal muscle.

None of the methods tried revealed specific, displaceable ligand binding for the [^{35}S]-TBPS (XLVI) experiment (Section 2.5.1.1). The amount bound in the presence or absence of 10^{-4}M cold TBPS was 1052 ± 137 dpm and 750 ± 106 dpm respectively. Even the denervation experiments showed no displaceable binding (Section 2.5.1.2). Thus the binding of [^{3}H]-glu (I) to sarcoplasmic membranes (Section 2.5.1.2.1) gave values with and without 10^{-3}M added cold glu of 421 ± 27 dpm and 442 ± 181 dpm for normal muscles and 212 ± 4 dpm and 226 ± 75 dpm for denervated muscles. Likewise, the results for the binding of [^{3}H]-muscimol to membranes prepared by the method Sharif and Roberts (Section 2.5.1.2.2) gave the following values when 10^{-3}M cold GABA (XVII) was either present or absent: 1479 ± 170 dpm and 1542 ± 51 dpm for normal muscle and 1072 ± 20 dpm and 1165 ± 86 dpm for the denervated muscles.

3.5.2. The binding of radio-labelled amino-acid to membranes derived from the central nervous tissue of insects other than P. americana.

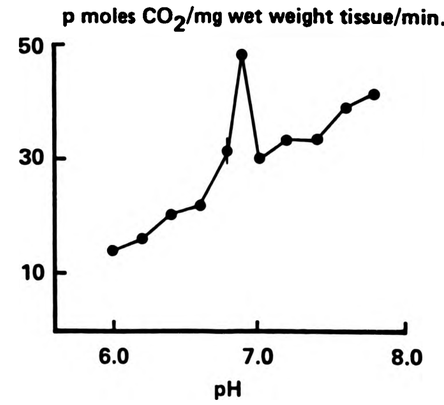
3.5.2.1. The binding of [^{3}H]-GABA to membranes derived from the heads of the green-bottle fly L. sericata.

The heads were added to the homogenisation medium at a concentration of 60mg (or 20 heads)/ml and the final membrane suspension was made up at a protein concentration of 0.25mg/ml (the equivalent of 50 heads/ml). From the initial experiments there was a clear indication of the presence of displaceable [^{3}H]-GABA binding and so a curve was constructed to show the displacement of [^{3}H]-GABA by the addition of extra cold GABA. The results of this experiment are graphically displayed in Figure 3.5. It was then clear that this preparation provided no improvement on the system using membranes derived from the central nervous tissue of P. americana and because of this, no further experiments were carried out on this preparation.

3.5.2.2 The binding of [^{3}H]-amino acids to membranes derived from the central nervous tissue of the locust L. migratoria.

Both the P_2 and the P_3 produced by the method described (Section 2.5.2.2) were used to try to reveal specific, displaceable binding of the [^{3}H]-labelled amino acids L-glu and GABA. It was found that both the P_2 and the P_3 did display such binding, but in neither case was the amount of displaceable binding seen in the P_3 a significant improvement upon that seen in the P_2. Thus for L-glu the amount of radioactivity bound in the presence or absence of 10^{-3}M cold L-glu was 2905 ± 93 dpm/100µg protein and 5478 ± 389 $\frac{dpm}{100\mu g}$ protein for the P_2 and 1857 ± 194 dpm/100µg protein and 6346 ± 1052 dpm/100µg protein for the P_3. The corresponding figures for the binding of GABA in the presence and absence of 10^{-3}M cold GABA were 1486 ± 141 dpm/100µg protein and 2302 ± 151 dpm/100µg protein for the P_2 and 1595 ± 222 dpm/100µg protein and 1882 ± 212 dpm/100µg protein for the P_3. Because the extra work seemed to produce no improvement in the performance of the binding assay, this preparation was not investigated further.

a.



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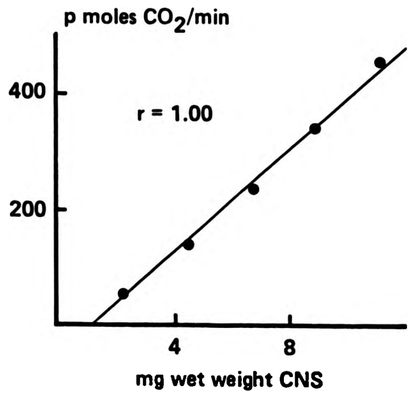


c.

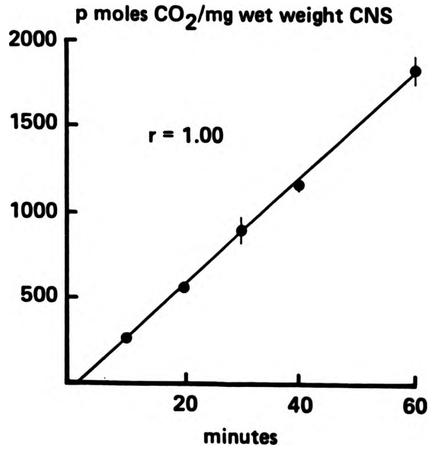


Figure 3.6

The properties of GAD derived from P. americana central nervous tissue.

This figure graphically displays the effect of various parameters upon the production of CO_2 (as measured by the amount of ^{14}C trapped on the hyamine wick) from radiolabelled L-glu (I) by the enzyme GAD produced from P. americana CNS. Each point represents the mean of replicate samples, the error bars, where they fall outside the representative point giving the standard error about that mean.

a) The effect of pH upon the production of CO_2. The maximum activity was obtained at pH 6.9 and was 48.75 $\pm$ 0.40pmoles CO_2/mg wet weight CNS/min.

b) The effect of enzyme concentration upon the production of CO_2. This gives a linear relationship with a very good fit, the slope of the line being 44.40pmoles CO_2/min/mg wet weight CNS.

c) The effect of time of incubation upon the production of CO_2. This gives a linear relation with a very good fit, the slope of the line being 33.70 p moles CO_2/mg wet weight CNS/min.

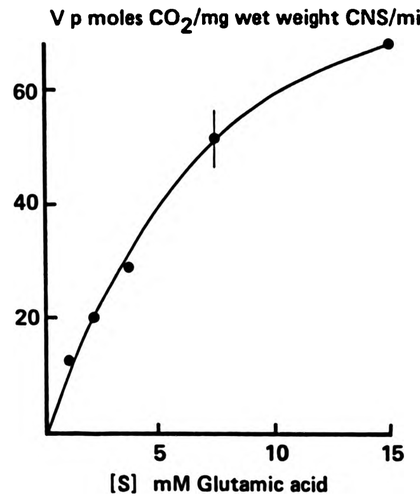
3.6 **The results from the study of the enzymes involved in the production and degradation of GABA in P. americana central nervous tissue.**

3.6.1 The properties of GAD derived from the central nervous tissue of P. americana.

Figure 3.6 shows the effect of pH, enzyme concentration and incubation time at 37°C upon the production of $^{14}CO_2$ from L-[U-^{14}C]-glu (I) by the enzyme GAD as prepared from the central nervous tissue of P. americana. The pH curve shows a distinct maximum at pH 6.9, with indications of a second maximum in the region of pH 7.6 - pH 7.8. The rate of production of $^{14}CO_2$ at the pH 6.9 maximum was 48.75 $\pm$ 0.40pmoles CO_2/mg wet weight tissue/min with a glu concentration of 5mM. This value for the pH was used for the subsequent investigations.

Linear relationships were found between the production of $^{14}CO_2$ and the amount of enzyme used and the production of $^{14}CO_2$ and the period of incubation. Between 1.2mg wet weight tissue/assay and 11.2mg/assay, $^{14}CO_2$ was produced at a rate of 44.40pmoles/mg/min in the presence of 5mM glu. The standard assay conditions of 5.6mg wet weight tissue/assay fitted nicely in this range. Likewise, $^{14}CO_2$ was produced at 33.70pmoles/mg/min at incubation times between 10 and 60 minutes in the presence of 5mM glu. The standard assay was incubated for 20 minutes.

Figure 3.7 shows the relationship between substrate concentrations and the rate of production of $^{14}CO_2$ by this preparation of GAD. From this raw data, the Eadie plot was calculated (Eadie; 1942). From such plots for replicate experiments, the Vmax for this preparation was found to be 88.52 $\pm$ 25.88pmoles CO_2/mg wet weight tissue/min, whilst the Km for L-glu was found to be 12.35 $\pm$ 0.07mM.



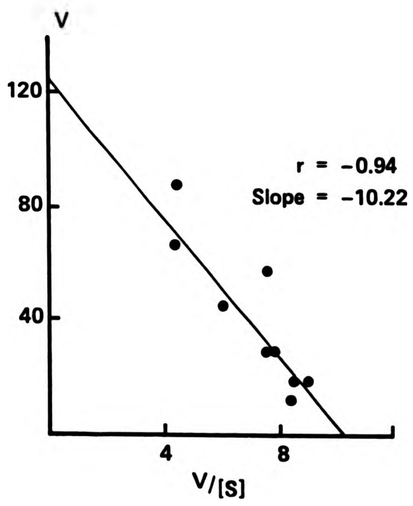


Figure 3.7

Calculating the Vmax and Km for GAD derived from P. americana central nervous tissue.

a) A plot of rate of production of CO_2 against glutamic acid concentration for GAD derived from P. americana. The points give the mean of replicate samples and the error bars the standard error about that mean.

b) An Eadie plot derived from the same data as that used in Figure 3.7(a). From this the Vmax is 125.11pmoles CO_2/mg wet weight CNS/min and the Km is 12.25mM.

The Eadie plot is derived from the Michaelis-Menten equation as follows:-

$$V = \frac{Vmax\ [S]}{[S] + Km}$$

from which:

$$V = Vmax - Km\ V/[S]$$

Thus a plot of V vs V/[S] will have L slope of -Km, and an intercept on the ordinate of Vmax.

Table 3.6

The fractionation of GABA-T derived from P. americana central nervous tissue.

The absolute and specific activities of various fractions sampled during the preparation of GABA-T from the central nervous tissue of P. americana. In each case, 50µl of the fraction (for P_3, the pellet was resuspended in 0.5ml buffer) were incubated with the reaction medium for 30 minutes at 37°C.

SAMPLE	GABA-T ACTIVITY-I pmoles GABA/50ul	PROTEIN-II ug/50ul	SPECIFIC ACTIVITY I/II	COMPARATIVE ACTIVITY pmoles GABA/CNS
S_1	1364	93.5	14.59	1091
S_2	794	22.5	35.27	1392
S_3	491	10.0	49.10	1291
P_3	230	15.0	15.30	252

3.6.2 The properties of GABA-T derived from P. americana central nervous tissue.

The background for the GABA-T studies was obtained using a blank assay which contained no enzyme. This gave a result of 1200 ± 100 dpm. This compared well with the typical enzyme incubations which were found to contain 4000-6000 ± 200 dpm. Assay samples lacking 2-oxoglutaric acid or containing 1mM aminooxyacetic acid gave levels of radioactivity statistically indistinguishable from the no-enzyme blanks, indicating that the increase in radioactivity seen on adding the enzyme fraction was due to the breakdown of [^{14}C]-GABA (XVII) by the enzyme GABA-T.

Initially, 50µl samples of S_1, S_2, S_3 and P_3 (resuspended in 500µl buffer) were assayed for their GABA-T activity and their protein content. From the results (given in Table 3.6) it was clear that the use of S_2 gave the best combination of enzyme specificity (in terms of [^{14}C]-GABA converted/mg of protein) and total activity (in terms of [^{14}C]-GABA converted/50µl homogenate). For this reason, the S_2 fraction was the one used to study some of the properties of the enzyme.

The effect of temperature, incubation time, and enzyme concentration upon the activity of this preparation of GABA-T were all studied using the S_2 and the results are graphically displayed in Figure 3.8. There was seen to be a clear temperature optimum at 37°C, where the rate of conversion of [^{14}C]-GABA to its products (initially succinic semi-aldehyde) was 2.59 ± 0.10pmoles/µg protein/min. The relationship between the conversion of [^{14}C]-GABA and incubation time or enzyme concentration were both found to be non-linear, the tail off in these lines probably being due to a limiting concentration of either 2-oxoglutaric acid or GABA.

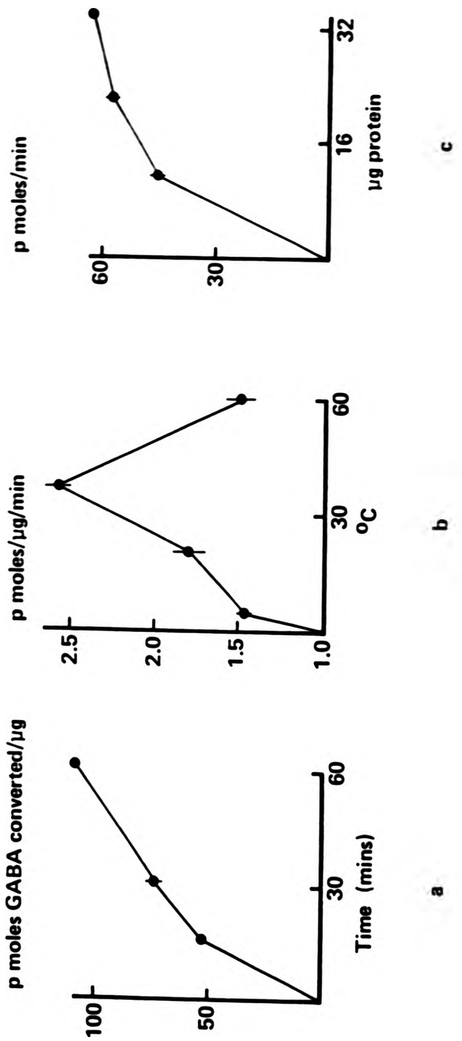


Figure 3.8

Various properties of GABA-T derived from P. americana central nervous tissue.

