Studies on the voltage-sensitive sodium channel of Manduca sexta

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STUDIES ON THE VOLTAGE-SENSITIVE SODIUM CHANNEL OF *MANDUCA SEXTA*

submitted by Bernard Verdon

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ABBREVIATIONS

AaHTII	*Androctonus australis* Mammalian Toxin II
AaIT	*Androctonus australis* Insect Toxin
ACN	Aconitine
ATX-II	Anemone toxin II
BjIT2	Buthotus judaicus Insect Toxin 2
B_{max}	Maximal specific binding capacity
BTX	Batrachotoxin
[^{3}H]BTX-B	[^{3}H]Batrachotoxinin A-20-α-benzoate
Ci	Curies
CNS	Central nervous system
μ-CTX	μ-Conotoxin
DDT	Dichlorodiphenyltrichloroethane
EDTA	Ethylenediaminetetraacetic acid
GABA	γ-aminobutyric acid
K_1	Kinetic association rate constant
K_{-1}	Kinetic dissociation rate constant
K_D	Equilibrium dissociation rate constant
Kb	Kilobases
KDa	Kilodaltons
Kdr	Knockdown resistance
Lqq	*Leiurus quinquestriatus*
LqqIT1	*Lqq* Insect toxin 1 (excitatory)
LqqIT2	*Lqq* Insect toxin 2 (depressant)

Mepsp	Miniature excitatory post synaptic potential
OD	Optical density
PCR	Polymerase chain reaction
Pfu/ml	Plaque forming units per ml
α-ScTX	α-Scorpion toxin
β-ScTX	β-Scorpion toxin
SD	Sample standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-Polyacrylamide gel electrophoresis
STX	Saxitoxin
TBPS	t-Butylbicyclophosphorothionate
TTX	Tetrodotoxin
VTD	Veratridine

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ABSTRACT

The voltage-sensitive sodium channel of *Manduca sexta* was studied using a variety of techniques with the intention of gaining a more comprehensive understanding of the Lepidopteran pyrethroid receptor.

Electrophysiological recordings demonstrated that pyrethroids induce a dramatic increase in the frequency of spontaneous transmitter release at the neuromuscular junction. This response could be eliminated by TTX, implicating the voltage-sensitive sodium channel as the probable target of pyrethroid action. VTD caused a response similar to the pyrethroids, but VTD had no effect on the pyrethroid induced response, which suggests that the *Manduca* sodium channel may possess novel properties.

A binding assay was developed to investigate the pharmacology of radiolabelled sodium channel toxins in preparations of *Manduca* excitable tissues. The binding of [^{3}H]STX to *Manduca* nerve cord homogenate yielded K_D and B_{max} values of 1.53 nM and 3.41 pmol/mg protein respectively. The K_D of this interaction is comparable with published values for mammalian brain, indicating that the STX receptor is highly conserved. Pyrethroid insecticides had no effect on [^{3}H]STX binding, confirming that the pyrethroid and STX binding sites are distinct entities on the sodium channel.

Insect heart tissue was found to possess a specific and saturable [^{3}H]STX binding site, with a K_D of 12.4 nM. This discovery may represent the first report of an insect

sodium channel subtype, although it is possible that these sites represent TTX-sensitive calcium channels.

The *Manduca* nerve cord preparation was shown to bind [^{3}H]BTX-B specifically, although this interaction was not saturable at concentrations of up to 150 nM. The inclusion ScVN completely abolished [^{3}H]BTX-B binding in this preparation. In contrast, a rat brain synaptosomal preparation was shown to bind [^{3}H]BTX-B in a conventional manner, with the addition of scorpion venom causing an apparent increase in [^{3}H]BTX-B binding affinity. Pyrethroids had no effect on [^{3}H]BTX-B binding to *Manduca* nerve cord, in contrast to literature reports that they increase [^{3}H]BTX-B binding affinity in nerve preparations from other species. These findings may indicate that the *Manduca* [^{3}H]BTX-B binding site has diverged considerably.

An attempt was made to clone the *Manduca* sodium channel from a developing brain cDNA library. The cloning strategy employed homology hybridization experiments using both an oligonucleotide designed specifically for *Manduca* sodium channel and mammalian cDNAs from highly conserved regions. Many of the techniques utilised were shown to be functional, since they were used to demonstrate specific hybridization of radiolabelled probes to Southern blots prepared from rat genomic DNA. The cDNA library was also shown to have been made from a suitable developmental stage. However, this study proved to be unsuccessful in isolating a *Manduca* sodium channel clone.

Taken together the results give an initial indication that the *Manduca* voltage-sensitive sodium channel may possess unique structural properties, which might in future be exploited as targets for Lepidopteran-specific insecticides.

CHAPTER 1

GENERAL INTRODUCTION

1.1. Voltage-sensitive sodium channels

1.1.1. Sodium channel function

Nerve cells communicate by means of an electrical signal which spreads in an all-or-none fashion along the length of the axon. This electrical signal usually takes the form of a self-sustaining spike-like depolarisation, referred to as the action potential. Action potentials are a function of the ability of the nerve membrane to change its permeability to various distinct ions. In most cases, the depolarising phase is the result of a rapid but transient increase in membrane permeability to sodium ions, while repolarisation to the normal resting membrane potential is achieved in part via a decline in sodium permeability back to its normal level, and in part by an increase in permeability to potassium ions. These ion fluxes are mediated through integral membrane proteins which open in response to voltage changes across the membrane to form channels, which allows the passive transmembrane flow of ions through a water-filled pore.

Voltage-operated channels are normally characterized in terms of their selectivity and gating properties. Selectivity refers to the ability of the channel to discriminate between ions, for example the sodium channel is approximately 10-fold more

permeable to sodium ions (and in fact lithium ions) than it is to potassium ions, which are the next most permeant ions. The application of molecular biology techniques has revealed that all types of voltage sensitive channel belong to a genetically related family, as subtypes of both the calcium channel (Tanabe *et al.*, 1987) and potassium channel (Tempel *et al.*, 1988) possess striking amino acid sequence homology to the sodium channel over regions which are thought to be of functional importance.

Gating describes the kinetics of the permeability changes, and no other ion channel has received as much attention in this respect as the sodium channel. Despite this however, its gating kinetics are still not fully understood. The concept of sodium channel gating is derived from experimental and theoretical results of Hodgkin and Huxley (1952). When the nerve membrane is depolarised under experimental conditions in a stepwise manner (voltage-clamp) to values which are sufficiently more positive than resting potential, the permeability of the membrane to sodium ions rapidly increases, then more slowly declines, despite the fact that the potential is maintained at the value required to open the channel. The simplest gating scheme necessary to describe this phenomenon involves three functionally distinct conformational states. A resting (closed) state, favoured at normal membrane potentials; a transient conducting (open) state, in response to rapid membrane depolarisation; and an inactivated (closed) state favoured in depolarised membranes. The normal sequence of events following sudden membrane depolarisation is therefore rapid sodium channel activation (resting to open states) followed by inactivation (open to inactivated states). Sodium channels usually open only once during a depolarising epoch, as inactivated channels cannot normally be reopened. The macroscopic decline in sodium current after activation therefore represents the entry of channels to the inactivated state after being open for random lengths of time. Upon membrane repolarisation the channel re-assumes the resting conformation. Although this three state scheme serves to illustrate the basic gating properties of the sodium channel, true channel kinetics are undoubtedly more complicated, with

multiple intermediary non-conducting pathways, as well as more than one channel conformation for each state.

The original Hodgkin and Huxley (1952) model assumed that the activation and inactivation processes were achieved via independent, kinetically distinct gates. Several lines of evidence have subsequently shown that activation and inactivation are in fact functionally linked, and a number of major revisions have been proposed to account for these new observations (see Yamamoto, 1985). However, it is worth noting that in the absence of a generally accepted more realistic scheme, the classic Hodgkin and Huxley (1952) equations are still considered to be the most useful framework for the analysis of activation and inactivation parameters (e.g. Stühmer *et al.*, 1989).

1.1.2. Sodium channel structure

Although the biophysical properties of the voltage sensitive sodium channel have been studied for decades, it has only relatively recently been possible to remove it from the membrane for molecular characterisation. This in part reflects the difficulty in identifying the channel after removal, but this problem has been largely overcome by the use of radiolabelled neurotoxins specific to the sodium channel as markers during purification. Since the molecular cloning of sodium channel subtypes, the process has been simplified as immunoprecipitation of solubilized channels can be achieved using antibodies raised to highly conserved segments of the amino acid sequence.

Detergent solubilized channels have now been isolated from a number of tissues from different species, purified by a sequence of conventional protein separation procedures and the molecular weight determined by SDS-PAGE. The principal component in all cases is a large peptide designated the α-subunit, with a molecular

weight of between 240 and 280 KDa. Hartshorne *et al.* (1982) have shown that in rat brain the α-subunit is additionally associated with two smaller nonidentical ß-subunits (ß1, 39 KDa; ß2, 37 KDa). Skeletal muscle sodium channels also contain one or two smaller peptides (Barchi, 1983). The human brain sodium channel appears to be made up of the α-subunit plus a single ß-subunit (De Rycker and Schoffeniels, 1990).

The function of the ß-subunits remains unclear. In rat brain sodium channels, removal of the ß2 subunit makes no difference to normal channel properties, whereas removal of the ß1 subunit results in non-functioning channels, indicating that the α-ß1 complex is necessary for the functional integrity of the channel (Messner *et al.*, 1986). However, sodium channels purified from eel electroplax (Miller *et al.*, 1983) and a number of different insect neuronal preparations (Gordon *et al.*, 1990; Moskowitz *et al.*, 1991) are composed only of the α-subunit. The purified eel sodium channel functions normally when reconstituted into artificial lipid vesicles (Rosenburg *et al.*, 1984). Furthermore, it has also been demonstrated that rat brain sodium channel mRNA coding for only the α-subunit is sufficient to direct expression of functional channels in *Xenopus* oocytes (Noda *et al.*, 1986b; Goldin *et al.*, 1986), which suggests that only the α-subunit is required, even in mammalian brain sodium channels.

The functional integrity of purified sodium channels incorporated into lipid bilayers has been evaluated using neurotoxin modified $^{22}Na^{+}$ flux measurements with channels purified from eel electroplax (Rosenberg *et al.*, 1984), rat skeletal muscle (Weigele and Barchi, 1982) and rat brain (Talvenheimo *et al.*, 1982). More recently, single channel currents have been analysed after reconstitution of purified sodium channels into planar lipid bilayers, which demonstrate an array of expected biophysical properties (Hartshorne *et al.*, 1986; Levinson *et al.*, 1986). This technique has allowed channel function to be monitored for the first time under fully defined conditions, and may in future prove to be an extremely useful system for pharmacological, biochemical and functional analysis of insect sodium channels.

The structural features of the sodium channel which have been revealed by the application of molecular biology techniques are considered in detail in Chapter 5.

1.1.3. Post-translational modification of sodium channels

Sodium channels purified from rat brain, eel electroplax and insects have been shown to be heavily glycosylated (Messner and Catterall, 1985; Miller *et al.*, 1983; Gordon *et al.*, 1990). The molecular weight of the α-subunit following enzymic deglycosylation indicates that the native α-subunit is 15% and 11% carbohydrate by weight in rat brain and *Schistocerca* α-subunit respectively. The reason for such extensive channel glycosylation is not yet clear, although the large predominance of sialic acid residues associated with rat brain and eel electroplax sodium channels has led to speculation that this dense array of negative charges may have significance in the functioning of the channel (Strichartz *et al.*, 1987). Insect sodium channels are however devoid of sialic acid (Gordon *et al.*, 1990).

Sodium channel α-subunits purified from rat brain and insect nervous systems act as substrates for cAMP dependent protein kinase (Costa *et al.*, 1982; Gordon *et al.*, 1990). The functional significance of sodium channel phosphorylation is not yet fully documented although it has been shown to reduce neurotoxin activated $^{22}Na^{+}$ flux into rat brain synaptosomes by between 16 and 26% (Costa and Catterall, 1984). More recently, whole cell voltage-clamp of rat brain neuroblastoma cells in the presence or absence of intracellular cAMP has revealed that approximately 20% of sodium channels inactivate at more negative membrane potentials in the presence of cAMP (Coombs *et al.*, 1988). These results suggest that phosphorylation enhances sodium channel inactivation.

1.2. Pyrethroid insecticides

1.2.1 The development of pyrethroid insecticides

The insecticidal properties of the crushed flowerheads of *Crysanthemum cineraraefolium* have probably been recognised for centuries. The active components, referred to as pyrethrins, are a mixture of esters, but the commercial value of these natural compounds is limited because they are highly photolabile. Since the elucidation of their chemical structures, efforts have been made to synthesize analogues with increased photostability and insecticidal activity. By the mid 1960s there was a wide variety of synthetic compounds, but although insecticidally active these were still too unstable for most applications. However, in the early 1970s, there began the development of more persistent compounds (Elliott and Janes, 1978) and since that time numerous more active analogues have been synthesized, so that the pyrethroids now represent one of the major classes of insecticide.

The diverse structural formulae of the modern pyrethroids often bear little similarity to the natural products from which they were derived. However, these structures do in fact retain a very close resemblance to pyrethrin I (the most active of the natural esters) in respect to the general shape and geometrical distribution of physical properties on the surface of the molecule.

The modern pyrethroid insecticides possess many favourable characteristics in comparison to the other major classes of insecticide. They are more active by at least one order of magnitude than most common organophosphates and carbamates. At the same time, their insect (topical) to mammalian (oral) toxicity ratio is much higher than that of the other classes of insecticide. Pyrethroids are highly lipophilic molecules, which assists cuticular penetration and makes them potentially rainfast.

Pyrethroids are thus extremely effective agricultural insecticides, and judged by any criteria the environmental properties are such that they represent a major advance on any other class of broad spectrum insecticide.

1.2.2. Molecular target sites of pyrethroid insecticides

In all species so far investigated, pyrethroids induce toxic symptoms which appear to be caused by the disruption of the normal functioning of the nervous system. This neurophysiological modification was first confirmed by Lowenstein (1942), who demonstrated with simple electrophysiological techniques that a mixture of natural pyrethrins induced spontaneous multiple action potentials in a cockroach giant axon preparation. This pyrethroid induced elevation in the firing frequency appears to be the principal feature of most nerve preparations so far examined, although some of the more recently developed pyrethroids, particularly those which possess a cyano group (type II pyrethroids) do not produce this effect in certain preparations, for example in cockroach cercal sensory neurons (Gammon *et al.*, 1981), cockroach giant axons (Pelhate and Sattelle, 1982) and crayfish stretch receptor (Chalmers and Osborne, 1986).

At the neuromuscular junction, pyrethroids cause a large increase in the rate of spontaneous transmitter release. This has been studied in greatest detail in insect preparations (Salgado *et al.*, 1983a,b) although this phenomenon occurs in vertebrate preparations also (Evans, 1976).

1.2.2.1. Sodium channels

The structure related effects regarding the ability of pyrethroids to induce repetitive activity in the nervous system was first reported by Clements and May (1977) using locust preparations. This relationship has been most extensively studied and

characterized using the lateral line sense organ and peripheral nerves of the frog (Vigverberg *et al.*, 1982a). Early information regarding the cellular mechanism involved in producing this type of activity was obtained by Narahashi (1962) using intracellular recording from cockroach giant axons. He showed that the production of multiple action potentials in the presence of allethrin was a consequence of an elevated depolarising after-potential which reaches the threshold for repetitive after-discharges. The molecular mechanism of this response has been studied using the voltage-clamp technique, which has demonstrated that pyrethroids affect the normal voltage-dependent conformational changes of action potential sodium channels in preparations from all species so far investigated, including crayfish and squid giant axons (Lund and Narahashi, 1981a,b), *Xenopus* node of Ranvier (Vigverberg *et al.*, 1982b, 1983) and cockroach giant axons (Laufer *et al.*, 1984, 1985). In each case the effects are essentially the same. Step depolarisation following application of pyrethroid has little effect on the activation of the sodium current, but macroscopic inactivation is drastically slowed. Furthermore, upon step repolarisation there is a marked sodium tail current which decays very slowly (tail currents decay very rapidly under control conditions). These effects are stereospecific, in that toxicologically inactive isomers fail to induce them (Vigverberg *et al.*, 1983; Lund and Narahashi, 1982; Laufer *et al.*, 1985). These results show that pyrethroids cause sodium channels to remain open much longer than normal, and it is probable that it is this effect which is directly responsible for the elevation of the depolarising after-potential which triggers repetitive discharges in the nerve. DDT has a very similar mode of action (Vigverberg *et al.*, 1982b).

A kinetic analysis of sodium currents under voltage-clamp conditions indicates that there are two populations of sodium channels in the pyrethroid modified axon: one group behaving identically to those in control conditions, and another which is stabilised in the open configuration (Lund and Narahashi, 1981a,b). In fact, the fraction of sodium channels which need to be pyrethroid-modified in order to produce

a 10 mV depolarising after-potential (a value which typically induces repetitive firing in squid giant axons) has been estimated as being considerably less than 1% (Lund and Narahashi, 1982) which may in part account for the remarkable insecticidal potency of pyrethroids.

Perhaps the most direct confirmation that pyrethroids interact with sodium channels comes from single channel recording experiments. Patch-clamp analysis of tetramethrin treated single sodium channels from mouse neuroblastoma cells has shown that a proportion of the channels have a much slower closing rate than normal, without affecting the channel conductance properties (Yamamoto *et al.*, 1983). Furthermore, upon step repolarisation, some pyrethroid modified sodium channels remain in the open state (Yamamoto *et al.*, 1984; Holloway *et al.*, 1989), thus corroborating the explanations given for tail currents in the earlier voltage-clamp experiments.

There is therefore a convincing body of evidence that pyrethroids maintain a fraction of nerve sodium channels in the open configuration for much longer than normal, but a more precise description of the mechanism by which this action is exerted is less clear. In frog node of Ranvier, the behaviour of the pyrethroid modified sodium current is explained simply by the prevention of the closure of the sodium channel activation gate (Vigverberg *et al.*, 1982b). Hille (1968) interpreted his results of the effects of DDT as being the prevention of closing of both the activation and inactivation gates in modified sodium channels from squid giant axon. Single channel analysis of mouse neuroblastoma cell sodium channels indicated that deltamethrin stabilises all the functional channel states by reducing the kinetic rates between them (Chinn and Narahashi, 1986).

There are also considerable discrepancies in the literature regarding pyrethroid affinity for open and closed sodium channels. In voltage-clamp experiments, the

magnitude of the tail current in pyrethroid modified frog node of Ranvier changed proportionally with the level as well as the duration of the step depolarisation, suggesting that pyrethroids interact preferentially with the open configuration of the sodium channel (Vigverberg *et al.*, 1982b). Chalmers and Osborne (1986) reached a similar conclusion, on the basis that pyrethroid poisoning symptoms occur much more rapidly in an actively firing preparation of the crayfish stretch receptor organ. Furthermore, measurements of $^{22}Na^{+}$ flux across mouse neuroblastoma cells showed that pyrethroid insecticides have little effect when applied alone, but greatly enhanced the $^{22}Na^{+}$ influx induced by neurotoxins which activate sodium channels (Jacques *et al.*, 1980). However, repetitive depolarisations of a pyrethroid-treated squid giant axon preparation did not increase the number of modified channels (Weille *et al.*, 1988), and single channel recordings of sodium channels in mouse neuroblastoma cells have shown that the probability of pyrethroid modification is independent of the length of time the channel remains open (Holloway *et al.*, 1989).

These differences in the precise mechanism of action are yet to be resolved, but may be related to the wide range of pyrethroid structures examined. Evidence for this has been presented by Nishimura *et al.* (1989) who describe the mode of action of bioresmethrin and kadethrin, which are in fact structurally very similar. Their results suggest that bioresmethrin preferentially interacts with open channels, whereas kadethrin binds more easily to the closed configuration.

The fact remains that because most of the literature covering pyrethroid mode of action shows that the effects on sodium channels are stereoselective and can be detected at low concentrations, it is generally agreed that the voltage-sensitive sodium channel is the primary target site of pyrethroid insecticides.

1.2.2.2. Alternative target sites

Although there seems little doubt that the voltage-dependent sodium channel constitutes the principal site of action of pyrethroid insecticides, it is perhaps not surprising that such highly lipophilic molecules interact with other membrane bound proteins also, particularly at high concentrations. Although the toxicological significance of such interactions has yet to be fully elucidated, many authors believe that the diversity of *in vivo* symptoms associated with pyrethroid poisoning is difficult to attribute to a single lesion in neurological function, and so the evidence for the involvement of alternative or additional neuronal target sites is briefly reviewed here.