The graphs in this figure show the relationship between the total amount of [^{14}C]-GABA (XVII) converted by GABA-T and various of the reaction parameters. Each point represents the mean of 3 replicates, with the error bars showing the standard error (if this falls outside the representational point).

a) The effect of time upon the breakdown of GABA.

b) The effect of temperature upon the breakdown of GABA.

c) The effect of enzyme concentration upon the breakdown of GABA.

Table 3.7

The effect of various compounds upon the levels of GABA in the CNS of P. americana.

120µg of DAPA (XXXVIII), γ-vinyl-GABA (XXXIX) and γ-acetylenic-GABA (XL) were injected into the haemocoel of P. americana. The insects were killed the next day and the levels of GABA estimated by micro-dansylation. Each sample gives the mean and standard error of five CNS.

x Significant difference between control and test levels.

COMPOUND	NUMBER	GABA PRESENT nmoles/ug protein
control	--	1.61+/-0.22
DAPA	XXXVIII	1.58+/-0.14
vinyl-GABA	XXXIX	2.41+/-0.37x
acetylenic-GABA	XL	3.00+/-0.28x

3.6.3 The effect of various compounds upon the level of GABA within the CNS of P. americana.

The three synthetic compounds 2-dimethylaminophenylacetonitrile (DAPA -XXXVIII), γ-vinyl-GABA (XXXIX), γ-acetylenic-GABA (XL) were tested for their effects upon the level of GABA within the CNS. The results are given in Table 3.7. DAPA was found to decrease the GABA levels by a small and statistically non-significant amount, whilst γ-acetylenic and γ-vinyl-GABA both significantly increased the levels of GABA. This was not too surprising, as both the latter compounds are potent inhibitors of GABA-T.

3.7 Studies into the release of radioactivity by synaptosomes derived from the central nervous tissue of P. americana.

Various compounds, mostly known or putative modulators of the GABA/Cl^- channel complex, were added alone or in various binary combinations, to investigate their ability to stimulate the release of radioactivity from synaptosomes produced as described in Section 2.7.

3.7.1 Calculations of results.

The results were calculated in terms of nmoles [^{3}H] released/CNS equivalent (nmoles [^{3}H]/CNS), this being the amount of radioactivity released due to the stimulating ability of the added compound and so the amount of radioactivity released above the basal (control) level of release. The control release was caused by a combination of the washing out of extra-synaptosomal radioactivity and the release of small amounts due to the leakage or rupture of some of the synaptosomes resting on the filter bed.

This value was calculated as follows (see Figure 3.9 for an example):

1. The total radioactivity released (in cpm) from the control (C) and the test (T) channels was summated from one fraction before the addition of the test compound to the end of the experiment (n fractions).

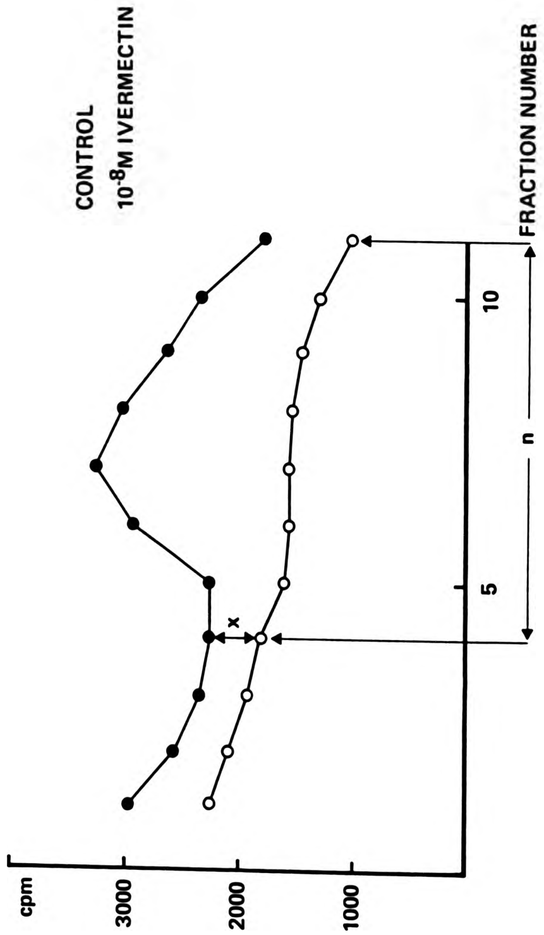


Figure 3.9

A worked example of the calculation of results from the study of the release of radioactivity from synaptosomes derived from the central nervous tissue of P. americana.

The graph opposite shows the result of a typical experiment involving 10^{-8}M ivermectin: The calculation of the ivermectin induced stimulated release from these profiles is given below.

1. Released radioactivity was summated for fractions 4 to 11.

   T = 20507 cpm
   C = 12017 cpm
   n = 8

2. C.S.R. = T-C = 8490 cpm

3. F = nx = 8.350 = 2800 cpm

4. T.S.R. = C.S.R-F = 5690 cpm

5. The counting efficiency for this experiment was 38%,
   Thus:
   Total stimulated release = $\frac{\text{T.S.R}}{0.38} \times \frac{1}{44.4}$

   = 337.25 fmoles [^{3}H]/CNS

o-o Control

●-● 10^{-8}M Ivermectin.

2. The crude stimulated release (C.S.R) was obtained by subtracting the control total from the test total.

$$C.S.R. = T-C.$$

3. A correction factor (F) was applied. This was required because the release profiles prior to the test compound induced stimulation did not superimpose, but ran closely parallel to each other. The correction factor was applied to account for this and give a true picture of the amount of radioactivity released due to the stimulating effect of the test compound. To obtain this factor, the difference between the test profile and the control profile at the initial fraction (x) was multiplied by the number of fractions used.

$$F = nx.$$

4. The true stimulated release (T.S.R.) was obtained by subtracting the correction factor from the crude stimulated release.

$$T.S.R. = C.S.R. - F.$$

5. For the final step, three factors needed to be known.

   (a) the number of CNS equivalents per channel, which was always four.

   (b) the activity of the [^{3}H]-choline chloride, which was 80Ci/mmole.

   (c) the efficiency of counting (E), which was between 30% and 40%.

To convert from cpm to dpm, the true stimulated release was divided by the efficiency and to convert from dpm to fmoles [^{3}H]/CNS, a factor of 1/44.4 was applied (as 1mmole = $80x2.22x10^{12}$ dpm) Thus:

$$\text{Total stimulated release} = \frac{\text{T.S.R.}}{E} \times \frac{1}{44.4} \text{ fmoles } [^3H]/\text{CNS}$$

3.7.2 The effect of ivermectin, alone and in combination with various GABA/Cl^- channel effectors, upon the release of radioactivity by superfusion from synaptosomes pre-loaded with [^{3}H]-choline chloride.

These studies were undertaken to confirm and extend the work described by Nicholson et al (1986).

Ivermectin (LIII) is the 22,23-dihydro derivative of avermectin $B_{1a/1b}$, the naturally occuring product from the mould Streptomyces avermectilis. It was obtained by small scale extraction of the MSD Agvet product 'Ivomec'. Figure 3.10 gives a brief dose-response curve. Nicholson et al (1986) showed this compound to give a maximum stimulation of release of 1164.4fmoles [^{3}H]/CNS, with an EC_{50} of 1.09×10^{-9}M. In the following experiments, 10^{-8}M ivermectin was used to cause stimulation of release. This gave a stimulation of 652.9 ± 120.0fmoles [^{3}H]/CNS, about 70% of the maximal stimulation.

Various compounds were tested in combination with 10^{-8}M ivermectin to see how they affected the ivermectin stimulated release. The results obtained are given in Table 3.8. None of the compounds tested were found to potentiate the response and the ones which were most effective at inhibiting the response were the putative Cl^- channel blockers such as PTX (XLII), dieldrin (XLIV) and lindane (XLV) (BHC). Of interest was the failure of TPBS (XLVI) to inhibit the ivermectin stimulated release, as it has been suggested that this compound is PTX like in its action.

All of the compounds tested with ivermectin were also tested on their own for their ability to perturb the release of radioactivity from superfused synaptosomes that had been previously loaded with [^{3}H]-choline chloride.

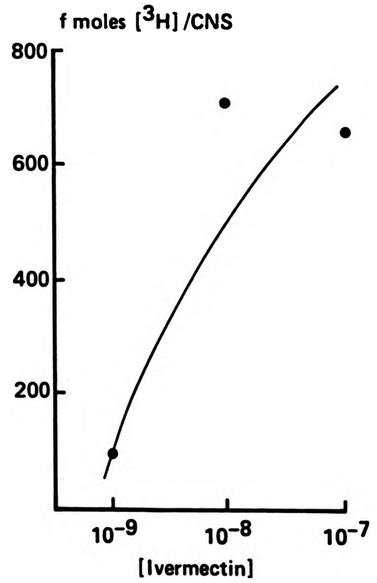


Figure 3.10

The effect of ivermectin upon the stimulation of release of radioactivity from synaptosomes pre-loaded with [^{3}H]-choline chloride.

A graph of the stimulated release caused by 10^{-7}M, 10^{-8}M and 10^{-9}M ivermectin (LIII): These results agree with those reported by Nicholson et al (1986).

Table 3.8

The ability of compounds to inhibit the stimulation of release of radioactivity induced by 10^{-8}M ivermectin.

Various compounds were tested for their ability to affect the stimulation of release of radioactivity by 10^{-8}M ivermectin (LIII). They were added at the same time as the ivermectin, and the percentage inhibition of stimulated release calculated. None of the compounds were found to stimulate release. The results for dieldrin (XLIV), lindane (XLV) and PTX (XLII) broadly correspond with those reported by Nicholson et al. (1986).

COMPOUND	NUMBER	CONCENTRATION (μM)	%INHIBITION OF IVERMECTIN RESPONSE
PTX	XLII	1.0	78
		0.1	29
dieldrin	XLIV	1.0	93
		0.1	56
lindane	XLV	1.0	78
		0.1	68
TBOB	XLVII	10.0	72
		1.0	6
GABA	XVII	2000.0	1
		500.0	0
TBPS	XLVI	10.0	15
diazepam	L	10.0	0

Table 3.9

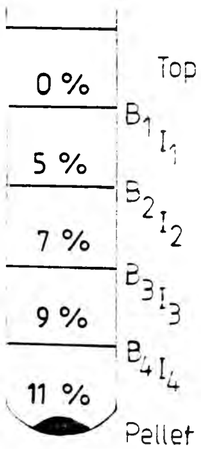
The binding of [^{125}I]-bungarotoxin to membranes derived from P. americana central nervous tissue.

(a)

FRACTION	[^{125}I]-BOUND(dpm) with 2mM nicotine	[^{125}I]-BOUND(dpm) without nicotine	[^{125}I] SPECIFICALLY BOUND(dpm)
S_1	2247+/- 58	25666+/-1426	23419+/-1285
S_2	358+/- 28	1250+/- 75	894+/- 89
P_2	3331+/- 56	34350+/-1765	30572+/-1864

(b)

FRACTION	[^{125}I]-BOUND fmoles	PROTEIN mg	SPECIFIC ACTIVITY fmoles/mg
top	0.050	0.14	0.357
B_1	0.031	0.06	0.571
I_1	0.033	0.02	1.650
B_2	0.086	0.04	2.150
I_2	0.073	0.04	1.825
B_3	0.121	0.06	2.017
I_3	0.166	0.06	2.767
B_4	0.352	0.12	2.514
I_4	0.743	0.20	3.715
pellet	1.104	0.54	2.044



a) This shows the crude results obtained from the S_1, S_2 and crude P_2 for this preparation. Specific, displaceable [^{125}I]-bungarotoxin binding was clearly seen in each fraction; the displaceable element being up to 90% of the total binding in the absence of cold nicotine added as a displacing agent. Each value gives the mean and standard error of triplicate samples.

b) The crude P_2 was further purified on a discontinuous Ficoll gradient containing bands of 0%, 5%, 7%, 9%, 11% (w/v) Ficoll. Each zone was tested for the ability to bind [^{125}I]-bungarotoxin in a specific, displaceable manner. The results are given for the fmoles of bungarotoxin so bound and the amount of protein present in each zone.

All compounds were tested at 10^{-5}M and at this concentration none were found to have any effect. Due to its higher solubility, GABA (XVII) was also tested at concentrations up to $2x10^{-3}$M. At 10^{-3}M, it consistantly failed to cause any stimulation of release, but at $2x10^{-3}$M it did cause stimulated release on one out of three occasions. The stimulation of release was at a level of 259.0fmoles [^{3}H]/CNS and this was inhibited by PTX (25% at 10^{-5}M) and pentabarbitone (35% at 10^{-5}M).

3.8 **Miscellaneous experiments.**

3.8.1 The binding of [^{3}H]-nicotine to membranes derived from the central nervous tissue of P. americana.

The binding of [^{3}H]-nicotine to a synaptosomal preparation gave results very similar to those for [^{3}H]-glu. Using 25µg of membrane protein per sample, with no added cold nicotine 920 ± 128 dpm were associated with the membranes, compared with 402 ± 32 dpm in the presence of 10^{-3}M cold nicotine. This gave a displaceable binding component of 0.267 ± 0.068fmoles/µg protein. The corresponding figures for [^{3}H]-glu using the same preparation were 1360 ± 59 dpm and 664 ± 59 dpm for the membrane associated radioactivity in the absence and in the presence of excess added cold glu, giving a displaceable component of 0.418 ± 0.049fmoles/µg protein.