Other voltage-dependent channels

Pyrethroids have been shown to depress potassium currents in voltage-clamp experiments involving a number of invertebrate nerve preparations (Narahashi and Anderson, 1967; Wang *et al.*, 1972; Kiss, 1988; Omatsu *et al.*, 1988). This suppression of the outward potassium current in combination with the delay in sodium current inactivation may contribute to an increase in the depolarising after-potential which initiates the repetitive firing associated with most pyrethroids. However, the toxicological relevance is questionable, since the concentrations required to cause these effects are high, and potassium currents in the cockroach giant axon were unaffected by most pyrethroids tested, even at very high concentrations (Laufer *et al.*, 1985).

Direct pyrethroid action on calcium channels has yet to be shown conclusively. Very low concentrations of permethrin are capable of producing intense repetitive firing in neurosecretory cells of the stick insect (Orchard and Osborne, 1979). However, according to Sattelle and Yamamoto (1988), although the action potential of the peripheral neurosecretory cell body is calcium dependent, it has not been established

that the inward current through the axon is carried exclusively by calcium. Until the possibility of the presence of sodium channels in the neurosecretory axons is studied the site of pyrethroid action in this system will remain unresolved.

Clements and May (1977) showed that some pyrethroids induced substantial muscle contractions in locust leg preparations, even after neurally evoked contractions were blocked. They suggested a direct action on the muscle membrane, thereby implying the involvement of calcium channels. However, this observation has never been confirmed by subsequent work.

Receptor-operated channels

It was initially proposed that certain pyrethroids interact with the GABA receptor-channel complex after the observation by Gammon *et al.* (1982) that the benzodiazepine diazepam (an allosteric potentiator of GABA function) delays the symptomology of poisoning by type II (but not type I) pyrethroids in both mouse and cockroach (see Section 1.2.3 for type I and type II pyrethroids). This interaction was confirmed pharmacologically using radioligands specific for the GABA receptor-channel complex. For example, Lawrence and Casida (1983) showed that type II pyrethroids inhibit the specific binding of [^{35}S]TBPS, (a non-competitive antagonist of the GABA receptor) in rat brain synaptosomes. Type I pyrethroids were much less potent. Furthermore there was an absolute correlation between mouse toxicity and *in vitro* inhibition of binding with all toxic pyrethroids, but none of their non-toxic stereoisomers were effective inhibitors. Electrophysiological experiments on rat muscle fibres and vagus nerve are consistent with binding studies in that they show that deltamethrin (type II) appears to reduce the resting membrane chloride ion conductance (Forsaw and Ray, 1990). Type II pyrethroids also inhibit the GABA induced $^{36}Cl^-$ uptake into rat brain microsacs (Eldefrawi and Eldefrawi, 1988). Despite the high concentrations of pyrethroid normally required to induce the

responses, these lines of evidence indicate that the GABA receptor/chloride channel complex may be a key site for pyrethroid action. However, the experimental evidence is gained mainly from mammalian sources, and studies on invertebrate systems have usually proved contradictory to this view, which may reflect the growing evidence that there are pharmacological differences between vertebrate and invertebrate GABA receptors (see Sattelle and Yamamoto, 1988). For example, the crayfish stretch receptor organ can be experimentally manipulated so that the effects of pyrethroids on both sodium channels and GABA-activated channels can be monitored simultaneously. Deltamethrin was found to have effects on sodium channels at picomolar concentrations whereas the GABA response was unaffected at concentrations up to 1 micromolar (Chalmers *et al.*, 1987). Furthermore, GABA induced current in the cell body membrane of an identified cockroach motorneuron was unaffected at deltamethrin concentrations up to 0.5μM (Sattelle, 1988, see Sattelle and Yamamoto, 1988). This data suggests that receptors on the GABA complex are unlikely to contribute to the toxicological effects of pyrethroids in insects.

Pyrethroids do not cause significant displacement of [^{3}H]acetylcholine binding from eel electroplax membranes, but interactions with the nicotinic acetylcholine receptor channel must occur because pyrethroids (especially type I) inhibit the binding of radiolabelled histrionicotoxin, a non-competitive blocker of this channel, (Abassey *et al.*, 1983). However, a poor correlation between effectiveness at inhibiting [^{3}H]histronicotoxin binding and pyrethroid toxicity was noted in this study. Furthermore, allethrin (a type I pyrethroid) had no effect on the amplitude of miniature end plate potentials in frog neuromuscular preparations (Wouters *et al.*, 1977). Deltamethrin depressed the current associated with iontophoretic application of acetylcholine in identified *Helix* neurons (Kiss and Osipenko, 1991), but the pyrethroid concentrations required were very high (up to 100μM). Any effects on acetylcholine receptors are therefore probably secondary.

ATPases

Pyrethroids at high concentrations have been shown to inhibit Ca^{2+}-ATPase activity in squid nerves (Clark and Matsumura, 1982). In contrast, pyrethroids were shown to stimulate Ca^{2+}-Mg^{2+}-ATPase which had been purified from rabbit sarcoplasmic reticulum and reconstituted into artificial lipid bilayers(Jones and Lee, 1986). In this study most of the pyrethroids tested, including non-toxic isomers, stimulated ATPase activity to about the same extent. The authors concluded that this lack of stereoselectivity presumably demonstrates that the effects are non-specific.

Effects of pyrethroid on protein phosphorylation

Deltamethrin has been shown to enhance the release of radiolabelled neurotransmitter from synaptosomes caused by a pulse of veratridine or elevated K^+ concentration. This increase in activity coincides with a prolonged elevation of the phosphorylation levels of proteins associated with transmitter release (Clark and Matsumura, 1991). In rat brain synaptosomes, tetrodotoxin and verapamil (a calcium channel blocker) were only partially able to inhibit the deltamethrin induced increase in phosphorylation activity, and there is some evidence that deltamethrin causes a direct release of calcium ions from intracellular storage sites (Enan and Matsumura, 1991).

In intact lobster leg nerve preparations, deltamethrin was found to inhibit the phosphorylation of a 260 KDa protein, which was considered most likely to represent the α-subunit of the voltage-gated sodium channel (Miyazawa and Matsamura, 1990). This is an intriguing observation, especially in the context of other recent research. After isolation using molecular biology techniques and expression in *Xenopus* oocytes, the α-subunit of the Rat IIA sodium channel displays slow macroscopic inactivation, but co-injection with low molecular weight rat brain RNA restores

normal channel inactivation (Auld *et al.*, 1988). This implies the existence of an additional, smaller protein which is required for rapid inactivation. The proposition that this low molecular weight protein is a sodium channel ß-subunit seems unlikely, since expression of the Rat IIA α-subunit alone in Chinese hamster ovary (CHO) cells (which lack endogenous voltage sensitive sodium channels) leads to the expression of functional channels with normal inactivation kinetics (Scheuer *et al.*, 1990). One interpretation of this experimental finding is that an endogenous low molecular weight component that regulates sodium channel function is present in CHO cells but not in *Xenopus* oocytes. This leads to the interesting possibility that slowing of sodium channel inactivation caused by pyrethroids may be due in part to the disruption of a modulatory event in the functioning of some types of sodium channel, rather than a conformational change caused by pyrethroid binding to a site on the channel. This proposal is at present merely speculative.

In conclusion, although there has yet to be shown a conclusive correlation between changes in nerve membrane permeability and insect mortality, the prolongation of the sodium channel opening time can explain neurotoxic activity as manifested by repetitive firing, membrane depolarisation, conduction block and facilitation of neurotransmitter release. The action of pyrethroids on some of the alternative target sites studied may require some further evaluation, but in general, these other sites appear less sensitive than sodium channels, and there is no firm evidence that they contribute to the toxicological effects.

1.2.3. Type I and type II pyrethroids

The possibility of the existence of two distinct groups of pyrethroid insecticides with different modes of action was first raised by observations made during toxicity studies on mammals. Treatment of rats with natural pyrethrins and the early synthetic pyrethroids resulted in symptoms characterized by hypersensitivity to sensory stimuli

(Verschoyle and Barnes, 1972). In contrast, deltamethrin (the first synthetic compound containing an α-cyanophenoxybenzyl) caused a quite different sequence, characterized by excessive salivation and irregular convulsions (Barnes and Verschoyle, 1974). Subsequent studies have confirmed that in general, this difference in symptomology corresponds to the presence or absence of the cyano group in the pyrethroid structure (Verschoyle and Aldridge, 1980; see Fig. 1.1).

Clements and May (1977) demonstrated that in most cases, pyrethroids without a cyano group caused repetitive discharges in the locust peripheral nervous system, whereas those with the cyano group did not. Gammon *et al.* (1981) designated the non-cyano pyrethroids type I and the cyano pyrethroids type II because of the differences in action on the electrical activity in the cercal nerves of *Periplaneta americana* recorded both *in vivo* and *in vitro*. Their results were consistent with Clements and May (1977), type I pyrethroids caused trains of sensory spikes whereas type II pyrethroids did not. Similarly, allethrin (type I) caused repetitive discharges in isolated *Periplaneta* nerve cords, whereas cypermethrin (type II) had no observable effect (Scott and Matsamura, 1983).

The proposal that the two categories of pyrethroid acted at different target sites was supported by electrophysiological studies which showed that pyrethroid resistant *Spodoptera littoralis* with a reduced neronal sensitivity to permethrin (type I) showed no cross resistance to the type II cypermethrin (Gammon, 1980; Gammon and Holden, 1980). Similarly, DDT resistance in *Blatella* extends cross-resistance to type I pyrethroids, but not to the type II deltamethrin (Scott and Matsamura, 1983). Binding studies have demonstrated that cypermethrin is a far less potent displacer of [^{14}C]permethrin than permethrin in house fly head membrane preparations, suggesting a different site of action on the pyrethroid receptor (Chang and Plapp, 1983a). In fact, there is electrophysiological and pharmacological evidence that type

II pyrethroids exert their toxicological effects by acting on a different membrane protein altogether (see Section 1.2.2.2).

However, the distinction between the two groups of pyrethroid is by no means clear cut. Permethrin resistant *Musca domestica* displayed cross resistance to all pyrethroids tested, with in fact a much higher resistance ratio for type II than type I (Ahn *et al.*, 1988). It is also the case that careful scrutiny of reports claiming two distinct structure-related pyrethroid actions reveals that there are virtually always exceptions. For example, Scott and Matsumura (1983) showed that permethrin (type I) and fenvalerate (type II) both caused type I and II effects, and in fact stated that the complete assignment of a single type of action to any pyrethroid is not possible. Gammon *et al.* (1981), who originally proposed the classification system based on the presence or absence of a cyano group, conceded that the structurally type II pyrethroid fenpropathrin is classified type I based on its action on cercal sensory nerves.

The original supposition that type I and II pyrethroids had different mechanisms of action was based on phenomenological aspects of nerve electrical activity. Since then the effects of a wide range of pyrethroids on sodium currents has been analysed using the voltage-clamp technique. Vigverberg *et al.* (1983) noted that the rate of decay of sodium tail currents after step- repolarisation of myelinated nerve fibres was at least an order of magnitude slower in the presence of type II pyrethroids compared with type I. The differences in tail current decay within each group was much smaller. Lund and Narahashi (1983) performed similar experiments on crayfish giant axon, and showed that the time constants of the tail current decay could be arranged in a continuous spectrum, ranging from milliseconds for DDT and its analogues to hundreds of milliseconds for type I pyrethroids and to seconds for type II pyrethroids. The amplitude of type II tail currents were however, comparatively small. Since the tail currents represented current through toxin modified channels, the rate at which

they decay represents the rate at which the channels leave the modified open state. Thus the differences in nerve poison symptomology caused by different pyrethroid structures can be explained solely in terms of the kinetics of their interaction with sodium channels: type I pyrethroids produce large transient depolarising after-potentials which reach the threshold required for repetitive discharge, whereas type II pyrethroids modify a smaller fraction of sodium channels, but these close much more slowly, resulting in a progressive nerve depolarisation and conduction block. This interpretation has been confirmed for cockroach axonal preparations (Laufer *et al.*, 1984).

1.3. kdr *resistance*

Insecticide resistance can be described as the ability of a strain of insects to develop tolerance to a chemical applied in a dose which would be toxic to the majority of the population of that species. Of the several types of resistance which are known to exist, there are two main mechanisms which affect susceptibility to pyrethroids. One of these involves an increased capacity to detoxify the insecticide by metabolism. This mechanism is well characterized (see Oppenoorth, 1985 for review) and can be overcome (at least in principle) by the addition of metabolic inhibitors to the insecticide before application. So called "knockdown resistance" (*kdr*), which is the other main category of tolerance to pyrethroids, refers to an insensitivity at the level of the nervous system and is the cause of growing concern because of its apparent increasing prevalence and the elusiveness of the mechanisms involved.

Strictly speaking *kdr* refers to a well characterized cross-resistance between DDT and pyrethroids found in certain strains of the housefly (*Musca domestica*), but the term is increasingly used in the literature to describe any resistance factor to pyrethroids in any species which cannot be attributed to an increase in metabolic detoxification.

Busvine (1951) first described the phenomenon when he noted that a strain of houseflies had developed cross-resistance between DDT, DDT analogues and pyrethrins which could not be explained in terms of metabolism or decreased rates of penetration. Detailed genetic investigation showed that this pyrethroid and DDT tolerance (*kdr*) was caused by an identical resistance factor (Farnham, 1977) and it is quite conceivable that the rapid development of resistance to a variety of synthetic pyrethroids in Lepidopteran field populations (Lui *et al.*, 1981; Gunning *et al.*, 1984) has been facilitated by previous exposure to DDT.

1.3.1. Determination of kdr *resistance*

One of the main criteria for establishing the presence of *kdr* resistance is the direct demonstration of nerve insensitivity to pyrethroids, since simple dose related toxicity studies may be complicated by a number of other resistance factors, such as increased metabolism, a decrease in cuticular penetration, an increased rate of excretion or perhaps an ability to partition the pyrethroid into unaffected tissues such as fat body. To this end, surprisingly few neurophysiological assays have been developed. Salgado *et al.*, (1983a) showed that pyrethroids cause a large increase in the frequency of spontaneous transmitter release in larval *Musca domestica* neuromuscular preparations due to a sodium channel mediated depolarisation of the nerve terminal. They demonstrated that compared to susceptible strains, preparations of *kdr* larvae required much higher concentrations of pyrethroid to induce this response. Pyrethroid application to the exposed thoracic ganglion of adult houseflies causes "uncoupling" of action potentials from paired flight muscles, which normally fire in synchrony. This is thought to arise from a disruption of the CNS pathway involved in the initiation and maintenance of motor pattern. *Kdr* flies require longer exposure times and higher concentrations to initiate this response (Miller *et al.*, 1979). Similarly, repetitive firing caused by permethrin application to a housefly femur motor nerve preparation is considerably delayed in *kdr* flies compared to a susceptible

strain. Pretreatment with oxidase and esterase inhibitors did not alter the latency of appearance or firing frequency. Cuticular penetration of [^{14}C]permethrin was similar in both *kdr* and susceptible strain preparations (Ahn *et al.*, 1988). Increase in firing rate could be elicited in abdominal nerves of susceptible houseflies by permethrin concentrations as low as 0.1 pM. The *kdr* preparation did not respond until the concentration was increased by more than 10,000-fold (Gibson *et al.*, 1988). A higher concentration of fenvalerate was required to elevate firing frequency in larval nerve cord preparations of a resistant strain of *Plutella xylostella* compared to a susceptible strain (Hama *et al.*, 1987). Ahmad *et al.* (1989) have characterized a pyrethroid induced response in the firing frequency of peripheral nerves of *Heliothis armigera*, and shown differences in a pyrethroid resistant strain from Thailand.

1.3.2. Kdr *resistance mechanisms*

Although the preparations described above may demonstrate the existence of a nerve insensitivity factor, they give little indication as to the mechanism employed. It seems likely that a modification in sodium channel structure, density, or subtype is involved, since the voltage sensitive sodium channel appears to be the primary site of pyrethroid action (see Section 1.2.2.1). The limited evidence for such alterations is reviewed below.

1.3.2.1. Reduction in sodium channel density

Chang and Plapp (1983b) found that head membranes from *kdr* houseflies had a lower binding capacity for [^{14}C]permethrin than from a susceptible strain, suggesting a reduced density of pyrethroid receptors in resistant flies. This evidence was later substantiated by Rossignol (1988) who demonstrated a 40% reduction in [^{3}H]saxitoxin binding (and therefore presumably sodium channels) in *kdr* flies compared with a susceptible strain. Compared with wild type, the *Drosophila* mutant

nap^{ts} possesses a reduced number of [^{3}H]STX binding sites by about 40% (Jackson *et al.*, 1984), and this *nap*^{ts} mutation confers some resistance to fenvalerate and permethrin (Kasbekar and Hall, 1988). Furthermore, these authors showed that pre-feeding wild type *Drosophila* with sub-lethal doses of tetrodotoxin (which presumably blocks a fraction of the sodium channels) also produces a pyrethroid resistant phenotype.

Exactly why a reduced number of sodium channels should convey pyrethroid resistance is not quite clear, although most authors who describe the phenomenon suggest that a decrease in the number of channels available for pyrethroid modification must necessarily result in a reduction in the effectiveness of a given dose. It is known that only a very small proportion of sodium channels need be activated by a pyrethroid insecticide to produce the characteristic neurophysiological effects of poisoning (Lund and Narahashi, 1982). If it is the case that an absolute number of channels must be modified, then a reduction in the size of the channel population would mean that a higher dose of insecticide would be required to cross this threshold. This view is supported by the fact that the *Drosophila* mutant *nap*^{ts} is much more resistant to feeding with the sodium channel activator veratridine than wild type (Hall, 1986), and larvae of *kdr* houseflies are 16 times more resistant to injection with aconitine (which has a similar action to veratridine) in comparison to susceptible flies (Bloomquist and Miller, 1986). However, using the same logic, a greater susceptibility to sodium channel blockers would be expected, and no difference in sensitivity to tetrodotoxin was observed between *kdr* and susceptible housefly larvae (Bloomquist and Miller, 1986). Furthermore, Grubs *et al.* (1988) reported that they could detect no difference in sodium channel density between *kdr* and susceptible strains of housefly, suggesting the results of Rossignol (1988) may be due to artefact. It is also worth pointing out that on a theoretical basis, it seems unlikely that a reduction in sodium channel density of approximately 50% (Chang and Plapp, 1983b; Rossignol, 1988) could account for more than a two-fold increase in

resistance, as opposed to the 21 to 28-fold difference observed for deltamethrin in *kdr* houseflies (Bloomquist and Miller, 1986; Grubs *et al.*, 1988; Rossignol, 1988). It remains unresolved whether the *kdr* trait in houseflies involves a reduction in channel density.

Another conceptually plausible mechanism for *kdr* resistance due to reduced sodium channel numbers could involve the rapid "down regulation" of sodium channels on exposure to pyrethroid, although no evidence has yet been presented to support this. Such a modification in channel number is observed in cultured chick muscle cells (Bar-Sagi and Prives, 1985) and cultured rat brain neurons (Dargent and Couraud, 1990) subjected to chronic exposure to batrachotoxin and other sodium channel activators. Measurement of surface sodium channels by [^{3}H]STX binding and $^{22}Na^{+}$ uptake experiments show a decrease of 75% for chick muscle and 50% for rat brain neurons, with a half life of 3-6 hours and 15 hours respectively. A similar mechanism in pyrethroid resistant insects would need to operate much more rapidly in order to prevent knockdown, but this fact does not preclude that such a mechanism is possible, and it could be easily tested experimentally.

1.3.2.2. Modification of sodium channel structure

Despite the fact that it is generally accepted as the most plausible explanation, there is remarkably little evidence to substantiate the proposal that *kdr* resistance is conferred by an alteration in sodium channel structure, thereby preventing the binding of pyrethroid molecules to their site. An analysis of the influence of pyrethroids on macroscopic and single channel sodium currents using neuronal tissue from susceptible and *kdr* insects may help to localise the site of *kdr* insensitivity to the sodium channel. This, however, would still not offer conclusive proof of a *kdr* structural change in the sodium channel protein, since the altered gating properties could result from an altered channel environment. In fact, Chialiang and Devonshire

(1982) have identified changes in the phospholipid membranes of pyrethroid resistant insects. *Kdr* resistance may therefore be due to the impaired partitioning of the pyrethroid molecule through the modified lipid phase to its site of action, thus otherwise normal channels may appear to be altered. To overcome this problem, the complicated procedure of sodium channel purification and reconstitution into artificial lipid bilayers would have to be undertaken.