3.8.2. The binding of [^{125}I]-bungarotoxin to membranes derived from the central nervous tissue of P. americana.

As well as assaying the various fractions of the step gradient for the ability to bind [^{125}I]-bungarotoxin in a specific, displaceable fashion, samples of the S_1, S_2 and crude P_2 were also assayed. Specific, displaceable binding was found in all the fractions tested and the results are given in Table 3.9.

3.8.3. The metabolism of [^{3}H]-TBOB.

Although TBOB is known to be an unstable compound in acid conditions, these experiments gave no evidence that the hydrolytic breakdown of the radiolabelled compound under the assay conditions in the presence of membranes derived from P. americana central nervous tissue was the cause of the poor results. Typical profiles for the optical density/radioactive content of the HPLC eluate of a test run and of an unincubated sample are given in Figure 3.11. The unincubated sample showed that 93% of the radioactivity co-elutes with the peak corresponding to TBOB. For samples incubated at 4°C, 21°C and 37°C the corresponding figures were 79%, 79% and 72%. There would appear to have been some degradation on incubation with the membranes, but this should not have been enough to have accounted for the failure to disclose specific, displaceable binding.

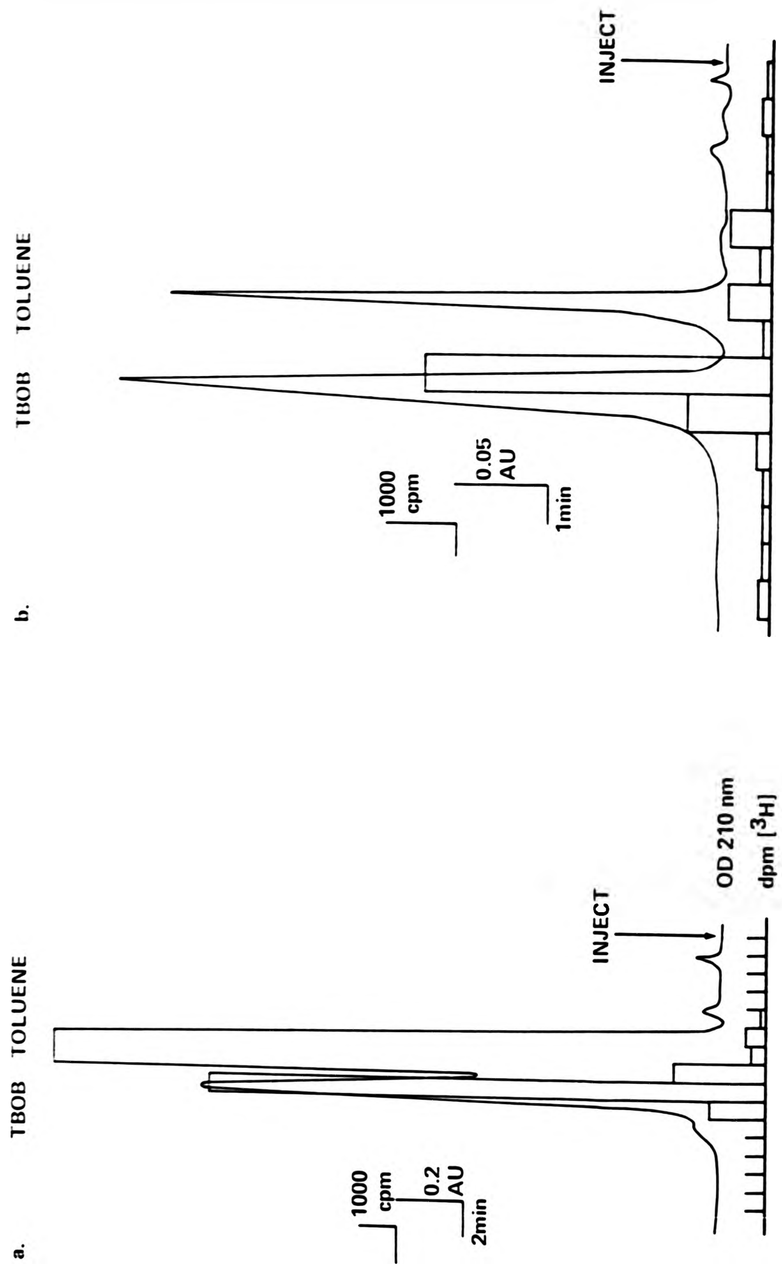


Figure 3.11

HPLC traces of [^{3}H]-TBOB.

Samples were run on a Chrompak - C_{18} reverse phase column with 70% methanol-30% water as the mobile phase. The optical density was read at 210nm and fractions were collected every minute for liquid scintillation counting 1µg of cold TBOB was added to each sample to act as a marker.

a) 1µCi[^{3}H]-TBOB (XLVI) was chromatographed to assay the purity of the stock solution. 93% of the radioactivity co-eluted with the peak corresponding to TBOB.

b) The elution profile for the sample which had been incubated at 4°C 79% of the radioactivity co-eluted with the TBOB peak.

CHAPTER 4

Discussion

4.1 Ligand binding experiments

The ligand binding experiments undertaken in the present study are discussed below in the context of similar ones carried out by other researchers. It should be noted that, where techniques have been adapted from use in other test species (usually the rat), the protocol was followed on the original models prior to their use in the insect. Results similar to those quoted in the literature were obtained for all techniques except for the binding of DPTX (LIII).

4.1.1. The binding of L-glu (I) to material from the CNS of P.americana.

The material used in these studies has been shown to consist mainly of membrane vesicles. For the most part these are empty, but some contain general cellular debris, whilst others contain smaller vesicles. There is also some general cellular debris outside the vesicles. An electronmicrograph of this material is given in Figure 4.1. The material used in the experiments on the GABA/Cl^- channel complex was found to be of a similar composition.

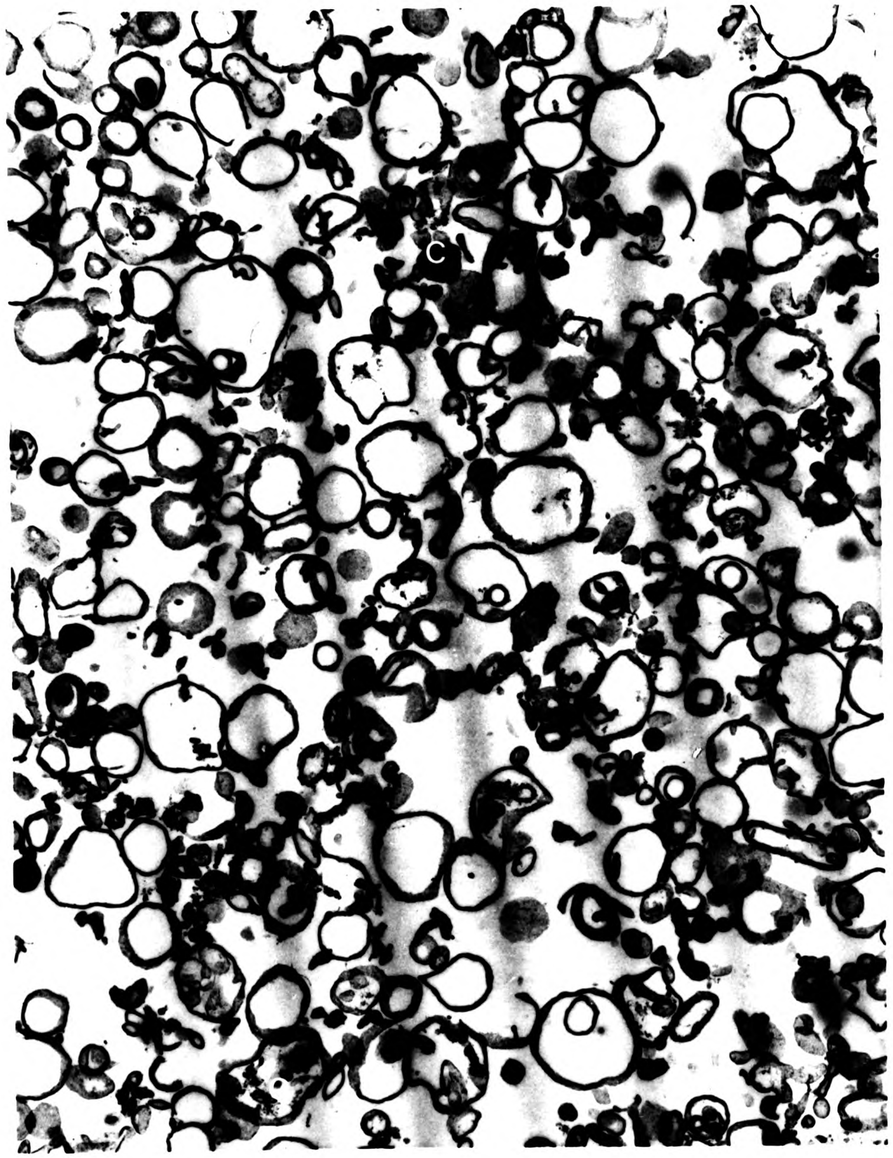
These membranes show two types of L-glu receptor, one independent of the presence of Na^+ ions and the other requiring the presence of these ions before it is revealed. The Na^+ ion dependent receptor only requires low concentrations of the cation to produce the maximal binding of L-glu, this being attained between 1 and 10 mM. The number of these Na^+ ion dependent receptors is far higher than that of the Na^+- independent receptors and it would swamp any attempt to study the latter in the presence of Na^+ ions. It is likely that this receptor represents a carrier system for the removal of L-glu from the external medium around the putative L-glu neuroreceptor. Such carrier systems have been shown in locust muscle (Briley et al.: 1982), roach muscle (Wright: 1984) and roach abdominal nerve cord (Evans: 1975), the L-glu being sequestered by the glial sheath close to the neuroreceptor site (Botham et al.: 1979).

Figure 4.1

An electromicrograph of the material used in the studies of L-[^{3}H]-glu binding to material derived from the CNS of the cockroach P.americana.

This is an electronmicrograph of part of the P_2 derived from the CNS of P.americana used in the current radioligand binding studies using L-glu (I) as the radioligand. The micrograph is at a magnification of 16250x and shows the preparation to consist mainly of empty membrane bound vesicles. There is also considerable cellular debris, both within some of the vesicles and in the surrounding area. The preparation used in the study of the binding of GABA (XVII) and muscimol (XXI) was very similar.

V-empty membrane vesicle
C-cellular debris



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V-empty membrane vesicle
C-cellular debris

Table 4.1

K_d and B_{max} values for the post-synaptic L-glu receptors reported by various workers.

Collated here are the values for the K_d and B_{max} of the post-synaptic receptors for L-glu (I) reported by various workers. The species and tissue involved are given in each case. This collation is by no means comprehensive, but it does give an idea of the spread of values obtained. More comprehensive reviews of values obtained can be found in Briley et al.(1981) and Honore et al.(1981a).

REFERENCE	SPECIES	TISSUE	K_d nM	B_{max} pmoles/mg protein
Biziere *et al.*;1980	rat	brain	11,0 60,0	- -
Honore' *et al.*;1981b	rat	brain	509,0 820,0	13,0 54,5
Michaelis *et al.*;1981	rat	brain	60-70 208-295	70-130 208-381
Redburn;1981	rabbit	retina	72,0	1,5
Werling and Nadler;1982	rat	brain	11,0 570,0	2,5 47,0
James *et al.*;1977a	*S.gregaria*	muscle	8000,0 50000,0	- -
James *et al.*;1977b	*S.gregaria*	muscle	530,0	25,0
Briley *et al.*;1979	*S.gregaria*	muscle	30000,0	52,0
Briley *et al.*;1981	*S.gregaria*	muscle	12,5 1300,0	0,57 10,9
Filbin *et al.*;1985	*M.domestica*	muscle	500,0	5-60
Current study	*P.americana*	CNS	184,0	10,6

The K_d and B_{max} of the Na^+ ion independent receptor, at $1.84x10^{-7}M$ and 10.6pmoles/mg protein respectively, are compared in Table 4.1 with those for putative post-synaptic receptors reported by various other workers from both the mammalian and the insect fields of research. On the whole the values obtained in this study seem to fit in well with the general trend. Although many of the receptors described by previous workers have a higher affinity for L-glu than the one descibed here, they are balanced by ones with lower affinity and the variation between these sets of results is well within that which one would expect when comparing the work of researchers in different laboratories using supposedly similar techniques. Due to the vague nature of the experimental detail in many of the papers, it is often difficult to assess the exact nature of any differences in the techniques that were used. But even when the same techniques have been used by the same workers (eg. James et al. 1977a and 1977b) quite large variation in the observed affinity for L-glu may occur. There are exceptions at both extremes. All those giving very low affinities were early reports and these can be put down to insufficiencies in the technique in a pioneering field of work. At the other extreme are those high affinity sites reported by Biziere et al. (1980) and Werling and Nadler (1982) in rat brain and Briley et al. (1981) in locust muscle.

Despite the variation found in the apparent affinity of the receptor sites for L-glu, all these results do agree on the slope of the Hill plot. All these investigators found Hill slopes close to unity, indicating a lack of cooperativity in the binding of the ligand to its receptor.

The overall variation in this set of results is so great that they provide no basis for making any firm conclusions about the nature of the receptor investigated in this study.

Besides Na^+, none of the ions tested had any effect upon the receptor binding of the L-glu. This is in contrast to certain workers (Baudry et al.:1981; Fagg et al.:1982) who have found that $CaCl_2$ in mM concentations increases the binding of L-glu to rat brain membranes by some three-fold and that the L-glu binding to these Ca^{2+}/Cl^- sensitive sites can be displaced L-APB (VI). The work by Fagg et al.

(1982) suggests that it is the presence of the Cl^- ion which reveals these sites and they have suggested that this response may be related to L-glu activated Cl^- channels. These may be similar to the H-receptors discovered by Gration et al.(1979). The absence of this response in the present study correlates well with the failure to reveal any specific binding of the radio-labelled ABP when that was tested on membranes prepared from roach CNS. From the current study it is not possible to say whether this absence of response is due to a lack of the appropriate receptors in this tissue, or the need to modify the conditions of the assay slightly. However, as this response does not seem to be part of the normal L-glu neurotransmitter response, its absence would in no way rule out this receptor as having neurotransmitter function in the insect CNS.

In trying to decide whether the receptor studied here is a candidate for an L-glu neuroreceptor in the insect CNS, it is necessary to compare its pharmacological profile with the standard receptor classes and the previous studies of binding characteristics of putative L-glu receptors. Table 4.2 compares the characteristics of the displacement of specifically bound L-glu of the receptor studied here with those of two studies of the putative L-glu neuroreceptor in the rat brain. As can be seen, the insect receptor differs quite markedly from that described by Roberts and Sharif (1981). The response to both stereo isomers of the short-chain excitatory amino acid aspartic acid (II) is greater in the insect that in the mammal, whilst the response to the D-isomer of glu is less. The response to cis-cyclopentane glutamic acid (X) is also weaker in the insect than in the mammal. Against this both these receptors show very little sensitivity to N-methyl-D-aspartic acid (IV: see note 'b' on Table 4.2) although the L-stereo-isomer does have moderate activity in the insect system. However, when the results from the study by Werling and Nadler (1982) are compared with those obtained in this present work, a much closer correlation is found in the rank order; this despite the large difference obtained in the value of the K_d from the two studies. The first thing to note is that the rank order for both the high and the low affinity sites found by Werling and Nadler is the same - at least for those compounds which were tested in both the mammalian and the insect systems. Secondly, the rank order for the receptor of Werling and Nadler and that for the insect are the same

Table 4.2

A comparison of the results for the displacement of specifically bound L-glu obtained for membranes derived from rat brain and for those derived from P.americana.

COMPOUND	NUMBER	INSECT %inhibition by 1mM	ROBERTS AND SHARIF IC_{50}(μM)	WERLING AND NADLER IC_{50}(μM) high	low
L-aspartate	II	123	42,1 (6)	0,77 (2)	1,2
L-glu	I	100	4,8 (1)	$0,45.10^{-3}$(1)	0,33
L-aminoadipate	III	87	26,3[a] (4)	1,1 (3)	1,5
D-aspartate	II	71	138,0 (7)	8,2 (5)	13,0
N-methyl aspartate	IV	61[b]	inactive[c](8)	---	---
DL-APB	VI	58	25,6 (3)	11,0 (6)	15,0
+/-cis-cyclopentane glutamate	X	56	18,5 (2)	---	---
D-aminoadipate	III	46	26,3[a] (4)	1,1 (3)	3,0
+/-2-aminophosphono-valerate	VII	44	---	27,0 (7)	56,0
D-glu	I	39	28,8 (5)	7,6 (4)	9,8
dihydrokainate	XV	1	inactive (8)	---	---

The Table only shows compounds which were tested in at least two of the systems. The final concentrations of the radioligand L-[^{3}H]-glu (I) were:

current study - 50nM
Roberts and Sharif (1981) - 800nM
Werling and Nadler (1982) - 2nM (high affinity)
100nM (low affinity)

The results are given as IC_{50} values in μM for those concerning mammals and as % inhibition of specific binding by 1mM displacer for those concerning the insect. The compounds are given in declining order of potency for this present study, with the rank order for the other studies given in brackets beside the IC_{50} values.

a: For the results of Roberts and Sharif, D,L-amino adipate was used

b: For the result on P.americana, the L-stereo isomer was used, NMDA was tested, but this compound failed to give consistant results. The indications were that it was inactive.

c: For the result of Roberts and Sharif, the D-stereo isomer was used.

except for the D-isomer of glu. This mismatch with D-glu is not unexpected when it is remembered that D- and L-glu are roughly equipotent in mammalian electrophysiological systems, whilst D-glu is virtually without effect in insect systems.

No other studies showing the specific binding of L-glu to insect CNS material seem to exist, and few using the binding of this ligand to muscle preparations are available. The original work undertaken by James et al. (1977a and b) on a proteolipid extracted from locust muscle can hardly be used as a comparison, as they used L-[^{14}C]-glu as the radioligand and described the specific binding as that component displaceable by high concentrations of cold D-glu. However, work has recently been carried out by Filbin et al. (1985) on a membrane extract from housefly thoracic muscles. As in the current study, this showed L-aspartic acid to be very active as a displacer of specifically bound L-glu, and D-glu to show moderate activity (1mM displacing 61% of such binding). Against this, they showed N-methyl-D-aspartate to have similar activity to that of D-glu, whilst the L-stereo isomer and D-aspartic acid showed little or no activity. Whether this reflects a true difference between the putative L-glu synaptic receptors for the muscle and the CNS, or whether one or both these receptors are not what they are claimed cannot be decided at this point.

These responses do not easily fall into classification as any of the three types of excitatory amino acid receptor commonly discussed. Binding L-glu with moderate affinity, they are clearly not of the kainate-type, as these are now generally accepted to be separate entities. However, they are clearly not of the NMDA-type either. Not only do they seem to show a low affinity for this compound, but they also have a low affinity for the two standard antagonists at this type of receptor - namely aminophosphonovaleric acid (VII) and D-aminoadipic acid (III). Neither are they simple quisqualate-type receptors, as L-aspartic acid is highly potent at the site revealed in these studies.

In general L-stereo isomers are more active than the corresponding D-stereo isomers. Thus the L-isomers of aspartic acid, N-methyl-aspartic acid and amino-adiptic acid, as well as of glu, show activity that is greater than their D-isomers. Further, comparing D,L-APB and D,L-aminophosphonovaleric acid with L-glu and L-aminoadipic acid suggests that the carboxyl analogues are more effective as ligands for the receptor sites than the corresponding phosphono compounds - especially as D,L-aminophosphonovaleric acid is less potent than the D as well as the L-isomer of aminoadipic acid. This suggests a receptor with considerable latitude in the size and shape of the compounds it will accept. As for the confirmation adopted by the ligands at the receptor site, the moderate activities of piperidine-2,6-dicarboxylic acid (VIII) and cis-cyclopentane glutamate with the lack of activity of 1-amino-1,2-dicarboxycyclobutane (XI) suggests that the two and three methylene amino acids (ie L-glu and L-aminoadipic acid) bind in a tightly folded configuration, whilst the one methylene amino acid (aspartic acid) binds in an extended conformation. This differs slightly to the partially folded conformation suggested by Usherwood (1981) as being the major receptor at the nmj in locusts.

Although it is not possible at this stage to show conclusively that the receptor site studied in these experiments is the neuroreceptor for L-glu in the insect CNS, various factors suggest that this may be the case. For these studies have revealed a receptor showing an affinity for L-glu that is well within the range expected for a neuroreceptor, that is Na^+ ion independent and that has an appropriate profile for the compounds that are active in displacing L-glu.

4.1.1.1 Other insect receptors for L-glu and related compounds.

The discovery of specific binding for L-glu in the CNS of locusts as well as in the cockroach suggests that the presence of L-glu as a central excitatory transmitter is widespread in insect species. However, on a superficial look at these receptors, they offered no advantages over that found in the cockroach for the general study of the insect CNS L-glu neuroreceptor.

The failure of kainic acid (XIV) and APB to show any specific binding was interesting. In the case of APB this was not unexpected - the failure to reveal extra L-glu sites on the addition of Cl^- and the relatively low ability of APB to displace L-glu from its receptor both suggested that this could happen. The failure to obtain specific binding of kainic acid was not too surprising. Although it is toxic to insects (LD_{50} for L.sericata of 20µg/fly) it appears to act at the peripheral nmj rather than at the CNS, as it does in mammals (R.P. Botham, personal communication).

The failure to obtain the binding of L-glu to either normal or denervated muscle tissue came as some surprise, as such receptors have been found in both locust (Briley et al.: 1979) and housefly (Filbin et al.: 1985). Both these studies have used the thoracic musculature. It is quite possible that the use of the coxal muscle in this study has some bearing upon the failure to notice such receptors, as this muscle provides less material than the thoracic muscle and may also be innervated to a lesser degree - the thoracic muscle includes the flight motor system and this is quite heavily innervated.

4.1.2 The binding of radioligands to the GABA/Cl^- channel in the insect CNS.

In contrast to the binding of L-glu and its putative insect neuroreceptor, considerable work has been undertaken in the study of the binding of ligands to their receptors in the GABA/Cl^- channel area in insects and other arthropoda - particularly in the binding of ligands to the Cl^- ionophore.

4.1.2.1 The binding of GABA and muscimol to a putative GABA neuroreceptor in the CNS of P.americana.

These studies revealed a single class of Na^+ ion concentration independent receptors for GABA (XVII) in membranes derived from the CNS of the cockroach P.americana. The K_d of these receptors was 1.18µM, with a density of 22.9pmoles/mg protein, and a slope for the Hill plot of close to unity - suggesting that these receptors are non-cooperative in their mode of action. Although these values are

similar to the ones reported above for the putative receptor for L-glu, values that were well within the normal range for such work, they are higher than the values normally associated with GABA-neuroreceptors, as can be seen from Table 4.3, where some examples from both the mammalian and the insect fields of study are given. The high values found in this study may be caused by the failure to remove all of the putative endogenous GABA-receptor modulator 'GABA-modulin' (Mazzari et al.:1980), although some of the very low values may be spurious, a factor which must be born in mind in the light of a comment made in the poster presented by Lunt et al. (1984) to the effect that the specific binding was very low in relation to the total, being only "10-30% but occasionally zero."

The differences between the electrophysiologically and the biochemically derived values for the Hill plot are probably caused by one of two reasons:

a) the binding of the radioligand in the biochemical experiments is to an inactivated form of the receptor having a high affinity for GABA. This certainly happens in the binding of acetyl choline to the nicotinic receptor (Weber et al.: 1975) and such a mechanism would explain the low values of the K_d when these are compared with the concentrations needed to produce effects in the electrophysiological experiments.

b) The interaction of the ligand with the binding site in vivo is truly non-cooperative, but more than one receptor-ligand complex is needed to activate the ion channel.

Until the channel is isolated and broken down into its component parts, it will be difficult to decide between these two options.

The interactions of various GABA/Cl^- channel effectors with this receptor were studied by measuring the displacement of specifically bound [^{3}H]-muscimol (XXI). This compound is generally accepted as a specific GABA agonist and is frequently used in radioligand studies in place of GABA because of its higher affinity for the GABA site in the mammalian CNS. There it is reputed as having an affinity for the neuroreceptor site ten-times greater than GABA. However, in two

Table 4.3

Values obtained for the K_d and B_{max} of the binding of GABA and muscimol to tissue samples from mammalian and insect sources.

This table shows the values obtained for most of the studies into the insect systems, with a few representative examples from the mammalian field for comparison.

REFERENCE	SPECIES	TISSUE	COMPOUND	K_d nM	B_{max} pmoles/mg protein
Meiners *et al.*(1979)	crayfish	muscle	muscimol	9.4	503 fmoles/g fresh
Lunt *et al.*(1984)	*M.domestica*	CNS	GABA	42.0	---
Breer and Heilgenberg(1985)	*L.migratoria*	CNS	GABA	100	2.2
Lummis and Sattelle (1985)	*P.americana*	CNS	GABA	384	1.42
Lunt *et al.*(1985)	*S.gregaria*	CNS	muscimol	10.0	0.06
	M.domestica	CNS	muscimol	40.0	0.02
Enna and Snyder (1975)	rat	brain	GABA	370	0.68
Schaeffer(1980)	rat	retina	muscimol	4.4	0.185
				12.3	0.497
Jordan *et al.*(1982)	rat	brain	GABA	20.0	0.48
				165	6.0
			muscimol	1.75	0.84
				17.5	4.8
Current study	*P.americana*	CNS	GABA	1180	22.9

cases in which the displacement of [^{3}H]-GABA by muscimol has been studied in insect CNS membranes that latter compound was found to be less active. The IC_{50} values for the displacement of specifically bound GABA given by Lummis and Sattelle (1985) were 130nM for GABA and 710nM for muscimol, whilst Breer and Heilgenberg (1985) found the K_d for GABA to be 100nM and the IC_{50} for muscimol to displace that GABA to be 90mM! There is also some doubt about its specificity, so that care must be taken in its use. Thus, de Feudis and his team have shown differences between the behaviour of the two ligands as regards numbers of sites and response to sera from patients with some neuropathological disorders (de Feudis et al.: 1979, Geffard et al.: 1979). Herschel and Baldessarini (1979) showed a differential effect of Triton treatment between the two compounds and Breer and Heilgenberg (1985) have shown muscimol to inhibit the uptake of endogenous GABA into synaptosomes almost as efficiently as the standard GABA uptake inhibitor di-aminobutyric acid (XX). Using the current system, the IC_{50} for the displacement of muscimol by cold GABA was 109nM. This compares with values of 125nM (Meiners et al.:1979), and 100nM and 40nM (Lunt et al.: 1985).