Conclusive evidence of an altered sodium channel receptor may come from the application of molecular biology techniques to clone the sodium channel gene in both susceptible and resistant strains, and compare the deduced amino acid sequences.

Whatever the nature of *kdr*, the developing resistance to a broad spectrum (if not the entire class) of pyrethroids at the site of action is an alarming prospect, and the elucidation of the precise molecular mechanism involved will be an important landmark in the combat of insecticide resistance. Otherwise, the pyrethroid insecticides may rapidly become impotent.

1.4. Natural toxins which act on the voltage-sensitive sodium channel

Sodium channels play a fundamental role in the generation and propagation of the nerve impulse throughout the animal kingdom. It is therefore perhaps not surprising that some of the most potent natural toxins known exert their effects by modifying the properties of this channel. The high affinity and specificity of action of these toxins for their receptor sites make them excellent tools to probe the structure and function of the sodium channel. There is a very large body of literature concerned with the interaction of these toxins with sodium channels, and there are numerous comprehensive reviews which cover this topic (see Catterall, 1980, 1984, 1986;

Strichartz, 1986; Strichartz *et al.*, 1987; Barchi, 1988). The main action of the various categories of toxin will be briefly summarised here.
The toxins have been categorised on the basis of their site of action on the channel protein molecule (Catterall, 1980). At present, four such sites are widely recognised, although others are now becoming apparent (see Fig. 1.2).

The so called guanidinium toxins tetrodotoxin (TTX) and saxitoxin (STX) are isolated from the ovaries of the puffer fish (several species of *Tetrodontidae*) and the dinoflagellates of the genus *Gonyaulax*, respectively. These toxins bind to a receptor site (site 1) at the external surface of the sodium channel, thus rendering it completely impermeable to sodium ions. The first description of this mechanism of action (Narahashi *et al.*, 1964) effectively proved the hypothesis of Hodgkin and Huxley (1952) that sodium permeability across the nerve membrane during the action potential is selectively mediated via discreet transmembrane protein channels. The small current associated with the conformational change during channel activation is unaffected in the presence of TTX (Armstrong and Bezanilla, 1973), and binding is independent of voltage (Catterall *et al.*, 1979). Therefore this blocking action is usually thought of as being the result of a simple physical occlusion at the mouth of the channel, with the guanidinium group entering the channel, but the rest of the molecule being too wide to pass through (Kao and Nishiyama, 1965). This idea is plausible, since guanadinium is a permeant cation through sodium channels. However, there has never really been any firm evidence to support this concept, and recent experiments suggest TTX may exert its action by binding some distance from the channel entrance and blocking it in an allosteric manner (Green *et al.*, 1987).

Receptor site 2 binds several lipid soluble toxins including grayanotoxin and the alkaloids veratridine (VTD) aconitine (ACN) and batrachotoxin (BTX). Of these, BTX, produced by several species of Columbian tree frogs (*Phyllobates* spp.) has been the most extensively studied. Modification by BTX causes sodium channels to

open persistently by preventing channel inactivation, and shifting the voltage dependence of activation to more hyperpolarising potentials. Binding to closed channels is extremely slow compared to open channels, which reflects the differences in the affinity of BTX for these channel configurations. This phenomenon has been exploited in the pharmacological study of sodium channel activators which bind to other sites on the sodium channel, by measuring their effects on the binding of radiolabelled BTX. Apart from the actions already described, BTX also increases the permeability of neuroblastoma cell sodium channels to other cations (Frelin *et al.*, 1981) and decreases single channel conductance (Quandt and Narahashi, 1982). The fact that BTX affects the gating, ion selectivity and conductance of the sodium channel may indicate that binding to site 2 considerably distorts the normal structure of the channel.

The competitive interaction of the alkaloid toxins at site 2, which was first demonstrated with ion flux experiments, has been confirmed with displacement studies of the radiolabelled form of BTX (Catterall *et al.*, 1981). Although recent evidence demonstrates that the detailed mechanism underlying their effects differ, the main distinctions between the actions of the alkaloid toxins appear to be quantitative rather than qualitative, relating to the number of channels affected at saturating concentrations. Thus, BTX is described as being a full agonist, being capable of opening all sodium channels whereas VTD and ACN are partial agonists, and can only open a proportion of the channels (Catterall, 1975, 1977).

Neurotoxin receptor site 3 binds a number of basic polypeptide toxins, the best characterized of which are the α-scorpion toxins (α-ScTX) isolated from the venom of North African scorpions. Of these, the most potent is contained in the venom of *Leiurus quinquestriatus quinquestriatus*. The action of α-ScTX is to specifically slow the rate of sodium channel inactivation (Catterall, 1979). Binding is highly voltage dependent, in that the affinity falls at a steady rate as the membrane potential becomes

less negative, as demonstrated in studies with ion flux (Catterall *et al.*, 1976) voltage clamp (Catterall, 1979) and binding using radiolabelled α-ScTX (Catterall, 1979; Catterall *et al.*, 1976).

Polypeptide toxins purified from the nematocysts of certain sea anemones have very similar effects to those of α-ScTX and appear to bind to the same site. Of these the best characterized is anemone Toxin II (ATX-II) from *Anemonia sulcata.* This toxin slows down sodium channel inactivation in the crayfish giant axon (Romey *et al.*, 1976). Depolarisation of the axons by current injection reduces the development of toxicity (as measured by the prolonged falling phase of the action potential) in the presence of either *Lqq* α-ScTX or ATX-II, indicating the action is voltage dependent (Warahashi *et al.*, 1981). ATX-II also competitively displaces the specific binding of radiolabelled *Lqq* α-ScTX (Catterall and Beress, 1978; Catterall, 1979). Therefore, despite the fact that none of the anemone toxins has sequence homology with the α-scorpion toxins, they appear to bind to a common (or at least overlapping) receptor site and affect sodium channel function in the same way.

Site 3 toxins interact cooperatively with site 2 to enhance the effects of BTX and VTD (see chapter 4).

Site 4 binds the ß-scorpion toxins, mainly from the new world species of scorpion *Centruroides* and *Tityus.* Classically, these toxins specifically affect sodium channels by shifting the voltage-dependence of activation to more negative membrane potentials (Cahalan, 1975). These actions are, however, rather complex and the mechanisms of action are not as yet completely clear (see Strichartz *et al.*, 1987; Strichartz, 1986).

There are numerous other natural toxins which are known to affect sodium channel function. Although they may have the same effect as the well characterized toxins

already described, their interaction with representative toxins from sites 1-4 has shown that they do not belong to any of these categories.

The μ-conotoxins are polypeptides purified from the venom of the piscivorous marine snail *Conus geographus*. This group of toxins preferentially blocks sodium channels of electric eel electroplax in a manner similar to TTX and STX (Kobayashi *et al.*, 1986). μ-Conotoxins inhibit [^{3}H]STX binding to muscle tissue from a number of sources, in a manner consistent with direct competition for a common binding site, but are ineffective as [^{3}H]STX binding displacers in tissues of neuronal and cardiac origin (Moczydlowski *et al.*, 1986; Ohizumi *et al.*,1986). These results suggest that the μ-conotoxins bind to a site which overlaps site 1 in the muscle sodium channel which is absent or altered in nerve sodium channels.

Brevetoxins are polyethers isolated from the marine dinoflagellate *Ptychodiscus brevis*. They exert their effects by activating sodium channels at normal membrane potentials. [^{3}H]brevetoxin binding is unaffected in competition experiments using representative toxins specific for sites 1 to 4 (Poli *et al.*, 1986) and so an additional site has been proposed. Ciguatoxin has a similar mode of action (Strichartz *et al.*, 1987) but has not yet been characterized with respect to the other binding sites.

The tropical frog alkaloid pumiliotoxin-B has recently been shown to increase sodium flux into mammalian brain synaptosomes (Gusovsky *et al.*, 1988). This effect was synergised by α-ScTX, ß-ScTX and brevetoxin. Pumiliotoxin-B had no effect on the binding of [^{3}H]STX or [^{3}H]BTX-B. Therefore, it appears to activate sodium influx by interacting with yet another site on the sodium channel.

In mouse neuroblastoma cells, the polypeptide goniopora toxins slow down sodium channel inactivation and enhance the effects of VTD in a manner similar to site 3

toxins. However, it does not inhibit the binding of radiolabelled α-ScTX, and so probably represents a new site (Gonoi *et al.*, 1986).

1.5. Interaction of toxins with insect sodium channels.

Most of the extensive literature covering the action of these natural toxins concerns voltage-dependent sodium channels of either cephalopod giant axons or preparations derived from mammalian excitable tissues. By comparison, very limited research has been directed towards the characterisation of toxin recognition sites on insect sodium channels. The electrophysiological analysis of such interaction has been confined almost exclusively to isolated axons of giant interneurons of *Periplaneta americana.* Other pharmacological studies have relied on neuronal membrane and synaptosome preparations derived chiefly from fly, locust and cockroach.

The limited available information would suggest that in general, the sodium channel toxin recognition sites in insects are similar to their vertebrate counterparts. Thus, TTX and STX (site 1) selectively inhibit sodium channel conductance in cockroach giant axons (Sattelle *et al.*, 1979; Pelhate and Sattelle, 1982). The binding of [^{3}H]STX also shows similar properties to vertebrate preparations (see Chapter 3). Aconitine (site 2) modifies a fraction of axonal sodium channels so that they do not inactivate (Pelhate and Sattelle, 1982). BTX, VTD and ACN mediate TTX sensitive $^{22}Na^+$ uptake into cockroach synaptosomes (Dwivedy, 1988). ATX-II and an α-ScTX isolated from *Androctonus australis* (site 3) cause a slowing of sodium channel inactivation in cockroach giant axons (Pelhate and Sattelle, 1982).

More extensive attention has been drawn to the so called insect-selective neurotoxins. Purification of the various protein components of North African scorpion venom has revealed the presence of several neurotoxic fractions. On the basis of injection

studies using blowfly larvae and mice, it was shown that certain fractions were highly specific towards either insects or mammals (see Zlotkin, 1986). The mammalian specific toxins are the α-ScTXs already described, which bind to receptor site 3 on the sodium channel. *Androctonus australis* insect toxin (AaIT) was found to have a similar mode of action to the site 3 toxins, in that the primary effect is the slowing down of sodium channel inactivation (Pelhate and Zlotkin, 1982). However, radioiodinated AaIT binds specifically to locust synaptosomes in a manner which is unaffected by α-ScTX and ATX-II, indicating that it recognises a site which is independent of site 3. ß-ScTX II from *Centruroides* (site 4) is also ineffective on AaIT binding (Gordon *et al.*, 1984). As well as the "excitatory" insect toxins, some North African scorpion venoms contain "depressant" insect- selective toxins also. The depressant insect toxins BjIT2 and LqqIT2 have been isolated from the venoms of *Buthotus judaicus* and *Leiurus quinquestriatus* respectively (Lester *et al.*, 1982; Zlotkin *et al.*, 1985). These toxins act primarily by suppressing sodium current in cockroach giant axon. Both the excitatory and depressant toxins from *Leiurus* venom displace [^{125}I]AaIT from insect synaptosomes with high affinity, and so may share a common binding site (Zlotkin *et al.*, 1985).

Insect specific scorpion toxins may prove to be valuable tools in the clarification of the structure and function of the insect sodium channel. However, certain aspects of this research require clarification. For example, the toxicological significance of excitatory and depressant toxins from the same venom acting competitively at the same binding site is unclear. It is also interesting to note that the mammalian Toxin II from *Androctonus australis* (AaHII, an α-ScTX) was completely inactive on blowfly larva by injection assay (see Zlotkin, 1986), and radioiodinated AaHII did not bind to locust synaptosomal preparations (Gordon *et al.*, 1984). AaHII nevertheless caused classical neurotoxin site 3 effects on the voltage-clamped cockroach giant axon (Pelhate and Zlotkin, 1982). This may represent real differences in sodium channel structure between *Periplaneta* and the other two insect species tested. However, it

would be interesting to test the effects of the insect-specific toxins on *in vitro* vertebrate preparations, which to date does not seem to have been done.

The work described in this thesis has employed a variety of techniques to study the functional and structural properties of the voltage-sensitive sodium channel of the Tobacco hornworm, *Manduca sexta* (L.). This insect has been widely used as a convenient experimental model for Lepidopteran pests. Characterisation of the sodium channel from a Lepidopteran species has significant implications for the insecticide industry, but has not previously been attempted.

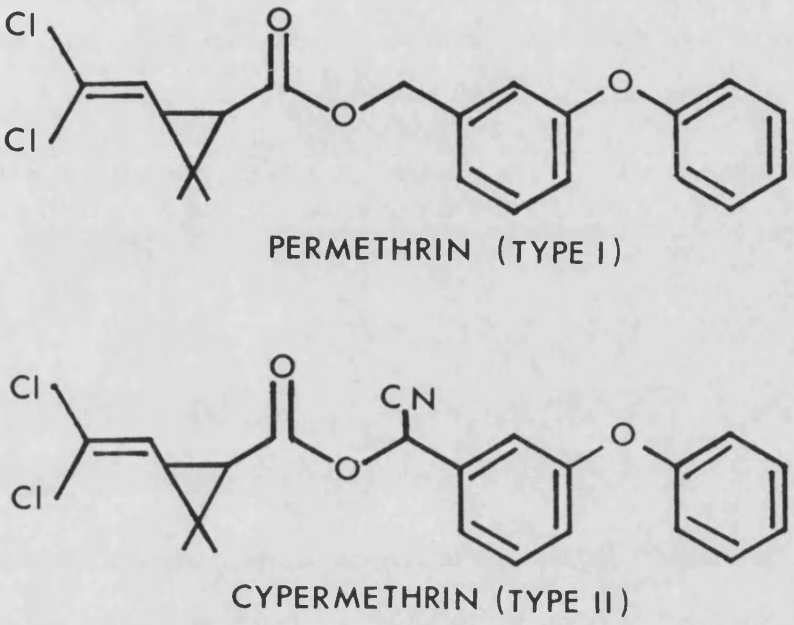


FIGURE 1.1. *Chemical structure of permethrin and its α-cyano derivative cypermethrin.* Despite the similarity in structure cypermethrin is approximately 10-fold more potent as an insecticide and causes different symptoms of toxicity, although the primary mode of action is probably the same (see text).

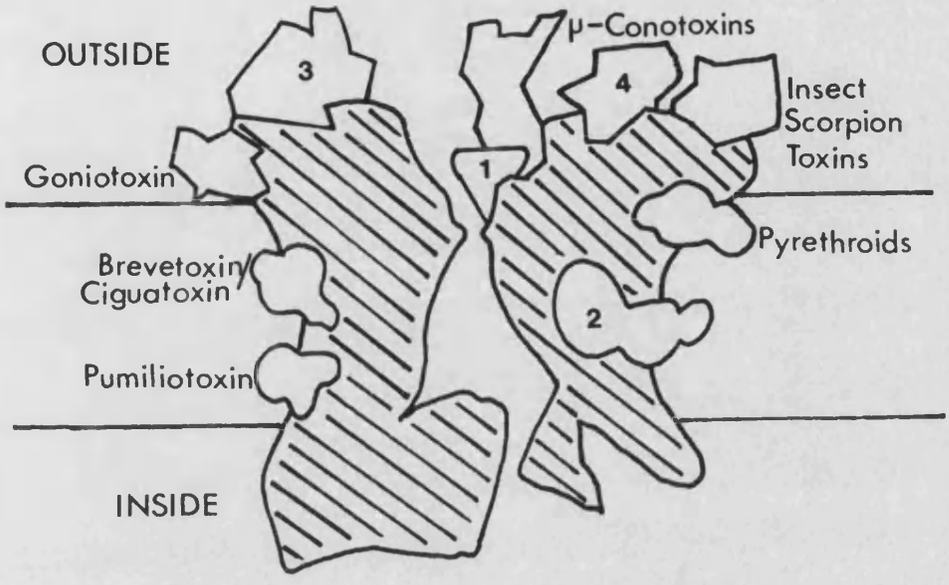


FIGURE 1.2. *Diagrammatic representation of the toxin binding sites on the voltage-dependent sodium channel.* The main receptor sites are numbered according to the scheme of Catterall (1980). The relative positions of the sites remain largely unknown, although the guanidinium (site 1) and polypeptide toxins bind to the extracellular portion of the channel, while the lipid soluble toxins are likely to bind at the membrane-channel interface. Modified from Hille (1984).

CHAPTER 2

THE SODIUM CHANNEL AS THE TARGET OF PYRETHROID ACTION ON THE NEUROMUSCULAR JUNCTION OF LEPIDOPTERAN LARVAE

2.1. Introduction

There is a general consensus that the principal target site for the pyrethroid insecticides is the voltage-sensitive sodium channel, although it also recognised that pyrethroids may exert an effect on other voltage-sensitive and ligand-gated channels, as well as other physiologically important membrane-bound proteins (see General Introduction, Section 1.2.2). At present it appears that these alternative targets are less sensitive to pyrethroids than are sodium channels, but it remains possible that one or more of the multiple target sites may constitute the major cause of pyrethroid toxicity in some insect species.

Most of the evidence for the sodium channel as the pyrethroid target has been obtained from preparations derived from a very restricted range of insect species, namely *Musca domestica* neuromuscular junction (Salgado *et al.*, 1983a,b) and *Periplaneta americana* giant axons (Laufer *et al.*, 1984, 1985). All other evidence is derived from non-insect preparations, for example cephalopod giant axons (e.g. Lund and Narahashi, 1981a,b) amphibian node of Ranvier (Vigverberg *et al.*, 1982b, 1983) and mammalian neuroblastoma cells (Jacques *et al.*, 1980; Yamamoto *et al.*, 1983). Therefore, it seemed worthwhile to examine the involvement of sodium channels in

pyrethroid toxicity to Lepidopteran larvae, especially as these insects are major economic targets of pyrethroids in agriculture.

Several studies have illustrated the sensitivity of the neuromuscular junction to pyrethroid action, and have implicated this site as the primary lesion in pyrethroid toxicity for a number of preparations (Adams and Miller 1979; Salgado *et al.*, 1983a,b; Irving, 1984). Synaptic activity can be monitored postsynaptically at the neuromuscular junction by recording the activity of miniature excitatory post synaptic potentials (mepsps) in insect preparations. Pyrethroid insecticides and sodium channel activating neurotoxins cause a massive discharge of mepsps in the housefly larva neuromuscular junction preparation (Salgado *et al.*, 1983a,b). These studies showed that the effects of these agents were due to a depolarisation in the presynaptic terminal, and were reversed in the presence of tetrodotoxin (TTX). This indicated that the increase in mepsp frequency was mediated by prolonged opening of the nerve terminal sodium channels, which is the accepted primary mode of pyrethroid action.

The effects of pyrethroids together with known sodium channel toxins on a *Manduca sexta* neuromuscular preparation was therefore studied, in order to investigate whether the mode of action of pyrethroids could be localised to the level of the voltage-sensitive sodium channel. The preparation could then lead to an assay for the detection and quantification of *kdr*-like resistance.

2.2 Methods

2.2.1 Chemicals

The pyrethroid insecticides permethrin and its α-cyano derivative cypermethrin were a gift from Dr. Philip Jewess (Shell Research Ltd, Sittingbourne). Tetrodotoxin,

veratridine, L-glutamate and pronase were purchased from Sigma Chemical Company. The insecticides were dissolved in acetone to a concentration of 10^{-3} M, which was diluted in saline to 10^{-5} M and then 10^{-8} M, giving a final acetone concentration of 0.001%. This concentration of acetone was added to all equilibration and wash salines, although preliminary investigations had shown that concentrations of up to 0.1% had no detectable effect on the preparation. Stock solutions of pyrethroid (10^{-3} M in acetone) were stored at -20°C and discarded after one month.

2.2.2. Preparation

Early third instar *Manduca sexta* larvae were used as experimental animals. Initial experiments had shown that the muscles of smaller larvae were too fragile to allow for long term intracellular recording, while mepsps could not normally be recorded from fourth or fifth instar larvae, probably due to the larger muscle dimensions and hence lower input resistance. Larvae were dissected in a 50 mm plastic petri-dish containing Sylgard resin (Dow Corning), which had been deliberately set at an angle (see Fig. 2.1A). A dorsolongitudinal incision was made under physiological saline (see Appendix I) and the cuticle was pinned out. The gut and salivary glands were removed, revealing the underlying nervous system and body-wall musculature. The thoracic region of the preparation, which was used for electrophysiological measurements, was virtually free of fat deposits at this stage of development.