These results agree with others working on the GABA neuroreceptor in the insect CNS in that it is clear that the receptor studied in this work is like neither the mammalian $GABA_A$ (bicuculline (XLI)-sensitive) nor the mammalian $GABA_B$ (baclofen (XXXVI)-sensitive) receptors. Thus whilst the $GABA_A$ agonists piperidine-4-sulphonic acid (XXV) and 3-aminopropane sulphonic acid (XVIII) show good activity in this system, 3-hydroxy-GABA (XIX), a compound having reasonable activity in the mammalian $GABA_A$ system, is totally devoid of activity in this system. Likewise, 3-phenyl-GABA (XXXVII), whilst not the best displacer of specifically bound baclofen, does have reasonable activity in the $GABA_B$ system, but shows none in this one. However, there is also little agreement in the exact displacement profile of the insect CNS GABA receptor. Thus, whilst this study makes piperdine-4-sulphonic acid and 3-aminopropane sulphonic acid highly active at the insect site, Lunt et al.(1985) make 3-aminopropane sulphonic acid orders of magnitude less potent than GABA. Lummis and Sattelle (1985) not only agree with this assessment, but also make piperidine-4-sulphonic acid totally inactive. On the whole, the results obtained in the present study

Table 4.4

A comparison of the IC_{50} values obtained for the displacement of $[^3H]$-GABA by various compounds in different test systems.

The following results are taken from Lummis and Sattelle (1985) and Lunt *et al*. (1985). The values for the rat brain systems are given in Lummis and Sattelle (1985), although some extra information has been obtained from Breckenridge *et al*. (1981) for the $GABA_A$ site and Bowery (1982) for the $GABA_B$ site.

The values quoted from the other works are for IC_{50} in terms of µM. For Lunt *et al*. this is for the displacement of radiolabelled muscimol (XXI), whilst for the others it is for the displacement of radiolabelled GABA (XVII). The values for the present study are for the % of the specifically bound radiolabelled muscimol displaced by 1mM cold compound. The compounds are given in order of activity at the receptor sites revealed in this study and the rank orders for the other studies are given in brackets below the appropriate values.

COMPOUND	NUMBER	%DISPLACED by 1 mM	LUMMIS AND SATTELLE IC_{50}-µM	LUNT et al IC_{50}-µM	$GABA_A$ IC_{50}-µM	$GABA_B$ IC_{50}-µM
piperidine-4-sulphonic acid	XXV	102	inactive (3)	---	0,034 (1)	inactive (4)
GABA	XVII	100	0,13 (1)	0,04 (1)	0,12 (3)	0,08 (1)
3-aminopropane sulphonic acid	XVIII	83	390 (2)	0,3 (2)	0,11 (2)	10 (2)
3-(piperidin-6-yl)-propionic acid.HCl	XXXI	12	---	---	110 (5)	---
3-phenyl-4-amino-butyric acid	XXXVII	6	---	---	---	10 (2)
2-(piperidin-6-yl)-ethyl sulphonic acid	XXXIV	-3	---	---	3500 (6)	---
nipecotic acid	XXVI	-14	inactive (3)	>100 (3)	inactive (7)	inactive (4)
3-hydroxy-GABA	XIX	-24	---	---	0,6 (4)	1,13 (3)

have disclosed a receptor that is closer in its profile to the mammalian $GABA_A$ receptor than to anything else. These comparisons are laid out clearly in Table 4.4.

4.1.2.2 The binding of [^{3}H]-muscimol and [^{3}H]-GABA to other insect receptors.

The presence of specific Na^+ ion concentration independent GABA binding to tissue derived from the CNS of both L.migratoria and L.sericata helps to confirm the presence of such putative GABA neuroreceptors over a wide range of insects. However, as these sites offered no clear benefits over the roach receptor in terms of amount of binding present or the ease of preparation of material, they were not followed up.

The failure to find specific binding of radiolabelled muscimol in roach coxal muscle membranes was not too surprising. The binding of ligands to the GABA receptor site (as opposed to the Cl^- ionophore) in such preparations has not been reliably found. Thus Briley et al. (1982) commented that the binding of GABA had been difficult to measure.

4.1.2.3 The binding of ligands directed at the Cl^- channel to membrane preparations from the muscle and the CNS of P.americana.

The failure to show the binding of the Cl^- channel directed ligands to either CNS or muscle membranes was disappointing. Thus no specific binding was found for [^{3}H]-DPTX(XLIII) or [^{3}H]-TBOB (XLVII) in the CNS, and none was found for [^{35}S]-TBPS (XLVI) in either the CNS or the muscle preparations.

The failure in the case of [^{3}H]-DPTX was not too surprising, as there have always been problems with the high levels of non-specific binding with this ligand. Thus Tanaka et al. (1984), a group with much experience in the use of this ligand, studied its binding to membranes derived from the CNS of P.americana. They quoted the level of specific binding of DPTX in the fraction giving the best binding characteristics as being 1783 ± 66 dpm/mg protein, whilst the total binding was only 16688 ± 104 dpm/mg protein - a specific binding

content of just over 10%. Such low levels of specific binding are very difficult to work with, and are of questionable validity.

In the case of [^{35}S]-TBPS, the failure to find specific binding in CNS tissue can be seen as confirmatory evidence for the thesis that this compound does not act at a central site in the insect. This will be discussed further in relation to the results of synaptosomal experiments. However, the failure to uncover such binding in the muscle membranes is not so easily understood as such sites have been found in the thoracic muscle tissue of other insects (Cohen and Casida: 1985, 1986). As with the binding of L-glu to similar membranes, the lack of binding may be due to a low level of innervation to the coxal muscles when compared with the thoracic muscles which are normally used to produce the membranes for use in binding studies in the insect muscle tissue.

Nor was it possible to uncover specific binding of the ligand [^{3}H]-TBOB to the membranes derived from the CNS of the roach. This newly available ligand is believed to bind at the same site as TBPS and DPTX (Lawrence et al.: 1985), but being less polar should have more chance of crossing any lipid barriers that may protect the insect CNS. Further, unlike TBPS, it is active in the synaptosome system discussed below. Investigations carried out to assess whether the ligand was being degraded under the experimental conditions failed to find any evidence for the metabolism or degradation of the TBOB in these conditions. It is possible that, being far less active than PTX (XLII) at the blockade of the Cl^- ionophore (this is so both chemically - see the work on the synaptosomes below; and electrophysiologically-G. Lees, personal communication), this ligand does not possess a high enough affinity for that site to be of use in the study of the insect receptor.

4.1.3 The binding of ligands to a putative acetylcholine receptor in material derived from the CNS of P.americana.

The goal in any study into receptor biochemistry is the isolation and characterisation of the receptor protein complex, ultimately leading to the reconstruction of that receptor from its component parts. This aim has been largely achieved with the nicotinic acetylcholine

receptor from Torpedo mamorata, the work being greatly aided by the use of the snake venom ∝-bungarotoxin (BTX). A receptor site bearing many similarities to the nicotinic receptor has been found in the nervous system of the housefly, M.domestica (March et al.: 1980).

When the nervous system of the cockroach was tested for its ability to bind [^{125}I]-BTX, specific binding of this ligand was clearly demonstrated - not only in the purified fractions that were enriched in synaptic membranes, but also in all the other fractions, including the crude homogenate. The ability of [^{3}H]-nicotine was then tested for its ability to reveal these sites. It was found that this ligand, which has an affinity for the acetylcholine receptor very similar to that of L-glu for its receptor, gave results that were qualitatively and quantitatively very similar to those obtained with L-glu in the studies described above. It would thus seem reasonable to conclude that little further progress will be made in studying the insect L-glu and GABA receptor sites until the discovery of a ligand for these sites bearing properties similar to those shown by BTX for the nicotinic acetylcholine receptor.

4.2 Studies using synaptosomes derived from the CNS of P.americana

In the study of the biochemistry of the mammalian nervous systems, much use has been made of transmitter release and radiolabelled ion fluxes from synaptosomes (e.g. the study of radiolabelled sodium fluxes to study the action of the pyrethroid insecticide; Soderland and Ghiasuddin: 1985). These pinched off nerve terminals possess many of the properties of the parent structures and are readily available for biochemical manipulation. Such preparations are now also available for insects (for locusts see Breer and Jeserich: 1980; for cockroaches see Nicholson et al.: 1985). The system developed by Nicholson et al. was used in this study, an electron-micrograph of the material present in this preparation is shown in the introduction.

Perfusion of synaptosomes previously loaded with [^{3}H]-choline with the macrocyclic lactone ivermectin (LIII) was found to cause a stimulation of the release of radioactivity (Nicholson et al.:1986), part of this stimulated release consisting of tritium labelled acetylcholine (Nicholson, personal communication). There is considerable evidence to suggest that the mode of action of ivermectin in insects is by pertubation of the GABA/Cl^- channel complex (for recent work see Duce and Scott: 1985; Tanaka and Matsumura: 1985; Wright: 1985). The ability of certain Cl^- channel blockers, such as PTX (XLII), lindane (XLV) and dieldrin (XLIV), to block the stimulatory action of ivermectin on insect synaptosomes (Nicholson et al.:1986) suggested that this would be a good system in which to study various GABA/Cl^- channel effectors. Work undertaken with dieldrin, lindane and PTX confirmed that the effects observed by Nicholson et al. were readily reproducable and the system was then used to study some of the other major GABA/Cl^- channel effectors.

Of the compounds studied, the bicyclo-octane TBOB (XLII) was shown to have moderate activity in blocking the effect of ivermectin. However, this compound was at least an order of magnitude less active than PTX and this low level of activity may account for its failure to reveal specific binding to the Cl^- channel when used in the preparation discussed above.

None of the other compounds tested showed any appreciable ability to affect the stimulation of release of radioactivity caused by ivermectin. The negligable activity of TBPS, even at 10μM, lends weight to the argument that this compound acts at peripheral rather than central sites in the insect. This compound has similar binding parameters to TBOB in mammalian CNS preparations (Lawrence et al.:1985), in stark contrast to the differential effects found in this system. Of the two remaining compounds, neither GABA (XVII) nor diazepam (L) perturbed the effect of ivermectin in this system. This profile of activity in the synaptosome system correlates very well with toxicity data for these compounds obtained by the Applied Biology Section at the Wellcome Research Laboratories at Berkhamsted Hill. These showed LD_{50} values for PTX, TBOB and TBPS to be 0.01, 0.5, >10μg/insect respectively for the co-treatment of the compound and the synergist piperonyl butoxide against the housefly, M.domestica.

When perfused alone, diazepam and the two bicyclic compounds always failed to give any response. GABA did sometimes give a small stimulation of release of radioactivity on its own when added at high concentrations, but this result was not readily reproducible. When the response was obtained, it was antagonised by PTX in a manner similar to the antagonism of the ivermectin response. Thus, although GABA would seem capable of producing a stimulation of release of radioactivity in this system, its low intrinsic activity explains why no enhancement of the ivermectin response was seen on cotreatment. Further, as the GABA response does not seem to be always present, in contrast to the ivermectin response which is, it would seem that, although GABA and ivermectin act in a similar manner at the GABA/Cl^- channel complex in this system, they do so at different receptor sites - the one for GABA being highly labile in the extraction procedures used in this investigation.

4.3 The presence and metabolism of GABA in insect nervous tissue

Having shown the presence of a putative GABA neuroreceptor , to help to confirm that these are pharmacologically relevant it is necessary to show the presence of the amino acid itself and that of the enzymes for its production and degradation.

4.3.1 The levels of GABA present in the CNS of P.americana

The control level of GABA (XVII) in the CNS of the roach of 1.61nmoles/mg protein works out to be approximately 12μmoles/g wet weight. This compares well with other values for the levels of GABA found in the insect CNS - e.g. 10-13μmole/g wet weight found in the cockroach by Pandey and Singh (1985) and the values given for the locust by Breer and Heilgenberg (1985) of between 10.7 and 21.5μmoles/g wet weight (dependent upon the region of the CNS assayed). Although these levels are higher than those found in the human brain where levels of 5.32μmoles/g wet weight have been quoted (Quastel: 1985), they are of the same order of magnitude. The fact that these levels were raised by the GABA-T inhibitors γ-vinyl- and γ-acetylenic-GABA (XXXIX and XL) helps to confirm that these levels are pharmacologically relevant and not merely extraction artifacts.

4.3.2. The activities of the enzymes concerned with the production and degradation of GABA in the CNS of P.americana.

The metabolising enzymes for GABA were also found in the CNS of the cockroach. The GABA producing enzyme, GAD, was studied in slightly greater detail, as in the mammalian system this enzyme is taken as the key one in GABA metabolism and is widely used as a marker for GABA-ergic neurones (Saito et al.: 1974).

4.3.2.1 The activity of GAD

The K_m value for GAD obtained in this study compares well with those obtained by Breer and Heilgenberg (1985). At 12mM it is slightly less than the value they obtained for the roach (28mM) but is the same as that obtained for the locust. The V_{max} of 125pmoles/min/mg wet weight is much lower than the values obtained by these two, which were 12.5nmoles/min/mg wet weight for the locust and 10nmoles/min mg wet weight for the cockroach. However, both these values are far higher than that found by Pandey and Singh (1985), who quote a GAD activity of only 0.4 pmoles/min/mg wet weight. In mammals values of around 150pmoles/min/mg wet weight have been found in the rat. This was the figure quoted by White (1981) in the paper which was basis for the procedure used in this study.

As with Breer and Heilgenberg, the current study indicated that there were two pH optima for the activity of GAD. Both studies showed one optimum to be close to pH 7 (6.9 in the current study, 7.1 in that undertaken by Breer and Heilgenberg: 1985), but there is a marked difference in the value for the second optimum. The German workers found this to be at pH 6.3 whilst this study indicates an optimum in the region of pH 7.6-pH 7.8. In mammals a single optimum is found around pH 7, White (1981) quoting a value of 6.8 in rat brain.

The other characteristics of this enzyme are as expected.

4.3.2.2 The activity of GABA-T

The activity for GABA-T in the roach CNS was found to be 17.1pmoles/min/mg wet weight tissue. Again this is lower than the value found by Breer and Heilgenberg (1985) in their study, but the ratio of GAD activity to GABA-T activity of roughly 10:1 is similar in both cases. This is in contrast to the situation found by Pandey and Singh (1985), who found the level of GABA-T activity to be some 1000x higher than that of GAD, which would also be the order of activity expected in mammalian tissues. This level is said to ensure the close control of GABA levels through the activity of GAD. However, even with the reverse levels of activity found in this study, there should be no problems in the control of GABA levels in the CNS of the roach because of the comparmentalisation of production and degredation within the CNS. Most models propose presynaptic production of GABA prior to release, followed by removal from the synaptic cleft by high activity uptake into the glial cells and degradation by GABA-T (eg. Turner and Whittle: 1983).

4.4 Conclusions

The aim of the study was the biochemical identification of receptors for L-glu (I) and GABA (XVII) in the CNS of the American cockroach, P.americana. After receptors relevant to the transmitter function of these two amino acids had been shown, the preparations could be used as tools to develop novel insecticides which would act through the modification of the neurotransmitter function of these two amino acids. How well were these objectives achieved?

4.4.1 The receptor for L-glu

The studies carried out in this thesis have shown the presence of radioligand binding sites for L-glu. The characteristics of these sites can be compared with the criteria for the acceptance for receptor sites as physiologically relevant laid down in the introduction:

a) Saturability: Saturable, Na^+ ion concentration independent binding sites for L-glu were clearly demonstrated, thus fulfilling the first of the criteria for the identification of a physiologically relevant receptor site.

b) Kinetics: Although detailed studies of the kinetics of the receptors were not carried out, the equilibrium dissociation constant and receptor concentration were well within the ranges expected for a neuroreceptor site and were similar to those described by previous workers.

c) Distribution: The fact that such receptors were found in the CNS of P.americana is one of the first pieces of evidence that L-glu may be acting centrally as well as peripherally in the insect nervous system. However, the failure to find these sites in the muscle preparations used was not as expected from the work of others in the field. This could well have been caused by the use of muscular tissue with less innervation than that normally used for such studies.

d) Pharmacology: The sites described in this study show a similiar pharmacological profile to putative neuroreceptor sites described by workers in both the insect and mammalian fields, although it would appear that there are some fine differences between that of the mammalian and insect receptors. However, such sites do not fit easily into any of the major excitatory amino acid receptor types as defined by electrophysiological studies, either in the insects or in the mammalian systems. This may well be a consequence of the grossly differing environments used in the two types of study.

If these sites prove to be relevant to the role of L-glu as a neurotransmitter, this will be one of the first firm pieces of evidence that this amino acid works as such in the CNS of an insect species and it will indicate that there are differences between the mammalian and insect systems at this level that may be exploited in the design of a novel, safe insecticide.

4.4.2 The GABA/Cl^- channel complex.

Electrophysiological evidence has already shown that GABA is active in the insect CNS, so that any GABA receptors that were revealed by these studies would be the more readily accepted as relevant to the role of this amino acid as a neurotransmitter. Comparing the results of these studies with the criteria in the introduction, the following conclusions can be drawn:

a) Saturability: The discovery of a saturable, Na^+ ion concentration independent site for this ligand was to be expected and so the first criterion was met.

b) Kinetics: Once more the kinetics were not studied in detail. The equilibrium values of the site studied in this report did not align well with those described elsewhere. However, other details of the results of this study suggest that this is a neuroreceptor site, so that the mismatch in this area was probably due to different preparation procedures.

c) Distribution: Once more, the type of membrane preparation used in the main studies would be expected to show GABA neuroreceptor binding if such were present in the CNS. The failure to show such receptors in the muscle preparation is probably due to reasons similar to those described for the L-glu receptor, although the evidence for a GABA receptor in the insect muscle has always been more equivocal than for the L-glu receptor.

d) Pharmacology: This receptor is clearly not a transport site - as shown by the lack of activity for the specific GABA transport blocker nipecotic acid (XXVI); and the profile of drugs able to displace radiolabelled muscimol (XXI) from the site is much as would be expected of a mammalian $GABA_A$ receptor.

Although the failure of receptor binding studies to show the presence of the binding of the recognised Cl^- channel receptors was disappointing, the results using the synaptosomal release system clearly show that the CNS of the cockroach contains Cl^- channels that

can be activated by the putative GABA-mimetic ivermectin. These Cl^- channels are blocked by the standard Cl^- channel inhibitors, but the results using GABA in this system suggest that they may not be that closely linked with the GABA receptor shown in the ligand binding studies.

Finally the presence of GABA and the related metabolic enzymes in the CNS at levels that would be expected for there to be a transmitter role for this compound lends further support to the identification of the site described above as being relevant to the neurotransmitter activity of GABA in the CNS of insects.

4.4.3 The use of these sites as simple screens in the development of new insecticides.

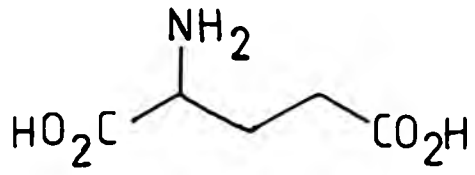
One aim of this project was met, in that receptor sites for L-glu and GABA have been identified and shown to be relevant to the role of those two amino acids as neurotransmitter substances in the CNS of an insect species. However, the second role of the project, the development of a high throughput screen for the testing of putative insecticides, has not been fulfilled. For although these preparations can discriminate between the activity of various compounds at the receptor sites, the length of the procedures, the small amount of material obtainable from the insects and the large variability in the amount of radioactivity bound preclude the use of these membranes as a high throughput screen with the current ligands. For further progress to be made, both in the use of this type of preparation in the screening of insecticides interacting with the L-glu or GABA receptor sites on a potential target species and in the further typing of the two receptor sites, ligands of much higher affinity will have to be found, ones on a par with BTX at the nicotinic acetyl-choline receptor.

Perhaps such a ligand for the L-glu receptor will be provided by one of the venoms now being tested (eg. the toxin from the wasp Philanthus triangulum, Clark et al.: 1982; the venom from the spider Nephila clavata, Yoshioka et al.: 1985). For the study of the $GABA/Cl^-$ channel site one of the bicyclooctane compounds recently produced by Casida and his team may prove useful in the study of the

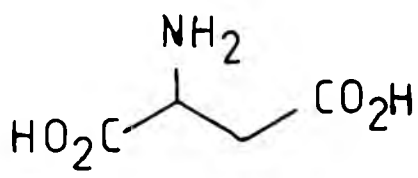
Cl^- ionophore. The results with the synaptosomes have shown one of these compounds - TBOB (XLVII) - to be active at this site in the insect CNS. This group of compounds is highly insecticidal, with many compounds giving much higher kill in the housefly M.domestica than TBOB. The cyano-derivative of TBOB (XLIX), has a toxicity to the flies that is 100x that of TBOB itself (Casida and Palmer: 1985) and can be readily tritiated. These factors make it a prime compound for use in such studies.

Compound Structures

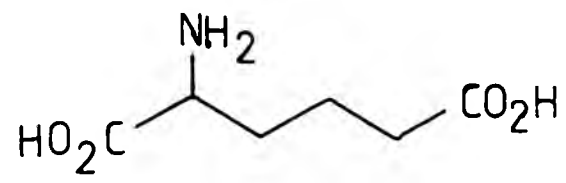
Glutamic Acid Analogues



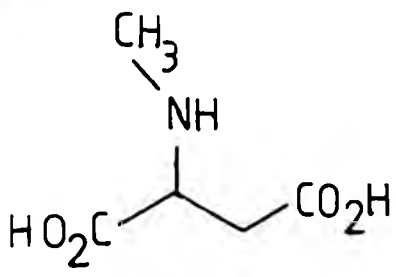
I: Glutamic Acid



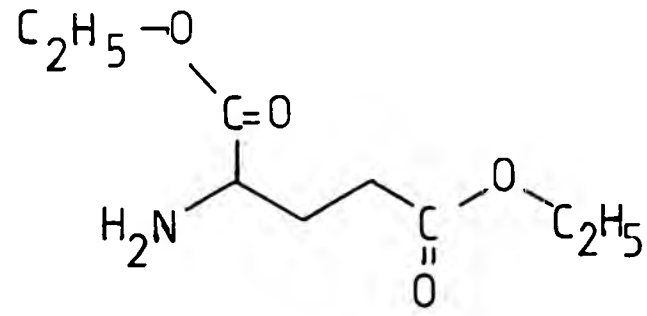
II: Aspartic Acid



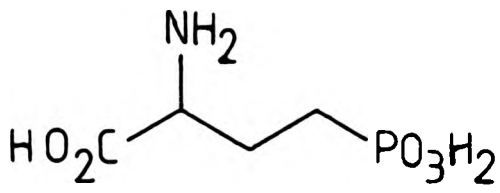
III: 2-Aminoadipic Acid



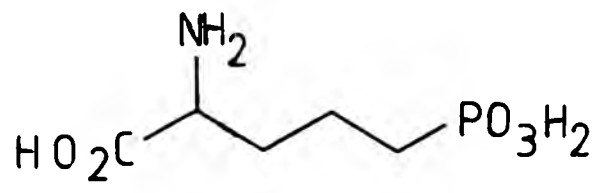
IV: N-Methylaspartic Acid



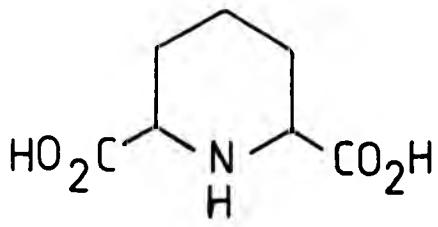
V: Glutamic Acid Diethyl Ester



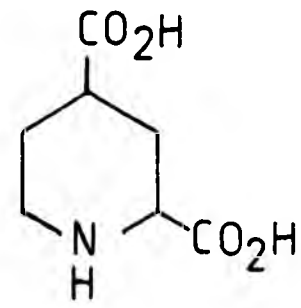
VI: 2-Aminophosphono-butyric Acid



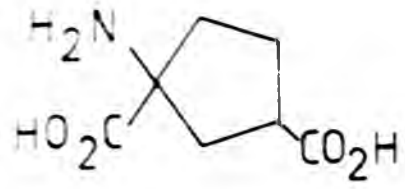
VII: 2-Aminophosphonovaleric Acid



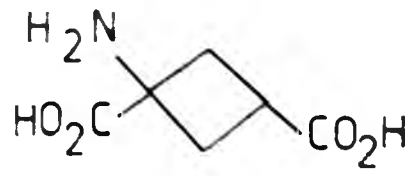
VIII: Piperidine-2,6-dicarboxylic Acid



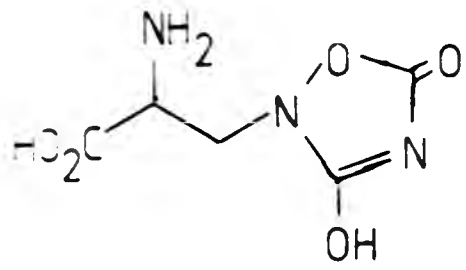
IX: Piperidine-2,4-dicarboxylic Acid



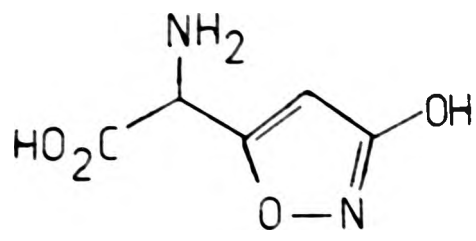
X: 1-Amino-1,3-dicarboxy Cyclopentane



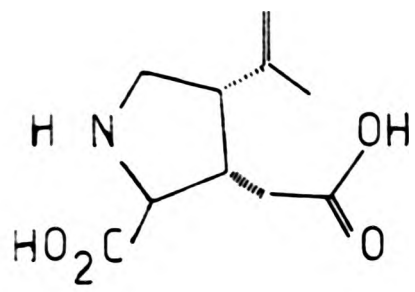
XI: 1-Amino-1,2-dicarboxy Cyclobutane



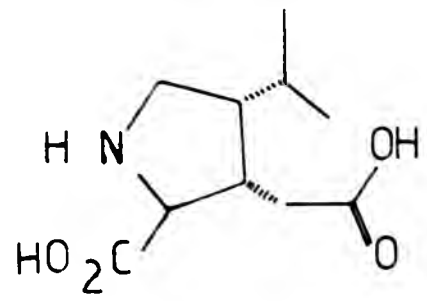
XI: Quisqualic Acid



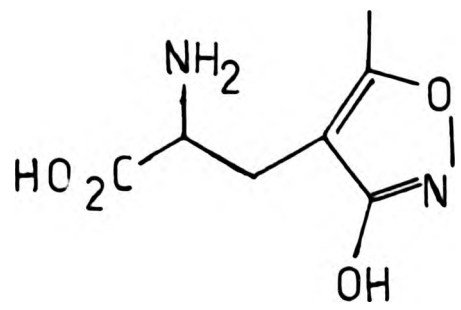
XIII: Ibotenic Acid



XIV: Kainic Acid



XV: Dihydroxykainic Acid

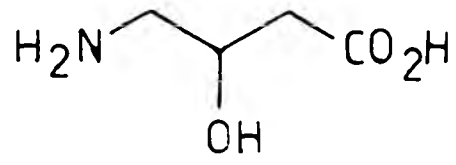


XVI: α-Amino-3-hydroxy-5-methyl-4-isoxazole-propionic Acid (AMPA)