2.2.3. Saline delivery

Saline was perfused directly over the preparation at a constant rate of 0.5 ml/min by using the apparatus represented in Fig. 2.1A. This method of saline delivery had several advantages over static bath application. The rapid turnover of the small volume of saline in contact with the preparation eliminates the possibility of effects caused by the accumulation of metabolites or the run-down of ionic gradients. A

change in saline composition is easily achieved by transferring the siphon-tube to an adjacent reservoir. The substitution over the preparation is almost immediate, and the air bubble preceding the new solution produces a recording artefact which acts as a marker without disturbing the recording electrode. The rate of flow can be easily changed by adjusting the height of the reservoir, and estimated by recording the volume of run-off with a measuring cylinder. Aeration of the saline was found to be unnecessary.

2.2.4. Electrophysiological measurements

Muscle fibres on either side of the third thoracic ganglion were used for intracellular recording (see Fig. 2.1B). Muscle potentials were measured using 3 M KCl-filled glass microelectrodes of 5-10 Mohm resistance, connected through a Neurolog NL102 DC preamplifier and NL125 filters, and displayed on a Gould OS4200 dual-beam digital-storage oscilloscope. The signal was split between both channels of the oscilloscope to allow for simultaneous monitoring of mepsps and membrane potential. Permanent recordings were made on photographic paper of a Medelec oscilloscope either directly, or recorded first on a magnetic tape-recorder (Data Aquisition) and played back later.

Excitatory synaptic potentials were evoked in the muscle by stimulating the motorneurons via a suction electrode applied to the connectives of the nerve cord in the segment anterior to the muscle. Isolated voltage pulses were generated by a custom-built stimulator. The excitatory synaptic input led to a regenerative response in the muscle. The evoked muscle potential, which overshot zero potential, produced a twitch contraction. Repetitive stimulation usually caused irreversible depolarisation and loss of signal, presumably due to microelectrode damage of the muscle membrane. Although the use of hypertonic (400 mM sucrose) saline (Hodgkin and Harowicz, 1957) prevented the contractions, this increased osmolarity was always

associated with poor membrane potentials and preparations which were viable for much shorter periods than was necessary for most experiments. Attempts to minimise muscle tear damage using "floating" electrodes were only occasionally successful. Therefore, the preparation was stimulated as infrequently as possible. Control experiments had shown that no damage occurs in preparations in which evoked muscle potentials were kept to a minimum.

The preparation was allowed to equilibrate for 30 minutes after dissection, after which time the response from a muscle fibre with membrane potential of -45 mV to -55 mV was monitored for a further 30 minutes to ensure uniformity of mepsp rate, membrane potential (E_m) and evoked response before proceeding with the experiment. All experiments were carried out at room temperature (20-22°C).

On completion of each experiment, all apparatus which had been in contact with pyrethroid was discarded, to avoid the possibility of cross contamination.

2.3. Results

2.3.1. Effect of Pyrethroid

Fig. 2.2 illustrates the stability of the control preparation, with mepsp frequency and evoked muscle potential remaining stable for several hours. In contrast, Fig. 2.3 shows the typical response to treatment with pyrethroid. This increase in membrane noise was evoked by both permethrin and cypermethrin, but never occurred spontaneously in control preparations. This increase in rate of mepsps could not be measured quantitatively, since individual potentials could not normally be resolved into discrete units, but rather summation of mepsps appeared as an increase in the

amplitude of the control level. However, the onset of the response occurred abruptly (often within seconds) and unambiguously.

The level of mepsp increase in frequency was indistinguishable over a range of pyrethroid concentrations spanning several orders of magnitude. Rather, differences in pyrethroid concentration were associated with the exposure time required until the onset of the response, and the number of fibres affected in a random sample. Preliminary investigations had shown that pyrethroid at concentrations as low as 10^{-10} M could induce the characteristic increase in mepsp frequency in 30-50% of muscle fibres (in a sample of 20) after 45 minutes. 10^{-8} M pyrethroid was routinely used in this study, since this concentration produced the effect in most muscle fibres within 15 minutes, thus allowing continuous monitoring of a single muscle fibre during the exposure time.

The pyrethroid induced increase in mepsp frequency was sustained for up to 1-2 hours, before slowly declining to subnormal levels over the subsequent 2 hours or so. After this time, the muscle action potential could no longer be evoked by nerve stimulation.

The effects of permethrin and cypermethrin could not be distinguished using the criteria used here. Both produced similar results (as described above), and both also caused bursting activity (high frequency multiple action potentials) in some fibres, particularly during initial exposure to the preparation.

2.3.2. Effect of Veratridine (VTD)

Veratridine had similar effects to those produced by pyrethroids, with certain distinguishing differences. The response was characterised by a flurry of mepsps which occurred after between 30 seconds and 5 minutes of exposure to 10 μM VTD

(Fig. 2.4). The increase in mepsp frequency appeared similar to that produced by pyrethroids, but it was relatively transient, lasting only a few minutes before declining to subnormal levels, after which time the evoked action potential could not be elicited. As with pyrethroids, the effects were not reversible by removal of VTD, and membrane potentials remained fairly constant throughout the experiment.

2.3.3. Effect of Tetrodotoxin (TTX)

The addition of 1 μM TTX to the perfusing saline immediately reversed the effect of pyrethroid in a proportion (approx. 35%) of the muscle fibres (Fig. 2.5). Increased mepsp activity was resumed within seconds of TTX removal, indicating that pyrethroid was mediating its effects by modification of presynaptic voltage-gated sodium channels. However, in many fibres, this effect of TTX was either absent or less dramatic and required prolonged exposure of up to 30 minutes to occur. Attempts to identify TTX- sensitive fibres proved unsuccessful. However, pre-treatment of the preparation with 0.01 mg/ml pronase for 5 minutes increased the proportion of TTX-sensitive fibres to about 60%. Increasing the pronase concentration to 0.1 mg/ml caused a massive contracture, followed by the virtually complete elimination of mepsps and evoked action potential, although membrane potentials remained normal. The action of pronase was shown to be presynaptic, since the membrane potential readily depolarised and repolarised on addition and removal of 1 mM L-glutamate. Pre-treatment with collagenase or trypsin up to 1 mg/ml had no effect on the normal preparation.

Some fibres could be protected against the action of VTD by the simultaneous perfusion of 1 μM TTX for up to 90 minutes (longest time tested). Removal of the TTX in these cases led to the immediate onset of the response described for 10 μM VTD alone. However, as with pyrethroids, protection from these effects by TTX was not consistent. In those cases where protection was achieved, it was not possible to

accurately assess the effects of re-applying TTX due to the rapid and irreversible nature of the response to veratridine.

2.3.4. Simultaneous application of pyrethroid and VTD

VTD and cypermethrin were made up to double the normal stock concentration to allow simultaneous perfusion of the drugs without increasing acetone concentration to more than normal control levels. When both agents were applied simultaneously, the response was identical to that produced by cypermethrin alone, characterised by a sustained increase in mepsp rate lasting much longer than the increase in frequency which would have been seen had VTD been used alone (Fig. 2.6). No synergism or antagonism were detected. The addition of VTD to a preparation after cypermethrin induced increase in mepsp activity had declined to subnormal levels produced no further effect (Fig. 2.7). Similarly, cypermethrin had no effect on the low level of activity associated with prolonged VTD exposure. Application of a pulse of 1 mM L-glutamate after prolonged exposure of either pyrethroid or veratridine (or both) caused immediate depolarisation and repolarisation of the muscle cell.

2.4. Discussion

The experiments reported here provide evidence that pyrethroids cause transmitter release from the presynaptic ending of motor nerve axons in *Manduca* larvae by their action on a TTX-sensitive site, probably the voltage sensitive sodium channel.

The mepsps recorded from control preparations of *Manduca* muscle fibres bear a close resemblance to those reported for other insect species. The variability in time course and amplitude of these randomly occurring spontaneous potentials was first reported by Usherwood (1961) in leg muscles of *Blaberus giganteus* and *Schistocerca*

gregaria, and are also evident in larval preparations from a range of insects (Irving, 1984). These fluctuations are presumably a consequence of the multi-terminal innervation of insect muscle, with synapses at different distances relative to the recording electrode. Multi-terminal innervation is evident in ultrastructural studies in *Manduca sexta* larval muscles (Shaner and Rheuben, 1985).

An increase in mepsp frequency induced by the presence of pyrethroid was first noted by Clements and May (1977) and is a common feature among all insect neuromuscular preparations so far tested. The latency of onset of this response is concentration dependent, which presumably reflects a more rapid rate of pyrethroid partitioning to the active site at the higher concentrations. The dose dependency of this response was studied in more detail by Salgado *et al.* (1983a) and is the basis for an assay to determine the sensitivity of a range of insect species to different pyrethroids (Irving 1984; Irving and Frazer 1984). Salgado *et al.* (1983a) demonstrated that the increased mepsp frequency was a direct consequence of nerve terminal depolarisation, but the reason why the elevated level is achieved as a sudden, stepwise change rather than as a gradual response remains unclear, and indicates some type of threshold mechanism.

VTD has been shown in a large number of studies to mediate its toxic effects by the persistent activation of the voltage-gated sodium channel (Catterall, 1980; see also General Introduction, Section 1.4). The fact that the initial effects of 10 μM VTD on mepsp frequency are so similar to those of pyrethroid supports the proposal that pyrethroids act directly on the voltage-gated sodium channel in this preparation.

Calcium channel blockade is another action of VTD at high concentrations in some preparations (Romey and Lazdunski, 1982). It was therefore possible that the rapid increase and decline in mepsp frequency caused by veratridine application is a manifestation of both toxic effects occurring asynchronously, due to different ligand

affinities for each type of channel. Thus sodium channel activation, followed by calcium channel occlusion was a possible explanation. This, however, cannot be the case, since pyrethroid induced mepsp frequency remained at a sustained level after the further addition of veratridine.

Although this result shows that VTD does not affect presynaptic calcium channels, it was nevertheless unexpected. The gradual decline of a cypermethrin induced increase in mepsp frequency has been shown by ultrastructural studies to be associated with a depletion of presynaptic vesicles (Shouest *et al.*, 1986). Assuming this is also the case for the veratridine induced response, it was expected that application of both toxins together would lead to a more rapid decline in mepsp frequency, characteristic of VTD poisoning, rather than maintaining an elevated level for the much longer time periods observed during pyrethroid application. This result is also inconsistent with other reports, which show that pyrethroids cause an increase in the fraction of mammalian sodium channels activated by alkaloid toxins (Jaques *et al.*, 1980). Since no synergism was observed on simultaneous application it is assumed that another, as yet unidentified interaction between VTD and pyrethroids exists in the caterpillar neuromuscular junction.

The strongest evidence that the sodium channel is the pyrethroid site of action is the fact that TTX can reversibly abolish the effects of pyrethroid on the mepsp frequency. However, despite the abundance of reports confirming the specificity of TTX for the voltage sensitive sodium channel, it has recently been suggested that heart tissue of *Manduca sexta* may possess a TTX-sensitive calcium channel (Brink and Tublitz, 1989). This raises the possibility that the response to pyrethroids and its reversal by TTX is mediated by direct action on similar voltage sensitive calcium channels in the presynaptic terminal, rather than the proposed secondary effects on calcium channels due to the level of nerve-terminal depolarisation caused by the interaction of these

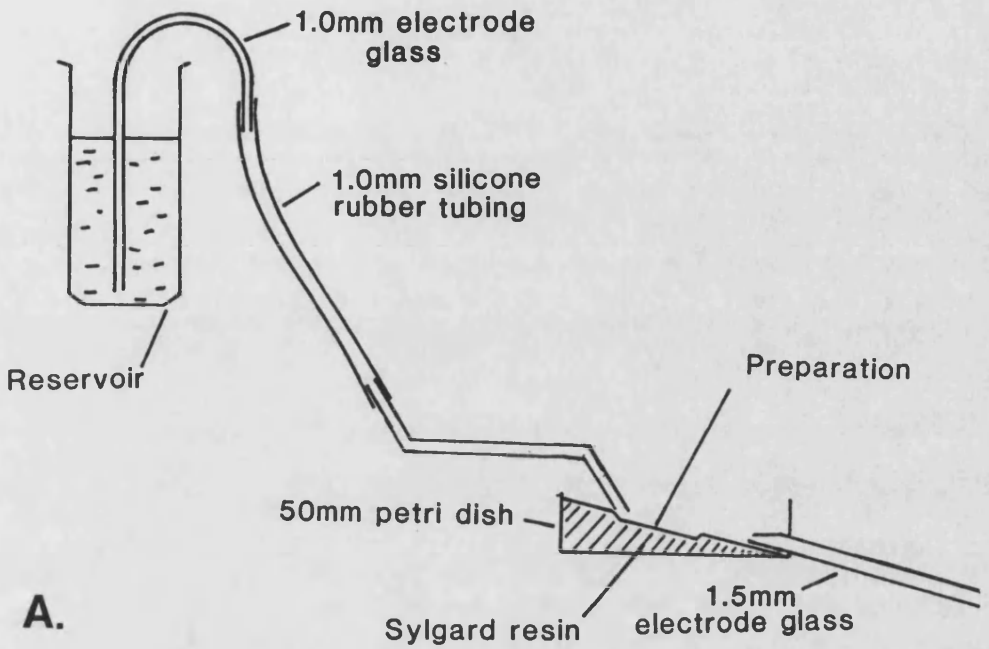
toxins with synaptic sodium channels. Although this proposal seems somewhat unlikely, it cannot at present be dismissed.

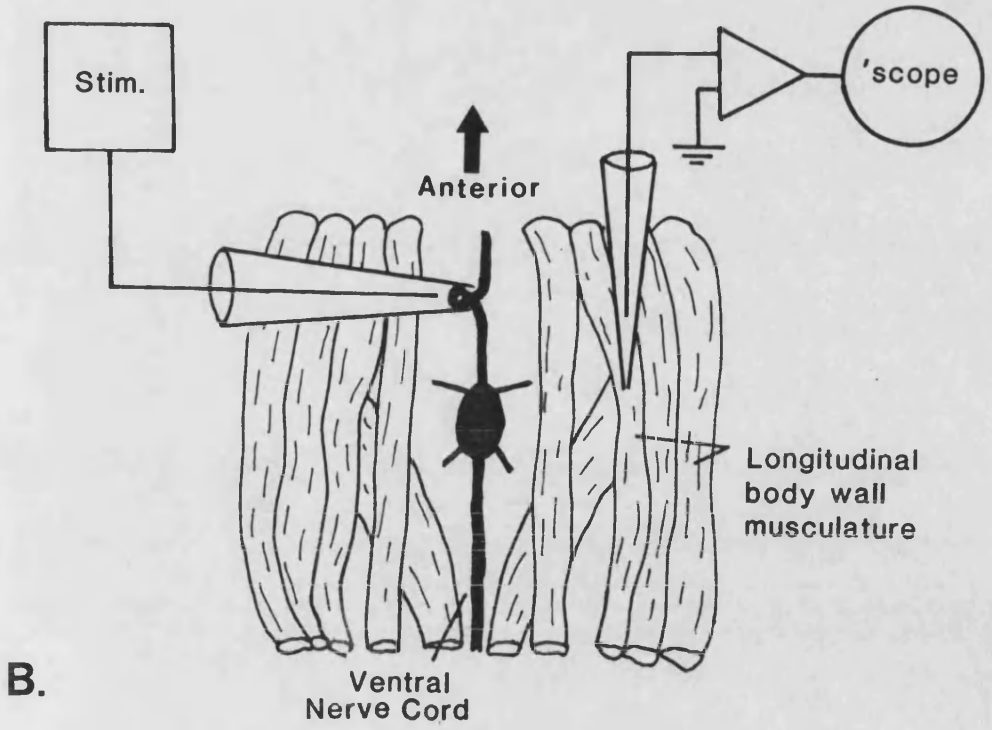
A recurring problem with this preparation was the lack of consistency in the effects of TTX, so the possibility that the toxin was physically obstructed from reaching its active site was considered. Detailed anatomical studies have shown that the membrane of *Manduca* larval muscle fibres is highly folded, with the neuromuscular junction forming within deep clefts (Shaner and Rheuben, 1985). In adult moths, glial processes also penetrate deep invaginations of the muscle surface in the area of the neuromuscular junction, and it has been proposed that these could function to anchor the nerve to the muscle fibres (Rheuben and Reese, 1978). The complete quiescence of membrane noise following muscle contracture after treatment with a high pronase concentration may possibly be explained by the physical extraction of nerve terminals after proteolytic digestion of the glial anchor. Lowering the pronase concentration presumably resulted in only partial digestion of the extracellular matrix that maintains this complicated anatomy, thus allowing TTX access to a greater proportion of muscle synapses. Although pre-treatment with low concentrations of pronase led to an increase in the proportion of TTX-sensitive fibres, this strategy did not produce the consistent responses that were hoped for. Since response to TTX could sometimes be demonstrated in untreated preparations, a systematic search was undertaken to try to locate muscle fibres with anatomically accessible neuromuscular junctions. Small muscles which were unlikely to possess the super-contractile properties of longitudinal body wall muscles were tested, but were found to be just as likely to be unaffected by TTX. It is evident that the body- wall muscles of caterpillars are less favourable preparations for this kind of work than the longitudinal ventrolateral muscles of *Musca domestica*, which respond readily to TTX application after pyrethroid poisoning (Salgado *et al.*, 1983a). The nerve terminals of this preparation are naked, possessing no glial cap (Hardie, 1976) and thus presumably facilitate easy chemical access.

No difference was observed in the effects of permethrin (type I pyrethroid) and cypermethrin (type II pyrethroid) in this study, although perhaps with further work an assay which distinguished between the two might have been developed. Other workers have attempted to attribute an "effective concentration" for a given pyrethroid (normally the concentration required to induce an increase in mepsp frequency in a given proportion of muscle cells in a given time). For example, Irving (1984) presents data for *Heliothis virescens* larvae showing very similar values of effective concentration of approximately 0.1 nM for both permethrin and cypermethrin. This is in agreement with the lowest concentration required to induce the response in this study. However, Salgado *et al.* (1983b) stated that another Lepidopteran, *Trichopisia ni*, was 100-fold more sensitive to type II compounds than type I. They also observed distinct differences in the concentration required to induce repetitive action potentials, as well as the duration of these bursts, between type I and II pyrethroids. These effects were not observed in this study, so they may be associated with differences between species.

The evidence of this study supports the conclusion that pyrethroids act at the level of the neuromuscular junction in Lepidopteran larvae. Assays involving pyrethroids alone have shown that it is possible to obtain very accurate and reproducible "effective concentration" data for a variety of species (Irving 1984), presumably because the high lipophilicity of pyrethroids make them easily accessible to otherwise well protected neuromuscular junctions. This type of assay has been used to show that effective concentration ratios correspond to resistance ratios in *kdr* houseflies and resistant mosquitoes (Salgado *et al.*, 1983b). This approach may prove to be the most important application of this preparation - as a method of determining the extent of *kdr*-type resistance, and distinguishing it from other resistance mechanisms.

FIGURE 2.1. *The* Manduca *neuromuscular preparation.* (A) Diagram of disposable perfusion apparatus used to deliver saline to the *Manduca* preparation. (B) Diagram of the *Manduca* neuromuscular preparation. (Innervation of the musculature was not visible under the binocular microscope).


1.0mm electrode glass
1.0mm silicone rubber tubing
Reservoir
Preparation
50mm petri dish
Sylgard resin
1.5mm electrode glass
A.


Stim.
'scope
Anterior
Longitudinal body wall musculature
Ventral Nerve Cord
B.