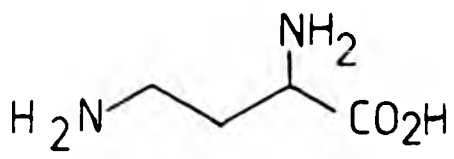
γ-Aminobutyric Acid Analogues

XVII γ-Aminobutyric Acid (GABA)

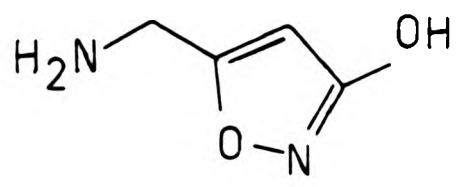
XVIII: γ-Aminopropane-sulphonic Acid



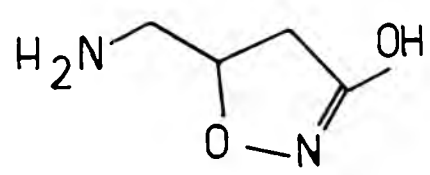
XIX: 3-Hydroxy-GABA



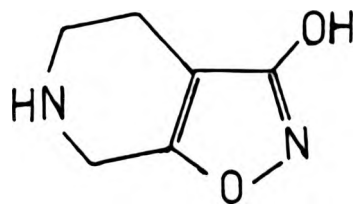
XX: Diaminobutyric Acid



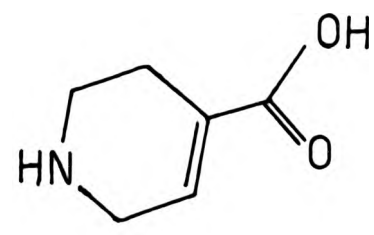
XXI: Muscimol



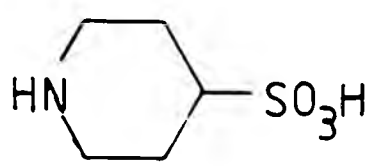
XXII: 4,5-Dihydromuscimol



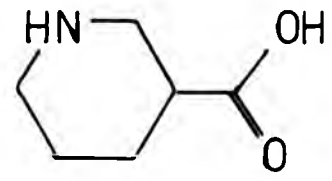
XXIII: 4,5,6-Tetrahyro-isoxazolo-[3,4-c]-pyridin-3-ol (THIP)



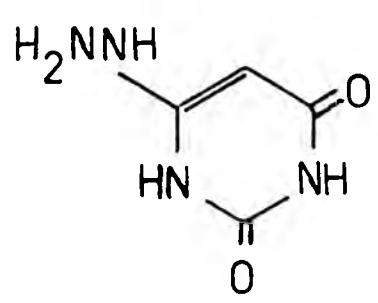
XXIV: Isoguvacine



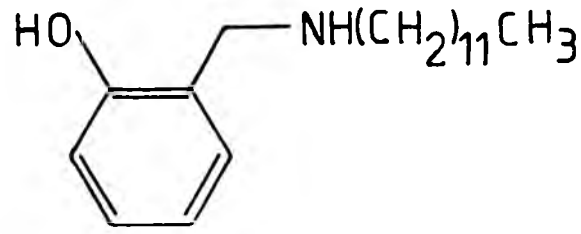
XXV: Piperidine-4-sulphonic Acid



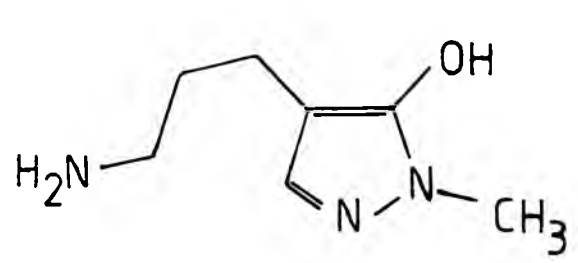
XXVI: Nipecotic Acid



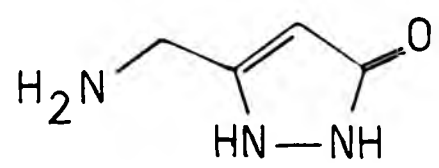
XXVII: N-(Pyrimidin-2,4-dione-6-yl) -hydrazine



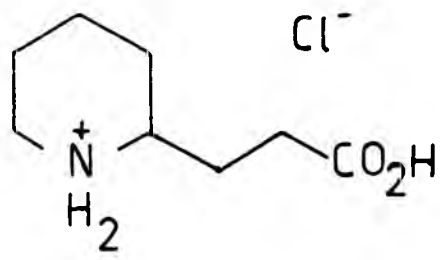
XXVIII: N'-(Dodecyl)-2-hydroxy-benzylamine



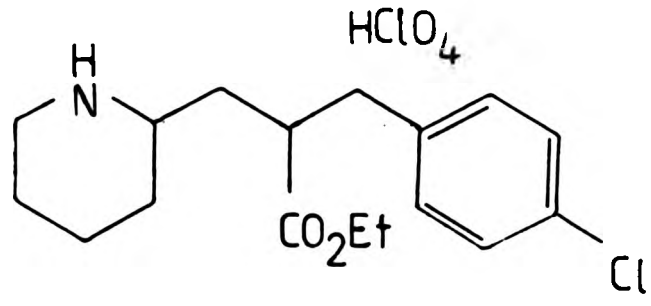
XXIX: 4-(Aminopropyl)-3-hydroxy-2-methyl-pyrazine



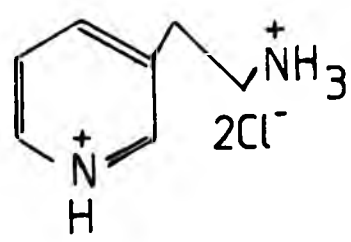
XXX: 5-(Aminomethyl)-3-hydroxy-pyrazine



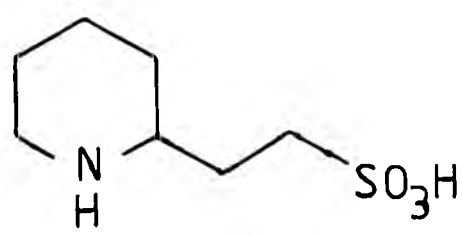
XXXI: 3-(Piperidin-6-yl)-propionic Acid HCl



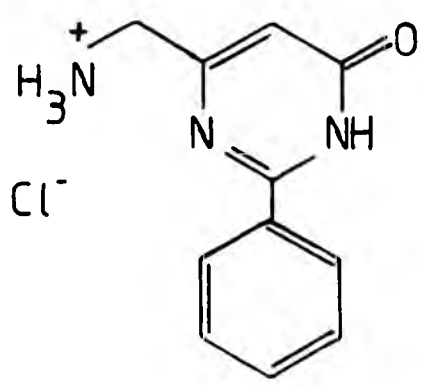
XXXII: Ethyl-2-(p-chlorobenzyl)-3-(piperidin-6-yl)-proprionate



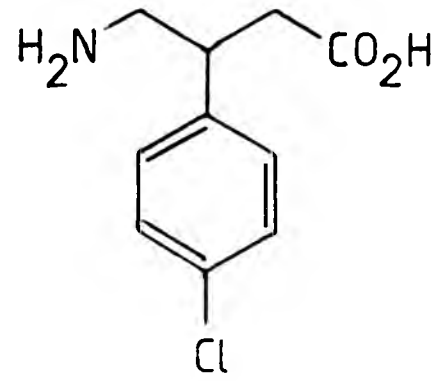
XXXIII: 3-Aminoethyl-pyridine HCl



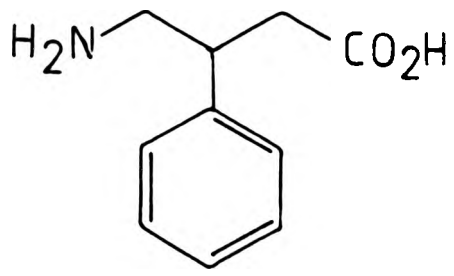
XXXIV: 2-(Piperidin-6-yl)-ethyl-sulphonic Acid



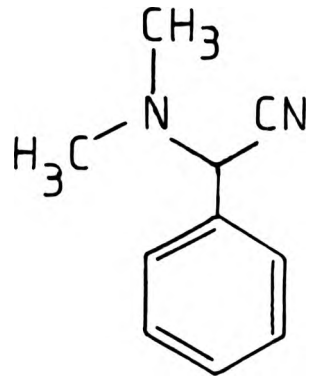
XXXV: 2-Phenyl-4-hydroxy-6-aminomethyl piperidine



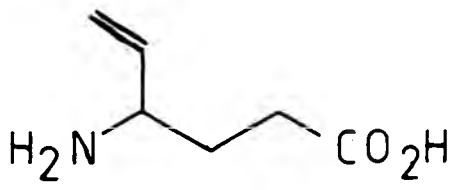
XXXVI: Baclofen



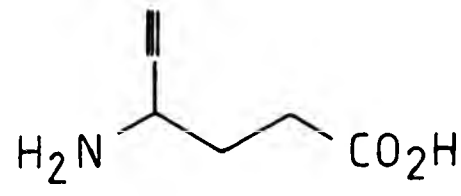
XXXVII: 3-Phenyl-GABA



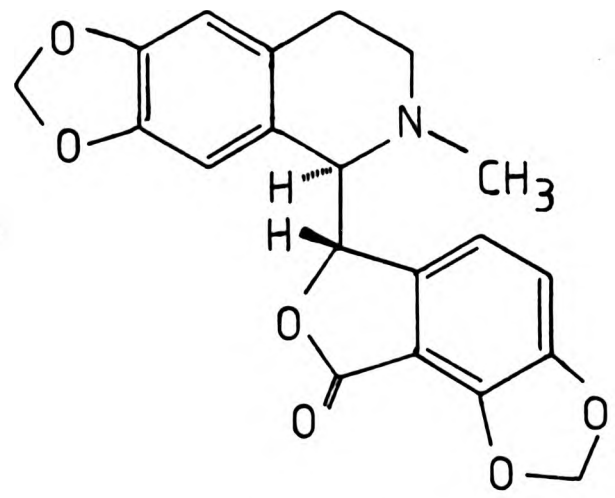
XXXVIII: 2-Dimethylaminophenyl-acetonitrile (DAPA)



XXXIX: γ-Vinyl-GABA

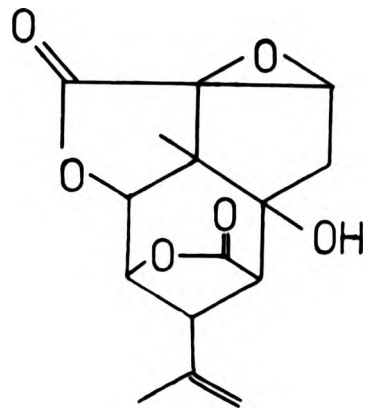


XL: γ-Acetylenic-GABA

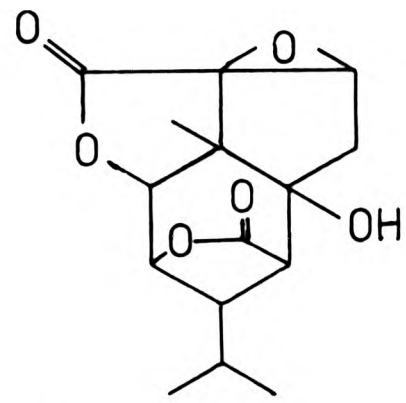


XLI: +-Bicuculline

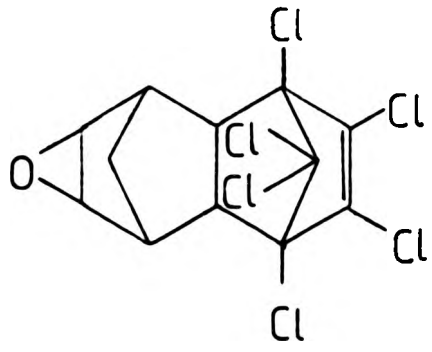
Chloride Ion Channel Blockers



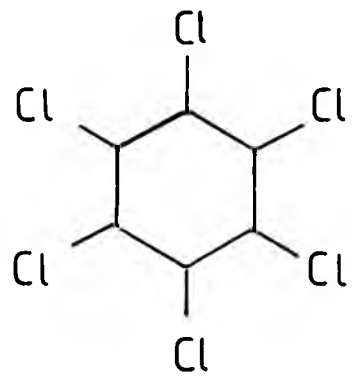
XLII: Picrotoxinin



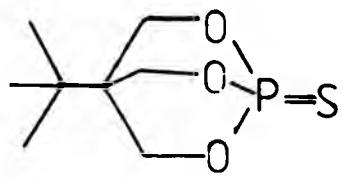
XLIII: Dihydropicrotoxinin



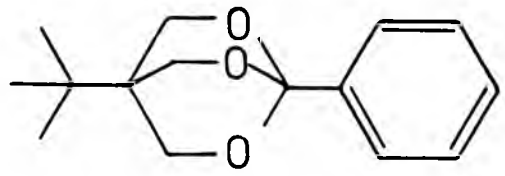
XLIV: Dieldrin



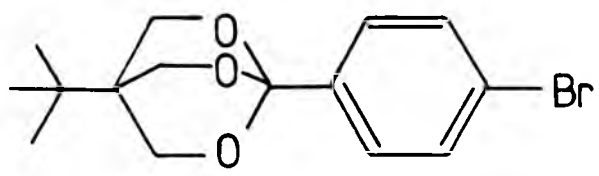
XLV: Lindane (γ BHC)



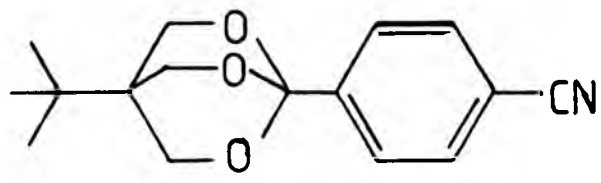
XLVI: t-Butylbicyclophosphorothionate (TBPS)



XLVII: 1-Phenyl-4-t-butyl-2,6,7-trioxabicyclo-[2,2,2]-octane (TBOB)

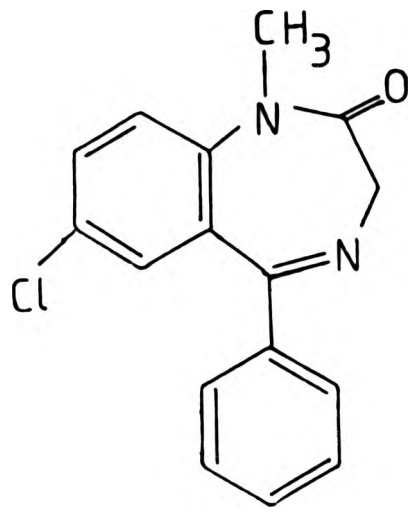


XLVIII: 1-Parabromophenyl-4-t-butyl-2,6,7-trioxabicyclo-[2,2,2]-octane

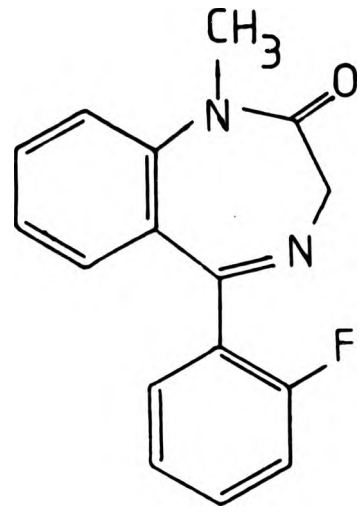


XLIX: 1-Paracyano-4-t-butyl-2,6,7-trioxabicyclo-[2,2,2]-octane

Miscellaneous Compounds

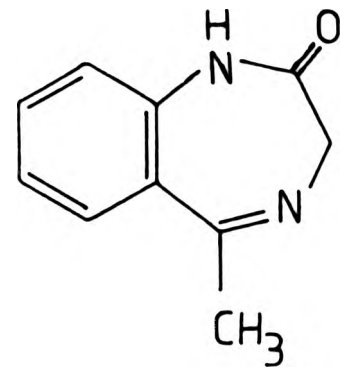


L: Diazepam

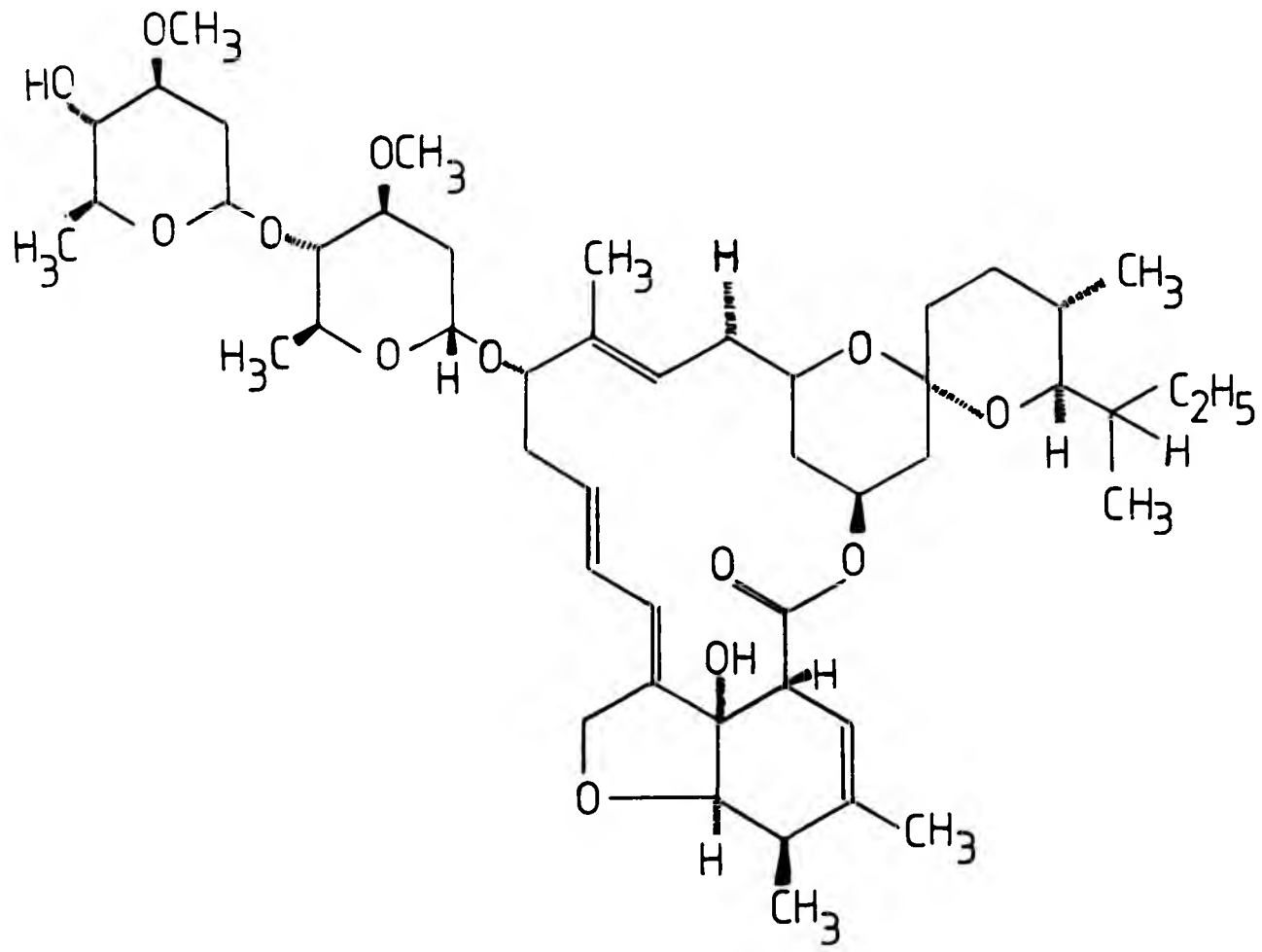


L-LII-Benzodiazepines

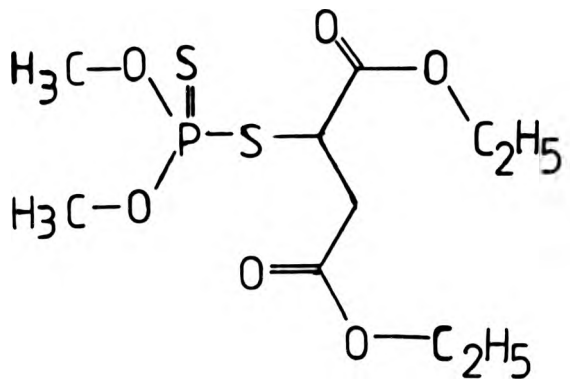
LI: Flunitrazepam

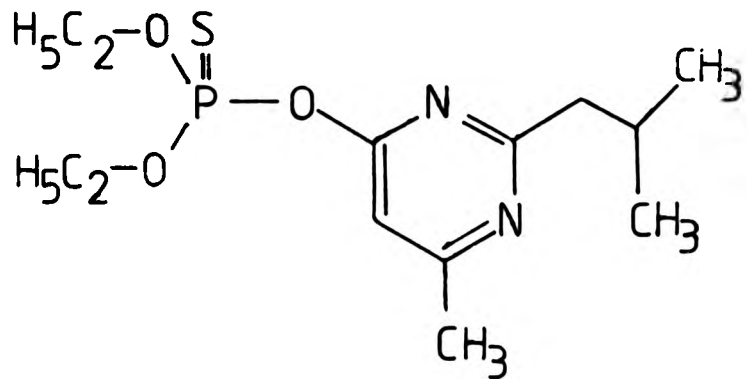


LII: Ro 5-3663



LIII: Ivermectin$_{B1b}$

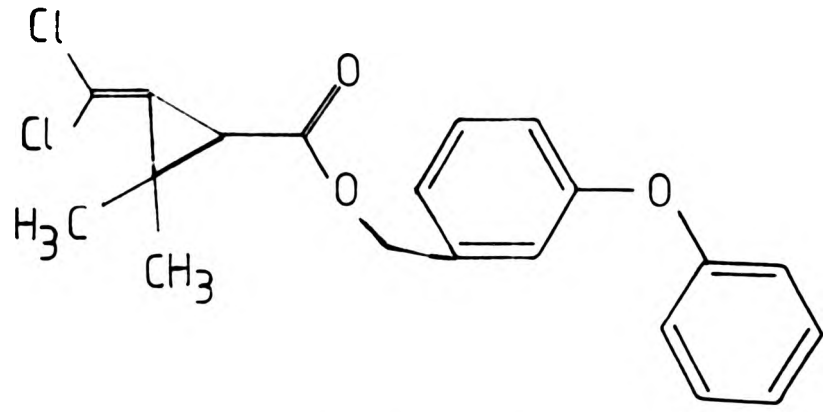




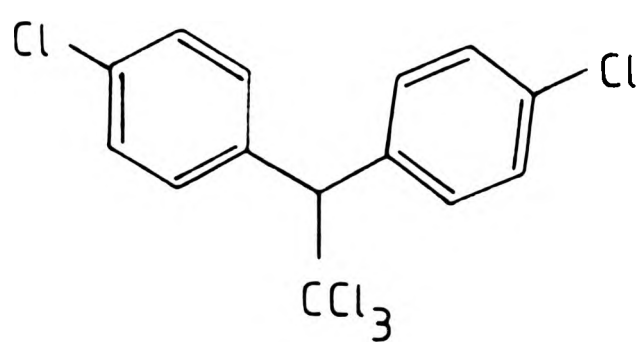
LIV-LV: Organophosphates

LIV:Malathion

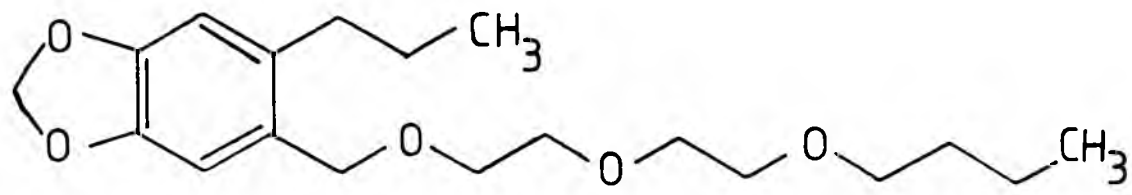
LV: Diazinon



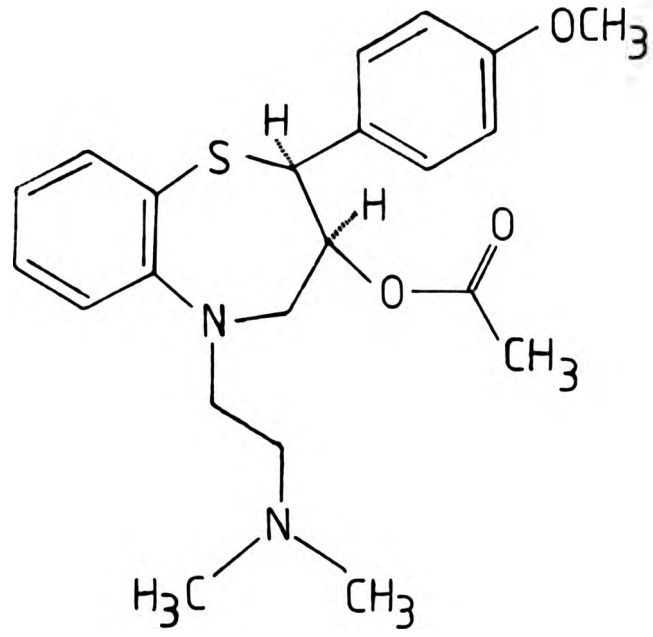
LVI:Permethrin



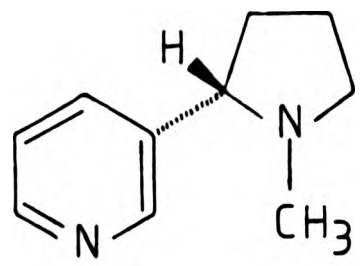
LVII:Dichloro-diphenyl-trichloromethane
(DDT)



LVIII:Piperonyl Butoxide



LIX:Diltiazem



LX:Nicotine

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