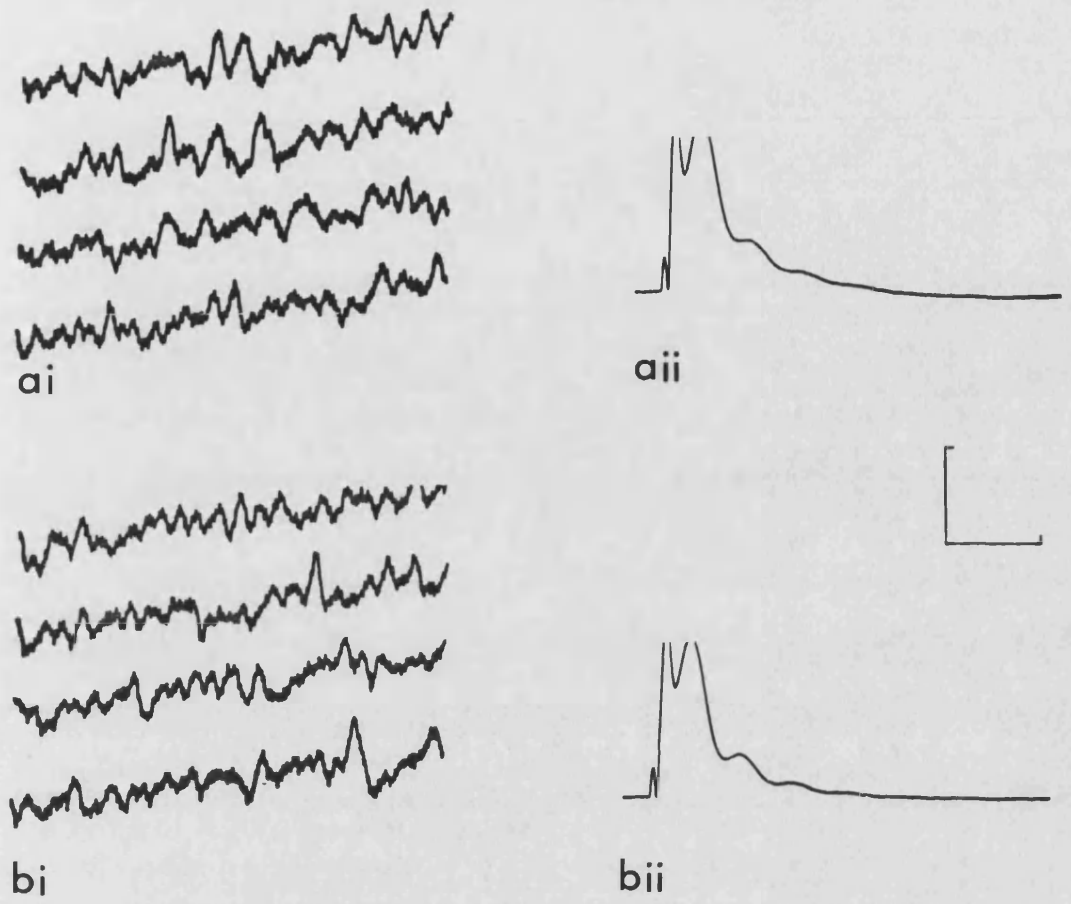


FIGURE 2.2. *Control recordings from the* Manduca *neuromuscular preparation.*
(ai) Mepsps recorded 30 minutes after dissection, and (aii) evoked muscle potential at this time. (b) Same preparation after 4 hours. (Note large contraction artefact following evoked muscle potentials).
E_m remained stable at -45 mV. Mepsp recordings read from bottom to top.
Calibration: ai, bi: 1 mV, 200 msec; aii, bii: 30 mV, 30 msec.

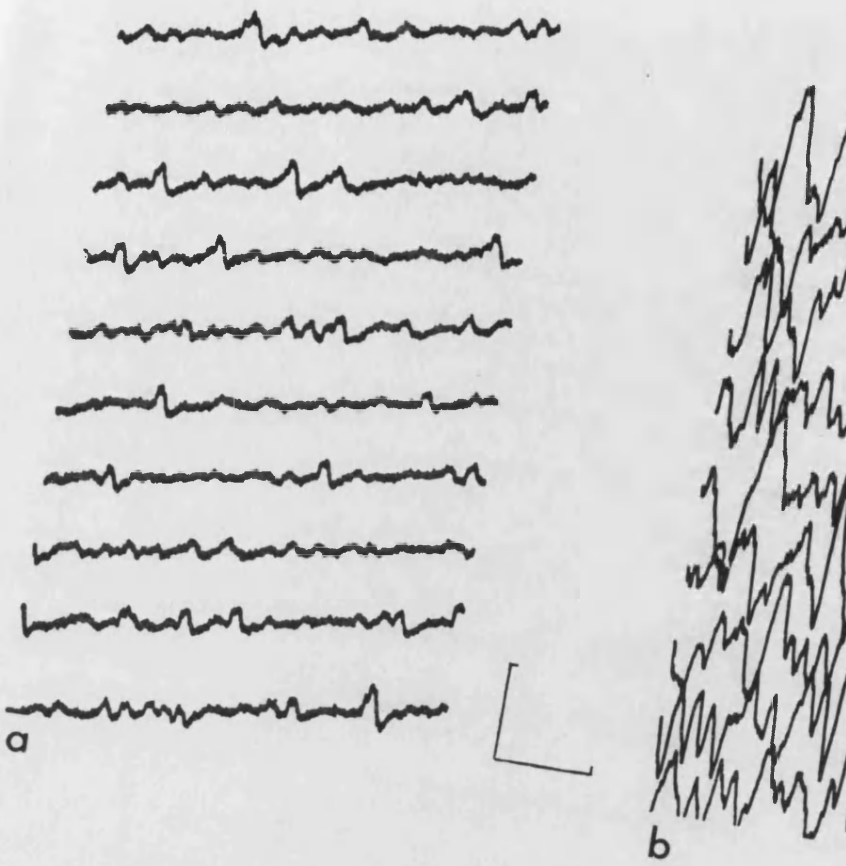
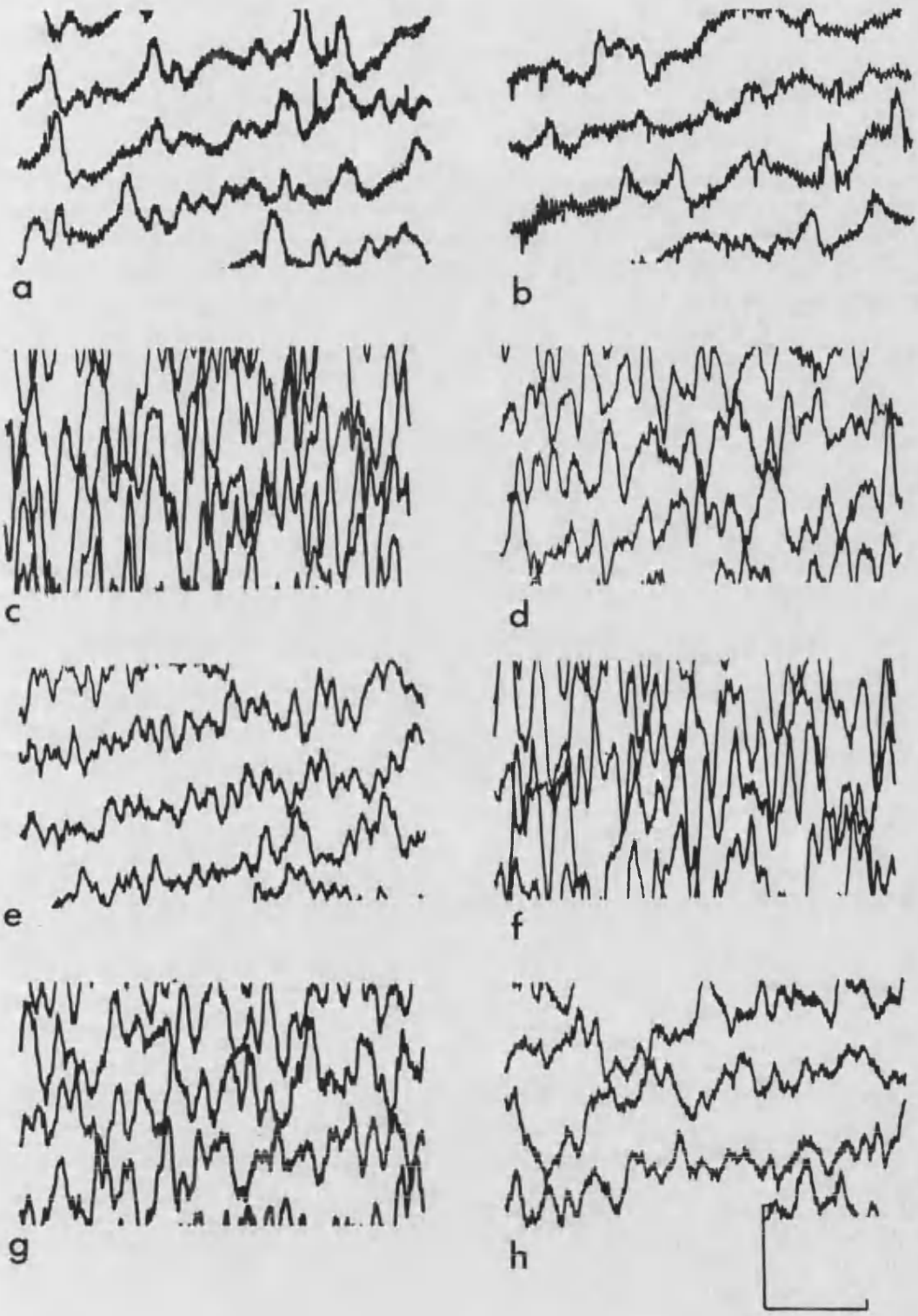


FIGURE 2.3. *Effect of pyrethroid on spontaneous miniature potentials.* Mepsps recorded before (a) and 20 minutes after application of 10^{-8} M cypermethrin (b). Calibration: 1 mV, 200 msec.

FIGURE 2.5. *Effect of 1 μM tetrodotoxin (TTX) on cypermethrin induced elevated mepsp rate.* (a) Control mepsp rate. (b) Control rate 5 minutes after treatment with 0.01 mg/ml pronase. (c) 20 minutes after the addition of 10^{-8} M cypermethrin. (d) 30 seconds after further addition of 1 μM TTX. (e) After 4 minutes TTX treatment. (f) 8 minutes after TTX removal. (g) 30 seconds after re-application of 1 μM TTX. (h) Mepsp rate 4 minutes after re-application of TTX.

Calibration: 1 mV, 200 msec.


a
b
c
d
e
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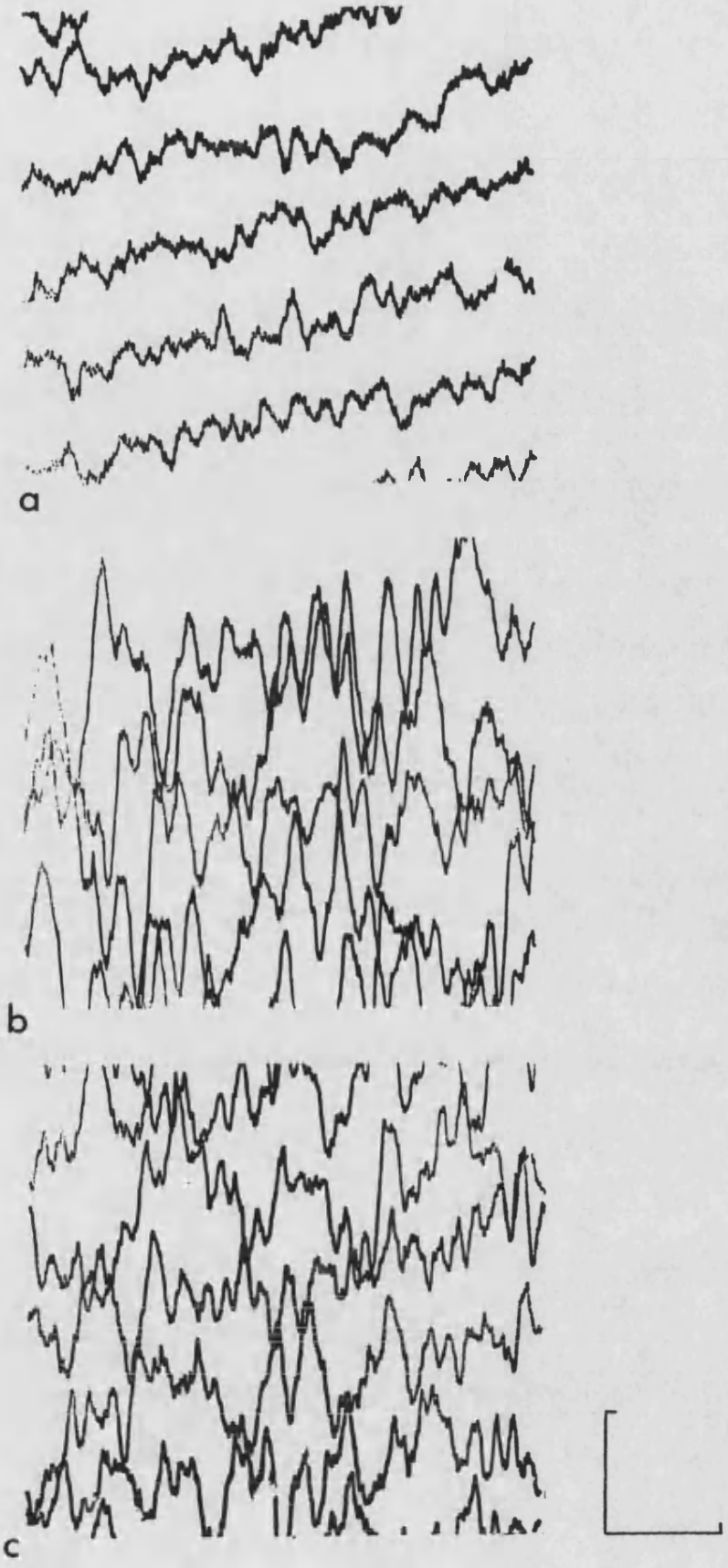
g
h

FIGURE 2.6. *Effect of simultaneous application of pyrethroid and veratridine.* (a) Control mepsp rate. (b) 30 minutes after the addition of 10^{-8} M cypermethrin. (c) Further application of 10 μM VTD had no detectable effect on the cypermethrin induced mepsp rate after continuous monitoring for 70 minutes.
Calibration: 1 mV, 200 msec.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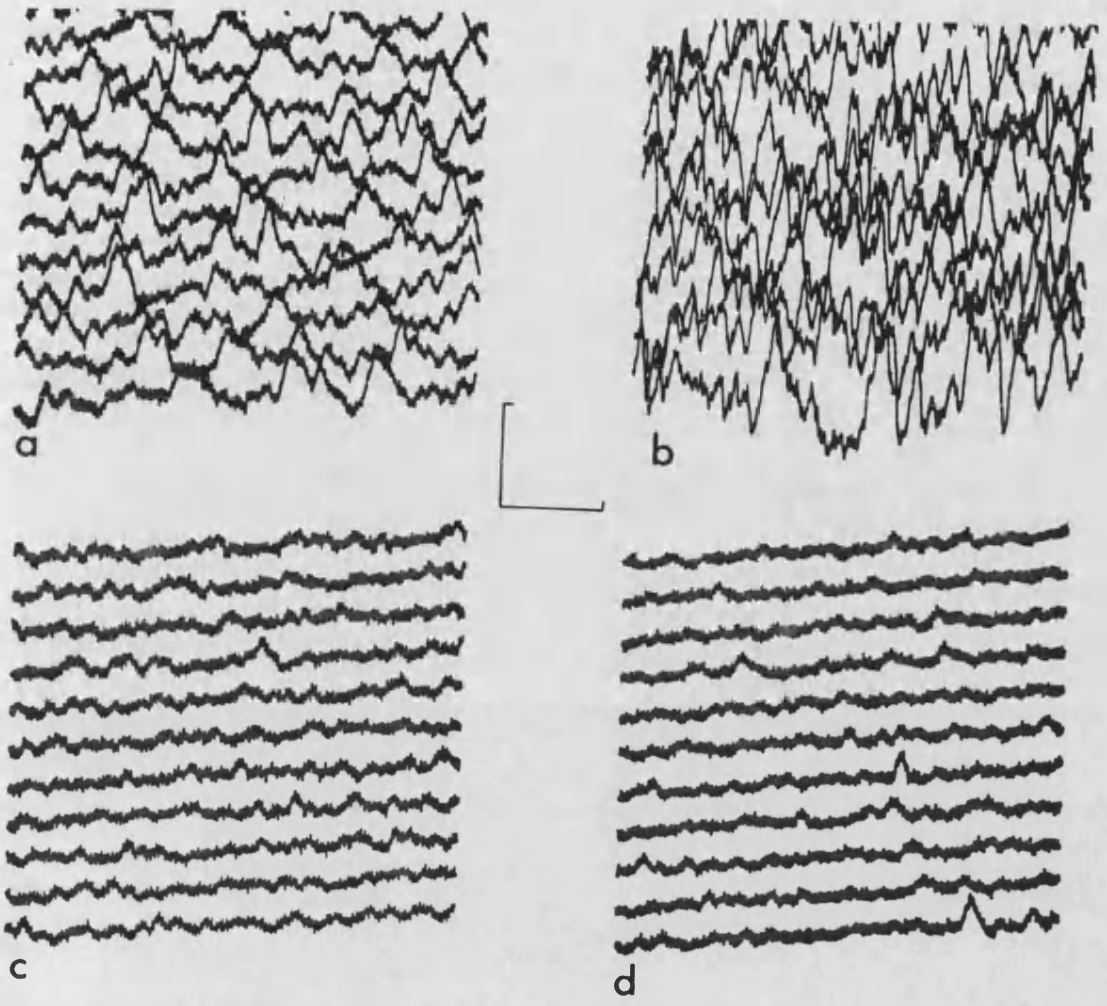


FIGURE 2.7. *Effect of application of VTD after prolonged pyrethroid exposure.* (a) Control mepsp rate. (b) 15 minutes after application of 10^{-8} M cypermethrin. (c) After 200 minutes exposure to cypermethrin, mepsp rate had diminished to subnormal levels. (d) Further addition of 10 μM VTD produced no change after 60 minutes. Calibration: 1 mV, 200 msec.

CHAPTER 3

THE BINDING OF [^{3}H]SAXITOXIN TO HOMOGENATES OF NERVE CORD AND HEART FROM *MANDUCA SEXTA*

3.1. Introduction

Saxitoxin (STX) and tetrodotoxin (TTX) are heterocyclic guanidinium toxins which bind to the action potential sodium channel and render it impermeable to sodium ions (Catterall *et al.*, 1980; see also General Introduction, Section 1.4). Their mode of action is so similar that a common binding site was suspected even before their structures were fully elucidated (see Kao, 1966) and this was eventually confirmed by the mutual competitive displacement of radiolabelled analogues (Colquhoun *et al.*, 1972; Henderson *et al.*, 1973).

These toxins are appealing as research tools because of their high binding affinity and remarkable specificity for sodium channels. Since the development of a labelling method which yields much purer samples with greater specific activity than previously (Ritchie *et al.*, 1976), [^{3}H]STX has been applied to the study of several diverse aspects of sodium channel research. For example, because these toxins bind monomolecularly to the channel (Hille, 1968), the maximal binding capacity of [^{3}H]STX has been used to calculate sodium channel density in nerve and muscle preparations from a variety of species (see Ritchie and Rogart, 1977a). Similar binding studies have revealed the distribution of sodium channels in mammalian

myelinated nerve, since the comparable maximum binding capacities between intact and homogenised tissue showed that the bulk of [^{3}H]STX binding sites are located at the nodes of Ranvier (Ritchie and Rogart, 1977b). Quantitative autoradiography with [^{3}H]STX has been used to map the density and distribution of sodium channels in mammalian brain (Mourre *et al.*, 1988). Another important application for these labelled toxins has been their extensive use as markers during the isolation and purification of sodium channels from several sources, including electric eel electroplax (Agnew *et al.*, 1978), rat brain (Hartshorne and Catterall, 1981) and various insect nerve tissues (Gordon *et al.*, 1990). Interspecies differences in the STX site have been made evident as a consequence of such purification studies, even if the affinities of the [^{3}H]STX binding are comparable. Selective removal of the ß1-subunit from rat brain sodium channels results in the complete loss of [^{3}H]STX binding, inferring that the ß1- subunit is part of the STX site or is needed to establish the physical integrity of the site on the α-subunit (Messner and Catterall, 1986). However, eel electroplax (Miller *et al.*, 1983) and insect nerve preparations (Gordon *et al.*, 1990) appear to lack the ß-subunits associated with mammalian brain sodium channels, and yet bind [^{3}H]STX with high affinity.

Perhaps the most interesting aspect of [^{3}H]STX binding work, with respect to the possibility of probing the mechanisms of pyrethroid resistance, involves its interaction with membrane preparations from various *Drosophila* mutants. Although they are genetically distinct, these mutants have all been selected for essentially the same phenotypic profile of temperature induced paralysis. However, they differ from wild type and each other regarding their [^{3}H]STX binding properties, and these differences give some clues as to the structure and regulation of sodium channels involved. For example, *nap*^{ts} (no action potential, temperature sensitive) displays normal binding properties, except for a reduction in the number of binding sites by about 40% when compared to wild type (Hall *et al.*, 1982; Jackson *et al.*, 1984). *Tip*-E (temperature induced paralysis, locus E) has essentially the same binding abnormality (Jackson *et*

al., 1986). This suggests that these mutants are defective in regulating the number of sodium channels without altering receptor structure or function. *Sei*$^{ts-1}$ (seizure, temperature sensitive 1) also has a reduced number of binding sites but this is far more pronounced at elevated temperatures, suggesting that under these conditions the *Sei*$^{ts-1}$ sodium channels are much more labile than normal sodium channels (Jackson *et al.*, 1985). *Sei*$^{ts-2}$ has normal channel density, but the receptors display a two-fold reduction in affinity for [^{3}H]STX at the elevated temperature, suggesting that the binding site is altered structurally in this mutant (Jackson *et al.*, 1984). *Ttx*^{s} is a mutant with a different phenotype in that it is abnormally sensitive to dietary TTX. However, its [^{3}H]STX binding properties are identical to wild type, thus implying a molecular defect which does not involve sodium channels in this mutation, such as detoxification enzymes or gut permeability (Gitschier *et al.*, 1980).

The fact that so many different mutations putatively involving sodium channels can be distinguished and defined in terms of [^{3}H]STX binding properties makes this technique a highly promising prospect in the field of pyrethroid resistance research. Indeed, it has already been claimed that there is a considerable reduction in [^{3}H]STX density in *kdr* resistant houseflies (Rossignol, 1988) although other workers have been unable to repeat these results (Grubs *et al.*, 1988). However, this approach is worth pursuing since *Drosophila nap*^{ts} mutants have been shown to be more resistant to pyrethroids than wild type (Kasbekar and Hall, 1988).

Evidence has already been provided which indicates that pyrethroid insecticides act at *Manduca* neuromuscular junctions, and that their action is blocked by TTX, demonstrating a pyrethroid interaction with sodium channels in this species (see chapter 2). It was therefore of interest to develop an appropriate assay in order to characterise the binding of [^{3}H]STX to *Manduca* nervous system, and to determine the effects of pyrethroids and other toxins known to affect sodium channels on this

ligand-receptor complex. Such an assay could in future be used to probe the pyrethroid resistance mechanisms of other Lepidopteran species.

A further aspect of this work involved the attempt to locate and characterise [^{3}H]STX binding sites on *Manduca* tissues other than nerve. Such receptors are relatively widespread in vertebrate tissues and often possess a population with a much lower affinity for [^{3}H]STX than neuronal tissue, suggesting a sodium channel subtype. For example, low affinity [^{3}H]STX binding sites are located on chick heart (Rogart *et al.*, 1983), denervated mammalian skeletal muscle (Rogart and Regan, 1985) and mammalian cardiac cells (Renaud *et al.*, 1983). Extraneuronal sodium channel subtypes may prove to be target sites for pyrethroid insecticides although they have not as yet been reported in any insect species.

3.2. Methods

3.2.1. Chemicals

[^{3}H]STX (68 Ci/mmol) was obtained from Amersham. Tetrodotoxin, veratridine and *Leiurus quinquestriatus* scorpion venom were obtained from Sigma Chemical Company. All pyrethroids were gifts from Shell Research Ltd., Sittingbourne.

3.2.2. Tissue preparations

Large pre-wandering fifth instar *Manduca sexta* larvae were dissected by dorsal incision. The gut was removed and the preparation flushed with 50 mM potassium phosphate buffer (pH 7.4). The entire nerve cord thus exposed was removed as quickly as possible to prevent drying, and transferred to fresh buffer held on ice. Nerve cord material was separated from adhering muscle and fat body, and either

used immediately or frozen rapidly over dry ice and kept at -40°C for future use. Unused frozen nerve cords were discarded after 4 months, although later experiments were to establish that [^{3}H]STX binding parameters were unaltered in material which had been frozen for over 18 months. Nerve cords were never re-frozen after thawing.

Cardiac tissue was isolated as follows: newly emerged adult moths were chosen for dissection. After cooling on ice, the abdomen was removed and pinned open after a lateral incision along its length. A microspatula was used to scrape away as much fat body as possible from around the heart before it was removed and transferred to sodium phosphate buffer (pH 7.4) held on ice. All other procedures for dissection and storage were the same as for nerve cords.

Larval body wall muscle and salivary glands were used immediately after removal.

3.2.3. Binding assays

Nerve cord homogenates were prepared using a tight fitting glass-glass hand held homogeniser and suspended without further fractionation in 50 mM potassium phosphate buffer (pH 7.4) to a final protein concentration of approximately 0.2-0.3 mg/ml. Binding was initiated by the addition of 10 µl of [^{3}H]STX (in buffer) to 100 µl of homogenate in 3 ml polypropylene tubes. The final assay volume was made up to 120 µl with the addition of 10 µl of buffer. After an incubation period of 20 minutes (except for association kinetics experiments), the reaction mixture was rapidly diluted with 3 ml of ice cold buffer, vacuum filtered through Whatman GF/C filters and washed with 5 ml ice cold buffer. This process of reaction termination and separation of bound [^{3}H]STX from free ligand was accomplished within 5 seconds, so dissociation of the ligand-receptor complex was not a limiting factor in most of the experiments performed using this procedure. Whatman GF/C filters were pre-soaked in 3 mg/ml polyethylenimine, which was found to yield a superior total/non-specific

binding ratio than filters soaked in assay buffer, 1 mg/ml BSA, or untreated filters. The filters were suspended in 5 ml scintillation fluid (Optiphase safe), and [^{3}H] radioactivity counted on a Rackbeta liquid scintillation spectrometer. Non-specific binding was determined in parallel experiments where 1 μM TTX was included in the buffer. Any specific binding to filters in the absence of homogenate was subtracted from all specific binding data. All experiments were performed in triplicate.

Association kinetics was determined by varying the length of incubation. Dissociation was followed by determining specific binding at intervals after effectively eliminating the forward reaction with a 100-fold dilution of a pre-equilibrated aliquot.

Experiments to test the effects of pyrethroids were performed in 3 ml glass assay tubes.

Binding data was analysed using the equations shown in Appendix II.

3.2.4. Measurement of protein quantity

The protein content of the homogenate was estimated using the modified Lowry assay of Markwell *et al.* (1978). The procedure was followed exactly apart from the volumes of protein samples and reagents used. Reagent A consisted of 2% Na_2CO_3, 0.4% NaOH, 0.16% potassium sodium tartrate and 1% SDS. This proved to be very stable at room temperature. Reagent B was 4% $CuSO_4.5H_2O$. Folin-Ciocalteu phenol reagent was diluted 1:1 with distilled water. Immediately prior to use, 100 parts Reagent A was mixed with 1 part reagent B to form C. 200 μl of homogenate was mixed with 600 μl of Reagent C and incubated at room temperature for 15 minutes. This was mixed vigorously with 60 μl of the diluted phenol reagent and incubated for a further 45 minutes. The 660 nm absorbence readings of the sample

were taken with a spectrophotometer. Experiments were performed in duplicate. Protein standards (BSA Sigma Fraction V) normally yielded linear regression coefficients >0.99 over the range 10-100 μg.

3.3. Results

3.3.1. [^{3}H]STX binding to nerve cord homogenate

3.3.1.1. Dependence on protein concentration

Estimates of specific [^{3}H]STX binding sites as a function of protein concentration were initially carried out to determine the upper limit of the usable concentration under the assay conditions employed. Fig. 3.1 shows that this relationship is linear up to at least 1mg/ml of protein. The concentration of 0.2-0.3mg/ml routinely used (corresponding to approximately 0.1 nerve cord per assay tube) was therefore well within the range of the assay.

3.3.1.2. Saturation experiments

The caterpillar CNS contained high-affinity, saturable [^{3}H]STX binding sites (Fig. 3.2), with specific binding constituting more than 95% of the total.
Scatchard analysis (Fig 3.3A) was consistent with a single class of binding sites giving an estimated equilibrium dissociation constant (K_D) of 1.53 ± 0.29 nM and maximal specific binding capacity (B_{max}) of 3.41 ± 0.91 pmol/mg protein (mean ± SD, n=3).
Hill plots were linear and had slopes close to 1.0 (Fig. 3.3B), indicating lack of cooperativity between binding sites.

3.3.1.3. Kinetic experiments

The kinetic data was linearised according to Weiland and Molinoff (1981, see Appendix II). Pseudo first-order conditions for association were satisfied, as less than 10% of the total [^{3}H]STX was bound at equilibrium.

Determination of both association and dissociation kinetics revealed that both on- and off-rates were rapid, with $K_1 = 11.3 \times 10^6\ M^{-1}sec^{-1}$ and $K_{-1} = 7.8 \times 10^{-3}\ sec^{-1}$ (Fig. 3.4). Hill coefficients of 1.0 and linear Scatchard plots do not necessarily mean simple bimolecular binding, since these results can be due to more complicated reactions (Weiland and Molinoff, 1981). However, the kinetic estimate of K_D (K_{-1}/K_1) = 0.7 nM is similar enough to the values calculated by linear transformation of the equilibrium binding data to confirm a simple second-order binding reaction. The comparative values also demonstrate an internal consistency for the assay methodology developed.

3.3.1.4. Competition experiments

Fig. 3.5 shows the competitive inhibition of [^{3}H]STX binding by TTX. The TTX concentration required to displace 50% of the bound [^{3}H]STX (IC_{50}) was estimated as 0.68 nM. The assay criteria which would have been necessary for the IC_{50} value to approach the true K_D value (see Lunt, 1985) required that the concentration of receptor sites be so low that the binding signal was effectively lost. Therefore, the IC_{50} is only an estimate of the K_D but nevertheless indicates a similar affinity between TTX and [^{3}H]STX for the common receptor.

No significant displacement of [^{3}H]STX binding was observed when the CNS homogenate was pretreated for 30 minutes with any of a number of insecticides that included representatives of most classes of compound but particularly pyrethroids

(Table 3.1 see also Fig. 3.5). The concentrations used in these experiments were several orders of magnitude greater than are necessary for insecticidal activity, since it has been shown that pyrethroids need only modify a small fraction of sodium channels in order to produce effects on nerve function (Lund and Narahashi, 1982). Similarly, binding of [^{3}H]STX was not affected by other sodium channel modifiers, namely veratridine, scorpion venom, or the local anaesthetics MS-222 and urethane.

3.3.1.5. Distribution of [^{3}H]STX binding sites

Apparent B_{max} values (i.e. specific binding at the saturating concentration of 15 nM [^{3}H]STX) were 2.95 (brain), 2.36 (ganglia) and 3.10 (connectives) pmol/mg protein, indicating a fairly uniform distribution of sodium channels throughout the caterpillar CNS.

3.3.2. [^{3}H]STX binding to heart homogenate

Homogenates of *Manduca* body wall muscle and salivary gland did not bind significant amounts of [^{3}H]STX. However, binding to heart homogenate was specific and saturable. Specific binding accounted for only 40-50% of the total, and the quantities bound were much smaller than would be expected for an equivalent amount of nerve cord homogenate. However, as with nerve cord experiments, triplicate determinations were normally uniform and so specific binding was readily discernible.

3.3.2.1. Dependence on protein concentration

[^{3}H]STX was linear over the range 1.25-10 mg/ml protein. However, binding above this protein concentration deviated markedly from the linear (Fig. 3.6). This was

probably caused by the large reduction in filtration rate due to the quantity of material at this concentration. The protein concentration of about 4.5 mg/ml (equivalent to approximately 0.5 hearts per assay tube) employed in subsequent experiments was thus within the linear range, and careful dissection and removal of fat body ensured rapid filtration of a comparable rate to that of CNS homogenate.

3.3.2.2. Saturation experiments

An example of a saturation binding isotherm is given in Fig. 3.7. Scatchard transformation (Fig. 3.8A) yields a K_D of 12.4 ± 3.81 nM and B_{max} of 11.25 ± 3.0 f mol/mg (mean of two experiments). Hill plots (Fig. 3.8B) yielded straight lines with slopes having values close to 1.0, indicating a single class of binding sites with no significant cooperativity.

3.3.2.3. Kinetic experiments

Association and dissociation rates were very rapid, with $K_1 = 2.42 \times 10^6\ M^{-1}sec^{-1}$ and $K_{-1} = 9.59 \times 10^3\ sec^{-1}$ (Fig.3.9). The K_D of the binding interaction calculated from these values was 3.96 nM. The discrepancy between K_D values obtained by saturation experiment compared with kinetics experiments might be accounted for by the fact that the very small quantities bound during the initial stages of association, and the particularly rapid rate of dissociation, were both approaching the limits of the assay capabilities under the conditions employed.

3.3.2.4. Competition experiments

[^{3}H]STX binding to heart and CNS homogenate could not be distinguished pharmacologically. Thus, no difference in binding to heart was observed in the presence of permethrin, cypermethrin, VTD, or scorpion venom. In addition to the

above sodium channel ligands, verapamil and destruxin, which bind to calcium channels, had no effect on either preparation. The effects of divalent cations in the form of $CaCl_2$, $CoCl_2$ and $CdCl_2$ could not be assessed since even at relatively low concentrations (10 mM) these caused precipitation of the buffer which effectively blocked the glass fibre filters.

3.4 Discussion

This report describes the first [^{3}H]STX binding study on a Lepidopteran species, and shows that the interaction of [^{3}H]STX with *Manduca* CNS shares many of the important characteristics associated with nerve and brain preparations from other species. Scatchard analysis of the saturation data showed high affinity binding with an average K_D of 1.26 nM, which is within the range of K_D values from 0.1-3.0 nM reported for the other insects which have previously been investigated: *Locusta migratora* (Gordon *et al.*, 1985) *Musca domestica*, (Grubs *et al.*, 1988; Rossignol, 1988), *Drosophila* (e.g. Gitschier *et al.*, 1980) and *Periplaneta americana* (Lummis and Sattelle, 1989; Dwivedy, 1990). K_D values for mammalian neuronal preparations also lie within these limits (e.g. Weigele and Barchi, 1978), indicating that the saxitoxin binding site is a highly conserved feature of the sodium channel structure. However, direct comparisons between species on the basis of [^{3}H]STX binding affinity cannot be made, since there are discrepancies in K_D values obtained from different laboratories using essentially the same preparation from the same species. For example, Grubs *et al.* (1988) found a K_D of 0.55 nM for a housefly head membrane preparation whereas Rossignol (1988) reported a value of 2.28 nM. Similarly, Lummis and Sattelle (1989) found a K_D of 0.84 nM for a *Periplaneta* nerve cord membrane preparation, compared with 3.0 nM for a synaptosomal preparation from the same species (Dwivedy, 1990). These examples perhaps

illustrate that exact duplication of the preparation and assay conditions are necessary before accurate comparisons can be made.

Maximum saturable [^{3}H]STX binding capacities (B_{max}) vary among the insects so far studied, as might be expected considering the different types of preparation utilised. However, the B_{max} value of 3.4 pmol/mg protein for *Manduca* CNS is particularly high, especially for a crude homogenate. Among the insect preparations so far investigated only a *Periplaneta* CNS membrane preparation surpasses this value with a B_{max} of 8.25 pmol/mg protein (Lummis and Sattelle, 1989). Sodium channel density may be of particular significance in terms of pyrethroid susceptibility, since it is claimed that *kdr* resistant houseflies possess a reduced number compared with wild type (Rossignol, 1988) and a moderate degree of pyrethroid resistance is conferred to the *Drosophila* mutant *nap^{ts}*, which has a reduced sodium channel density (Kasbekar and Hall, 1988).

High concentrations of pyrethroid insecticides did not cause any significant displacement of [^{3}H]STX binding from the *Manduca* CNS preparation. Since the pyrethroid deltamethrin has also been shown to be ineffective at displacing [^{3}H]STX in neuronal preparations from *Periplaneta* (Lummis and Sattelle, 1989) and *Musca domestica* (Grubs *et al.*, 1988), it is concluded that pyrethroid activity does not rely on interaction with the STX binding site.

Although it generally agreed that the STX/TTX binding site is a remote entity on the sodium channel, with no interactions with other defined sodium channel toxin receptor sites (Catterall, 1980; 1984),
there is electrophysiological evidence that in some preparations a degree of interaction between this site and other sites does occur, suggesting a more complex interaction than was at first proposed. For example, anemone toxin II (ATX-II) binds to sodium channel site 3 and has an essentially irreversible action on crayfish giant

axons, causing a prolonged plateau, instead of the normal falling phase of the action potential (Romey *et al.*, 1976). However, initial co-application with TTX (which abolishes the action potential) followed by wash, results in the appearance of the normal action potential, suggesting occupancy of the TTX site prevents the interaction of ATX-II (Romey *et al.*, 1976). Similarly, voltage-clamp studies on *Xenopus* nerve preparations have shown that nominal concentrations of TTX prevent the modification of sodium channel inactivation by an α-scorpion toxin (another site 3 ligand) from *Buthus tamulus* (Siemen and Vogel, 1983). Furthermore, Brown (1986) showed that the affinity of [^{3}H]BTX-B for its sodium channel binding site (site 2) is decreased in the presence of STX or TTX under certain circumstances. However, representative ligands for site 2 (veratridine) and 3 (*Leiurus quinquestriatus* venom) did not affect [^{3}H]STX binding to the *Manduca* CNS homogenate. Similar results have been reported for other insect preparations (Lummis and Sattelle, 1989; Grubs *et al.*, 1988) and so it appears that there is no competitive interaction between site 1 and either site 2 or site 3 on the insect sodium channel. The present findings thus reinforce the long held view that TTX or STX binding is not modified by most other known sodium channel toxins (Catterall, 1980). However [^{3}H]STX binding studies cannot rule out the possibility of non-competitive interactions, in which occupancy of the TTX/STX binding site produces conformational changes thereby decreasing the ability of site 2 and 3 ligands to bind. This type of interaction is thought to occur between sites 1 and 2 in rat brain synaptasome sodium channels (Brown, 1986; Garritsen *et al.*, 1988).

Homogenates of *Manduca* body wall muscle and salivary gland did not bind significant quantities of [^{3}H]STX. Although there is no information regarding the possible presence of sodium channels in insect salivary gland, the result for *Manduca* muscle is in accord with electrophysiological evidence that insect muscles do not possess sodium channels, as the inward current in Lepidopteran muscle is carried by calcium ions (Deitmer and Rathmayer, 1976).

However, binding of [^{3}H]STX to *Manduca* heart homogenate was both specific and saturable. This is the first report of an STX/TTX receptor to be located in any insect tissue other than in the nervous system. Since the density of the binding sites is so low (B_{max} = 11.25 fmol/mg protein) it may be argued that the observed [^{3}H]STX binding is to sites associated with axonal innervation of the myocardium. However, if such innervation exists in *Manduca* heart, it seems unlikely that the nerve sodium channels would occur in much greater preponderance than they do at the neuromuscular junction. There is good evidence that sodium channels are located here (see chapter 2) although they were not detectable by [^{3}H]STX binding, presumably because they are few in number.

The heart binding site has a lower affinity for [^{3}H]STX than the CNS as determined by saturation experiments (K_D = 12.4 nM, compared with 1.53 nM in the CNS) and it is important to realise that direct comparisons can be made between these values since [^{3}H]STX binding was determined in both tissues under identical conditions. Thus on the basis of [^{3}H]STX binding affinity, this study may represent the first description of sodium channel subtypes within the same insect species, although it remains possible that the differences in [^{3}H]STX affinity may be caused by differences in channel microenvironment.

It is interesting, although probably coincidental, that mammalian cardiac cells (Renaud *et al.*, 1983) and avian heart (Rogart *et al.*, 1983) also appear to possess a sodium channel subtype with low affinity for STX. Chick heart has only one class of channel whereas rat heart possesses both high and low affinity sites. The rat heart low affinity site has already been cloned (Rogart *et al.*, 1989) and its deduced amino acid primary sequence is highly homologous to, although quite distinct from, that of rat brain sodium channel clones. The changes required to confer TTX resistance may not be quite so extensive in some subtypes, since a single amino acid substitution on a

rat brain sodium channel clone reduces the sensitivity of the expressed channel to STX and TTX by more than 3 orders of magnitude (Noda *et al.*, 1989). The functional role of sodium channels with low STX/TTX affinity in vertebrate heart and other tissues, for example denervated skeletal muscle (Rogart and Regan, 1985) has not yet been identified, since they have been extensively characterised and their electrophysiological properties are essentially normal (see Trimmer and Agnew, 1989). There is one report however, concerning the electrophysiology of *Manduca* heart, which indicates that the inward current of the cardiac action potential is carried by calcium ions through a TTX sensitive voltage-gated channel (Brink and Tublitz, 1989). Although primary sequence data suggests overall structural similarity between sodium and calcium channels (Tanabe *et al.*, 1987), TTX blockade of calcium channels has never been previously reported. However, addition of the vasoactive atrionatriuretic peptide (ANP) to mammalian ventricular myocytes renders sodium channels highly permeable to calcium without significantly altering their TTX sensitivity (Sorbera and Morad, 1990). This indicates the structural modification necessary to alter the ion selectivity of the sodium channel may be a minor one, and shows that the existence of a calcium conducting TTX-sensitive channel in *Manduca* heart is quite plausible.

It was not possible to distinguish between the putative heart and CNS sodium channels on pharmacological grounds, since none of the chemicals tested affected [^{3}H]STX binding in either preparation. However, although there appears to be no competitive interaction between the STX binding site and any of the other sodium and calcium channel binding sites investigated here, the polypeptide μ-conotoxins (μ-CTXs, isolated from the venom of *Conus geographus*) do seem to act competitively at the TTX receptor (Moczydlowski *et al.*, 1986; Ohizumi *et al.*, 1986). Furthermore, this interaction is capable of distinguishing between certain sodium channel subtypes, with high μ-CTX affinity for STX receptors from skeletal muscle, whereas [^{3}H]STX binding in mammalian brain and heart preparations was virtually unaffected even at

high μ-CTX concentrations. It would have been interesting to test the effects of μ-CTX on [^{3}H]STX binding in both CNS and heart homogenate to further characterize the STX site. However, μ-CTX was not commercially available and the cost of custom synthesis proved prohibitive.

The results presented here confirm the presence of a [^{3}H]STX binding site in the caterpillar CNS with a similar [^{3}H]STX affinity and in a similar density to the majority of other nerve tissues studied from a broad selection of species. The lack of interference from pyrethroids confirms that the pyrethroid binding site is remote from, and does not interact with, the [^{3}H]STX receptor.

The binding assay described here is rapid and simple in that it does not require extensive purification of a membrane preparation to generate reproducible results. The application of this assay to the comparison of [^{3}H]STX binding properties among populations of insects could detect resistance due to changes in channel density and may also detect alterations in sodium channel structure on the basis of altered [^{3}H]STX binding characteristics.

It would be interesting to pursue the discovery of low affinity [^{3}H]STX receptors, perhaps initially by investigating the effects of TTX and pyrethroids on the beat frequency and amplitude of a semi-isolated heart preparation. If these receptors are associated with channels which have a functional role in the generation or regulation of the insect hearteat, then it is possible that they may be susceptible to disruption by pyrethroid insecticides. This could have important implications regarding the events which lead to insect death after pyrethroid exposure, which at present remain almost totally obscure.

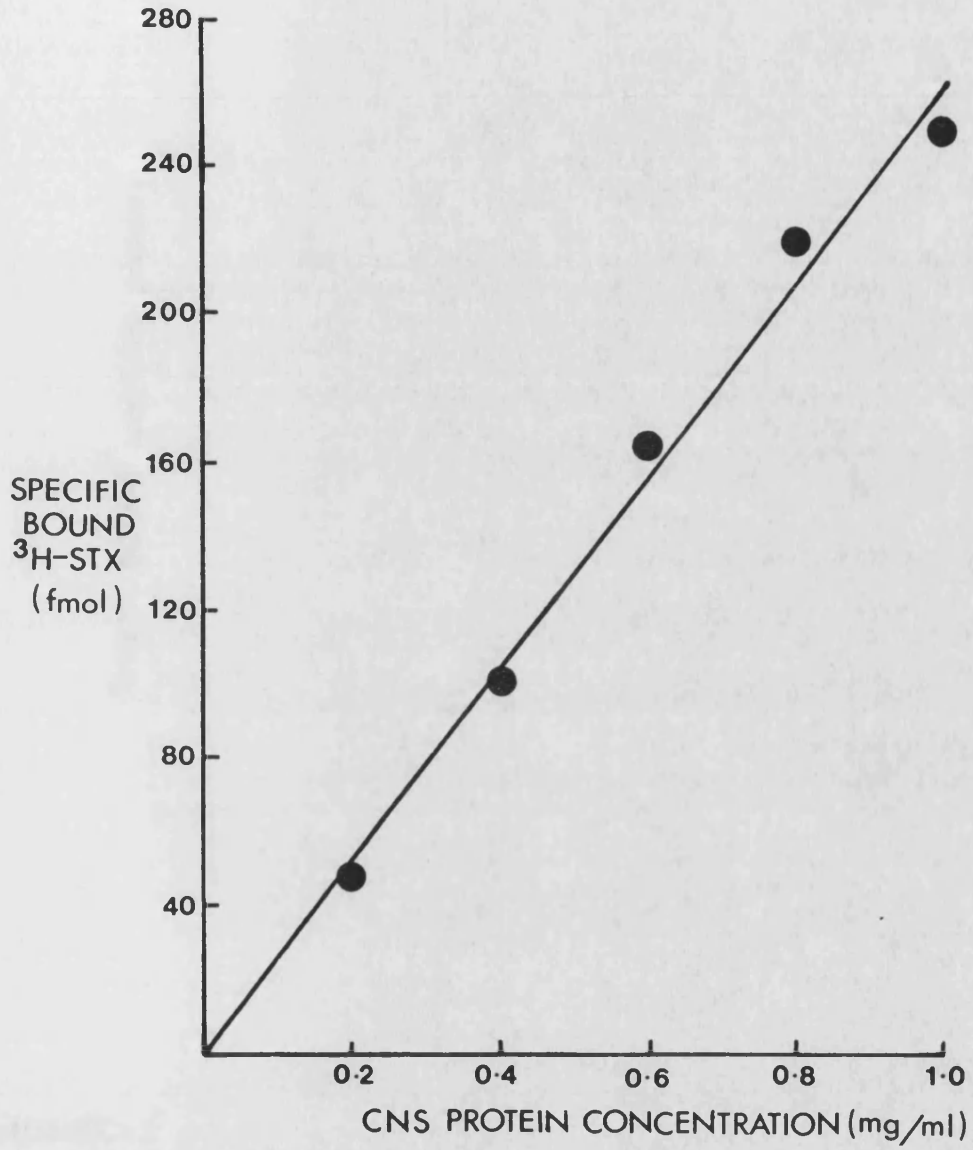


FIGURE 3.1. *The dependence of specific [^{3}H]STX binding to* Manduca *CNS homogenate on total protein concentration*. The assay is independent of protein concentration up to at least 1 mg/ml. A protein concentration of 0.2-0.3 mg/ml was used in subsequent experiments. The relationship was determined using 5 nM [^{3}H]STX.

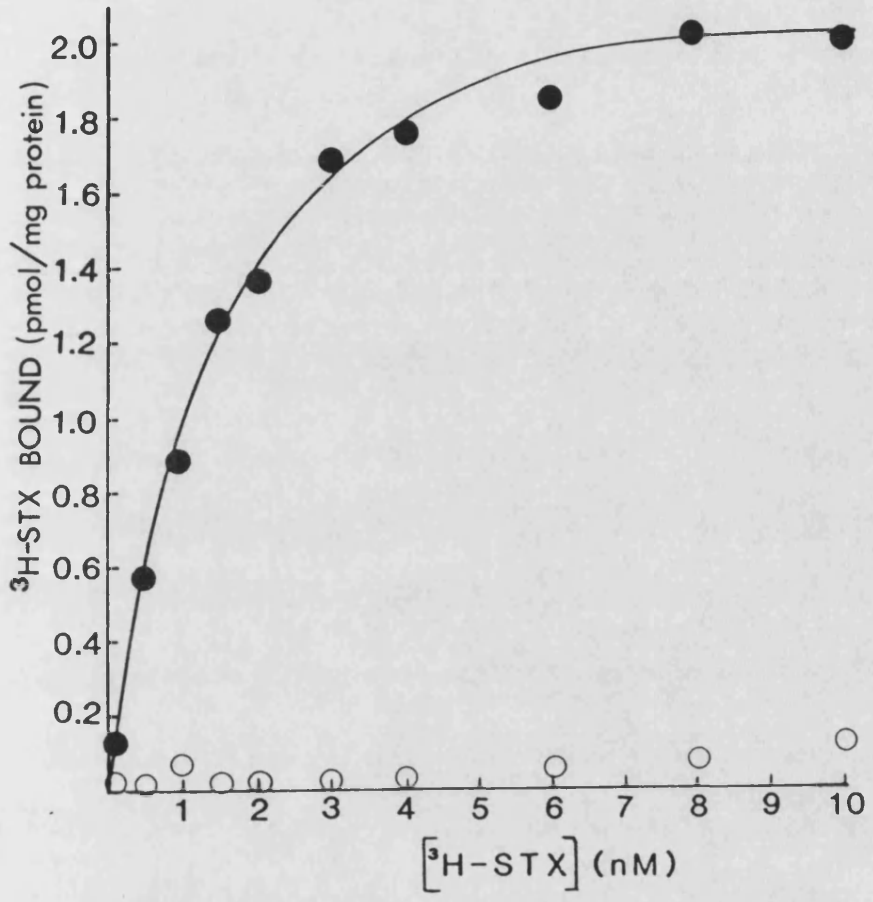
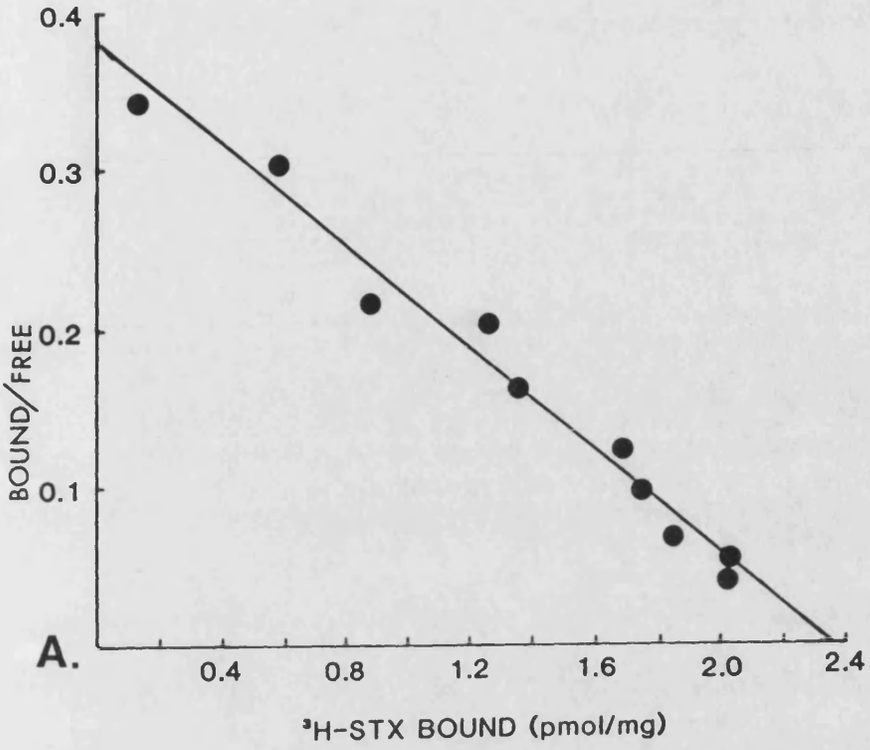


FIGURE 3.2. *Saturable binding of [^{3}H]STX to* Manduca *CNS homogenate.* Specific binding (●) was determined as the difference between total binding (not shown) and non-specific binding(○). The data points are the means of triplicate determinations from a single representative experiment, which was repeated three times.

FIGURE 3.3. *Linear representation of the equilbrium binding of [^{3}H]STX to* Manduca *nerve cord.* (A) Scatchard transformation of the specific [^{3}H]STX binding data shown in Fig. 3.2. This experiment exhibited K_D and B_{max} values of 1.26 nM and 2.37 pmol/mg protein, respectively. (B) Hill plot of the same data. Hill coefficient of 0.98 is indicative of a single population of non-interacting binding sites. The lines drawn represent the best fit as determined by least squares linear regression analysis.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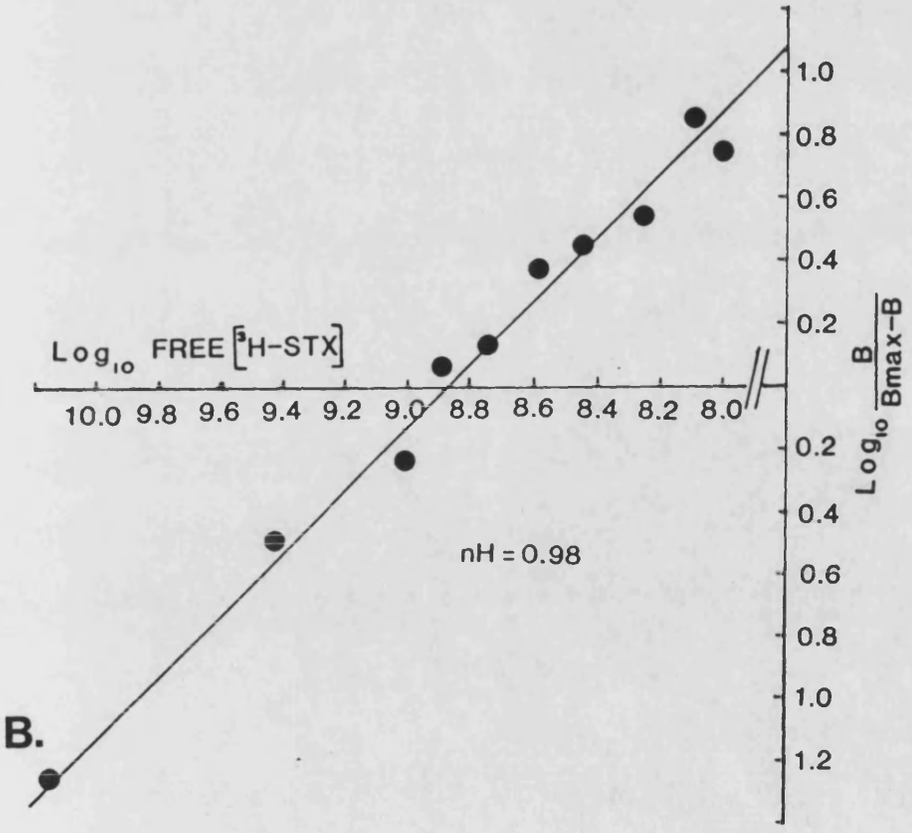
Log₁₀ FREE [³H-STX]
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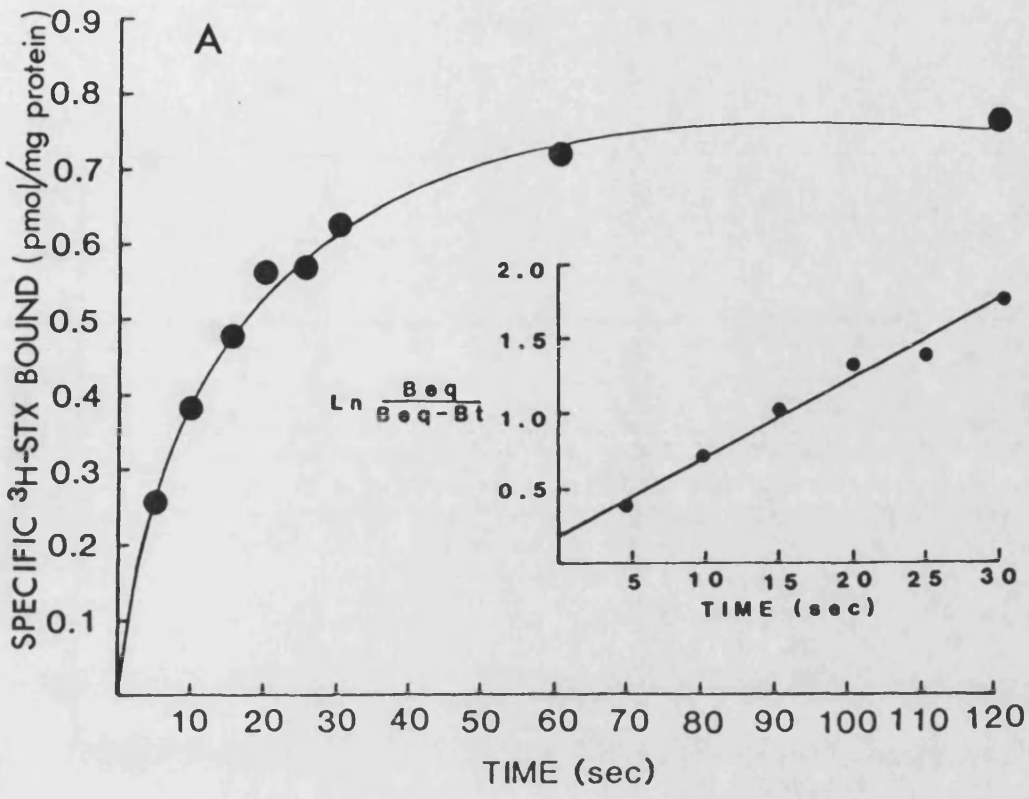
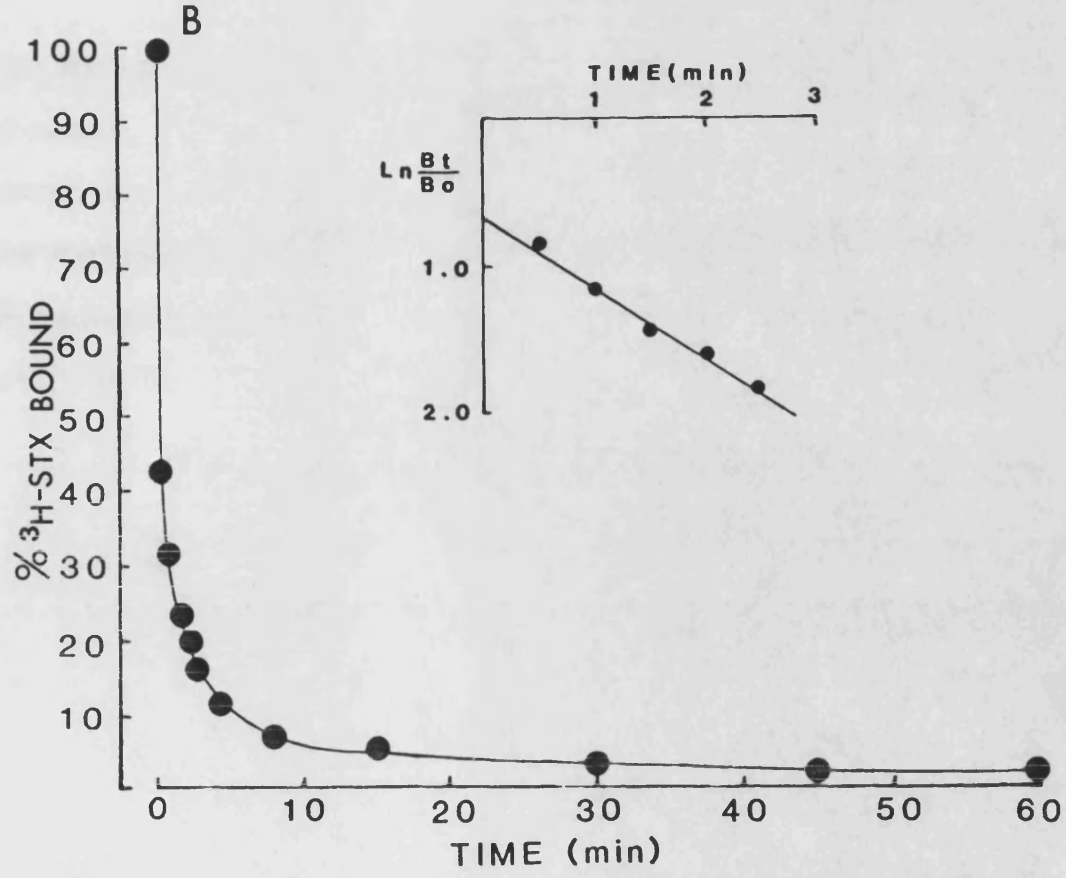
Log₁₀ B/(Bmax−B)

FIGURE 3.4. *Kinetics of the [^{3}H]STX interaction with the* Manduca *CNS receptor.* (A) Association time course for the specific binding of [^{3}H]STX to *Manduca* CNS. Linearised representation of the data (inset) gives $K_1 = 11.3 \times 10^6\ M^{-1}sec^{-1}$. (B) Dissociation time course for [^{3}H]STX binding. Linearised representation of the data (inset) gives $K_{-1} = 7.8 \times 10^{-3}\ sec^{-1}$. The K_D calculated from this kinetic data (K_{-1}/K_1) = 0.70 nM. These experiments were performed using 1 nM [^{3}H]STX.


A
SPECIFIC ^{3}H-STX BOUND (pmol/mg protein)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
10 20 30 40 50 60 70 80 90 100 110 120
TIME (sec)
Ln Beq / Beq-Bt
2.0
1.5
1.0
0.5
5 10 15 20 25 30
TIME (sec)


B
% ^{3}H-STX BOUND
100
90
80
70
60
50
40
30
20
10
0 10 20 30 40 50 60
TIME (min)
Ln Bt / Bo
TIME(min)
1 2 3
1.0
2.0

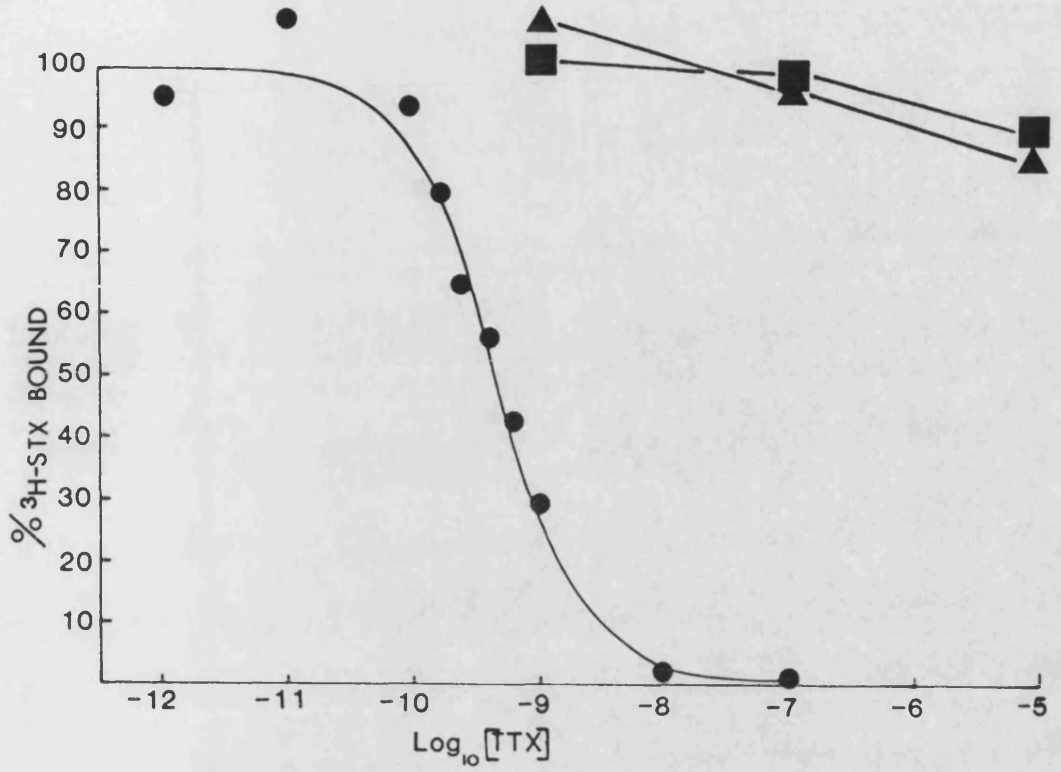


FIGURE 3.5. *Competitive inhibition of [^{3}H]STX binding to* Manduca *CNS by tetrodotoxin.* TTX (●) displaces bound [^{3}H]STX with an IC_{50} of 0.68 nM. In contrast, deltamethrin (▲) and permethrin (■) have little effect on [^{3}H]STX binding, even at relatively high concentrations.

The results were normalised to 100% of specific binding, which was determined with 1 nM [^{3}H]STX.

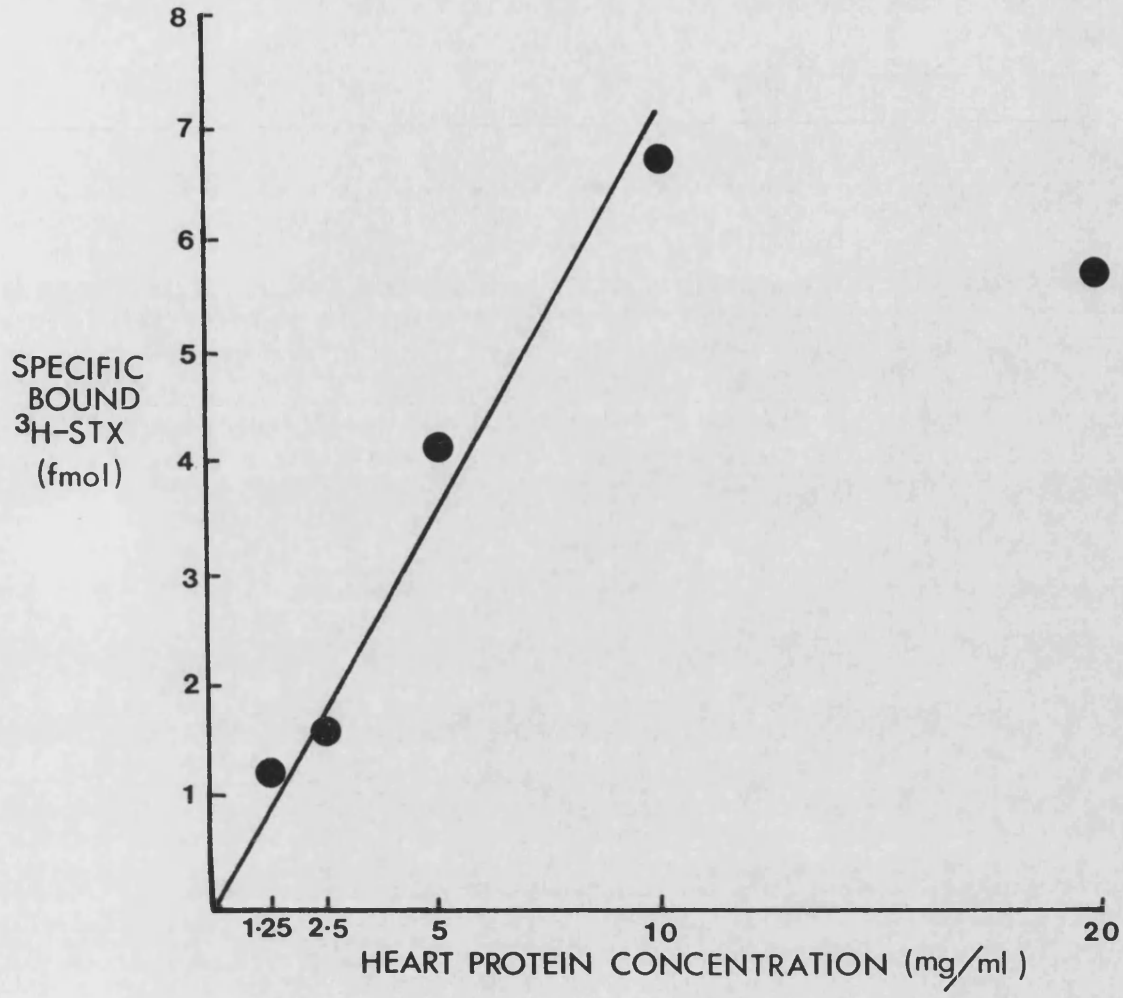


FIGURE 3.6. *The dependence of specific [^{3}H]STX binding to* Manduca *heart homogenate on the total protein concentration.* The binding of [^{3}H]STX (10 nM) is linear up to a concentration of 10 mg/ml protein. A concentration of 4-4.5 mg/ml was used in subsequent experiments.

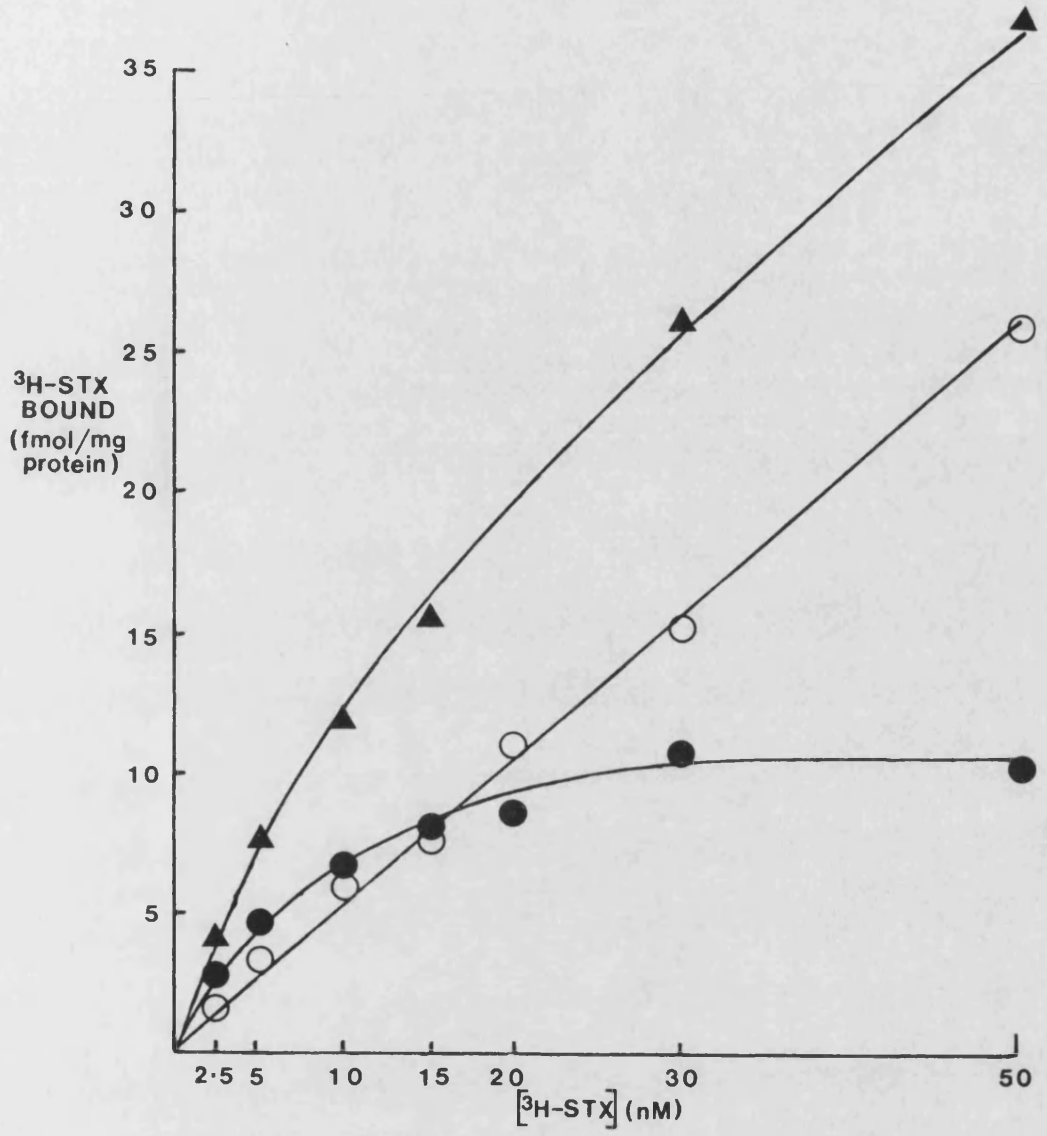
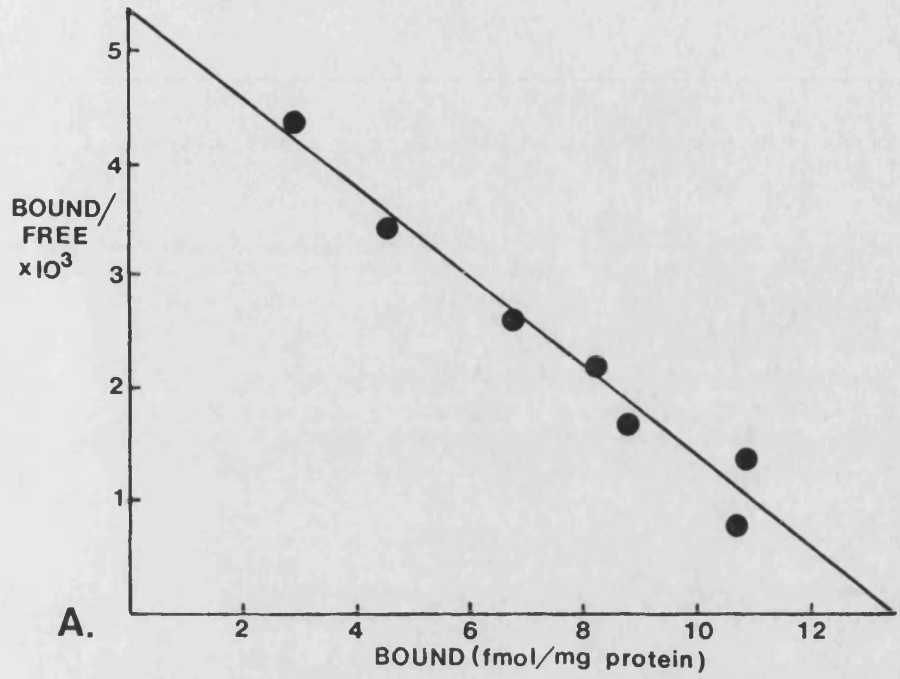


FIGURE 3.7. *Saturable binding of [^{3}H]STX to* Manduca *heart homogenate.* Specific binding (●) was determined as the difference between total binding (▲) and non-specific binding (○). The data points are the means of triplicate determinations from a single representative experiment, which was performed twice.

FIGURE 3.8. *Linearised representation of the equilibrium binding of [^{3}H]STX to* Manduca *heart.* (A) Scatchard transformation of the specific [^{3}H]STX binding data shown in Fig. 3.7. For this experiment K_D and B_{max} values were 9.7 nM and 13.4 fmol/mg protein, respectively. (B) Hill plot of same data. A Hill coefficient of 0.97 indicates a single class of non-interacting binding sites.


BOUND/
FREE
x10³
5
4
3
2
1
A.
2
4
6
8
10
12
BOUND (fmol/mg protein)

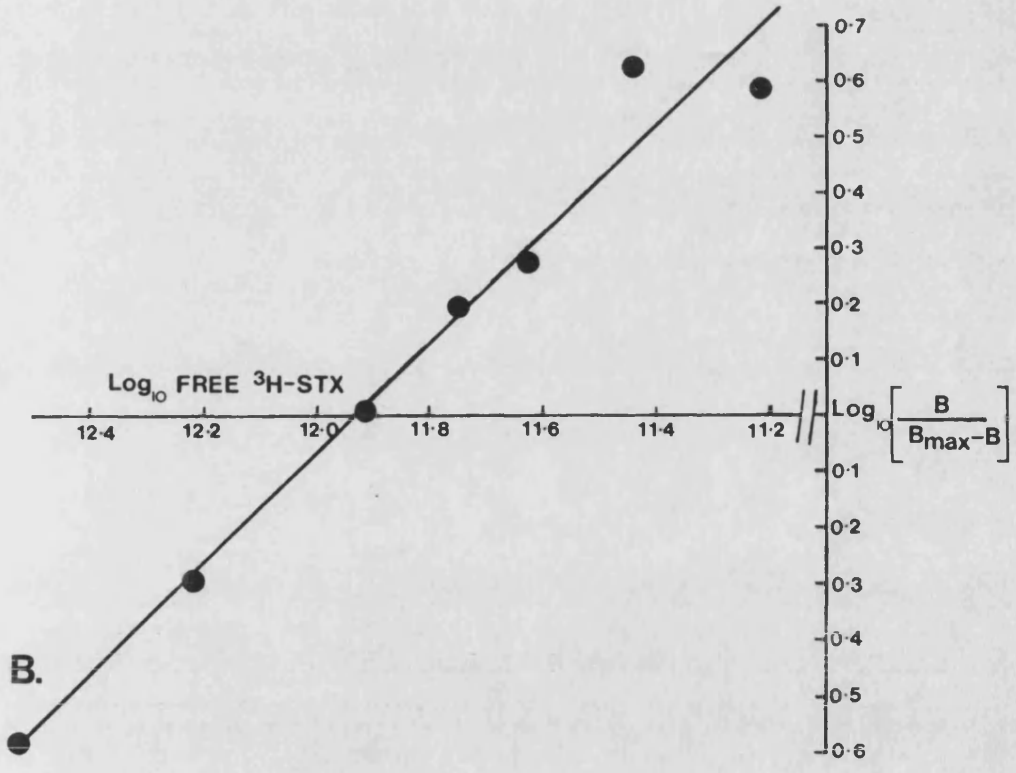
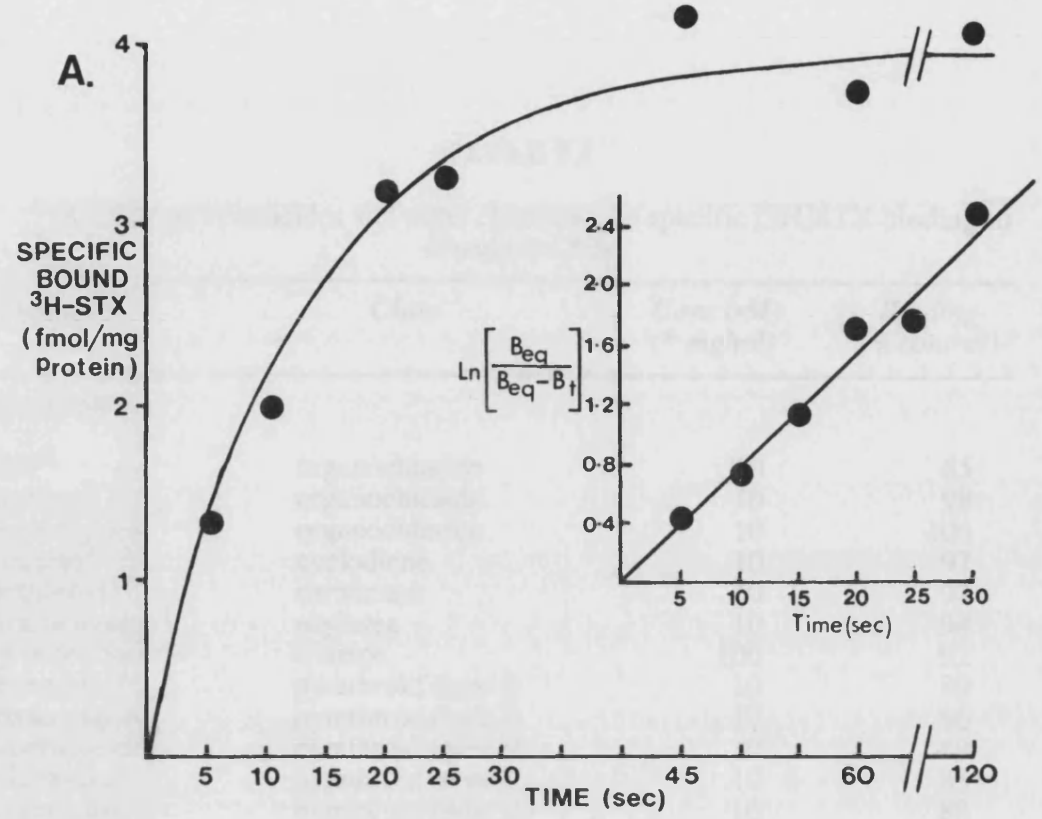

Log₁₀ FREE ³H-STX
12·4
12·2
12·0
11·8
11·6
11·4
11·2
Log₁₀ [B / Bmax−B]
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0·1
0·2
0·3
0·4
0·5
0·6
B.

FIGURE 3.9. *Kinetics of the [^{3}H]STX interaction with the heart receptor.* (A) Association time course for specific binding of [^{3}H]STX to *Manduca* heart homogenate. The results shown are from a single representative experiment, which was performed twice. Linearised representation of data gives $K_1 = 2.42 \times 10^6$ $M^{-1}sec^{-1}$, which was calculated as the mean of both association experiments. (B) Dissociation time course for [^{3}H]STX binding to the same tissue. The graph represents the pooled data from three independent experiments. Linearised representation of data (inset) gives $K_{-1} = 9.59 \times 10^{-3}$ sec^{-1}. (Although only a single regression line is indicated, K_{-1} was in fact calculated independently for each experiment to yield a mean value). The K_D calculated from the kinetic data (K_{-1}/K_1) = 3.96 nM.

All experiments were performed using 10 nM [^{3}H]STX.


A.
SPECIFIC BOUND ^{3}H-STX (fmol/mg Protein)
4
3
2
1
5 10 15 20 25 30 45 60 120
TIME (sec)
$Ln\left[\frac{B_{eq}}{B_{eq}-B_t}\right]$
2·4
2·0
1·6
1·2
0·8
0·4
5 10 15 20 25 30
Time (sec)

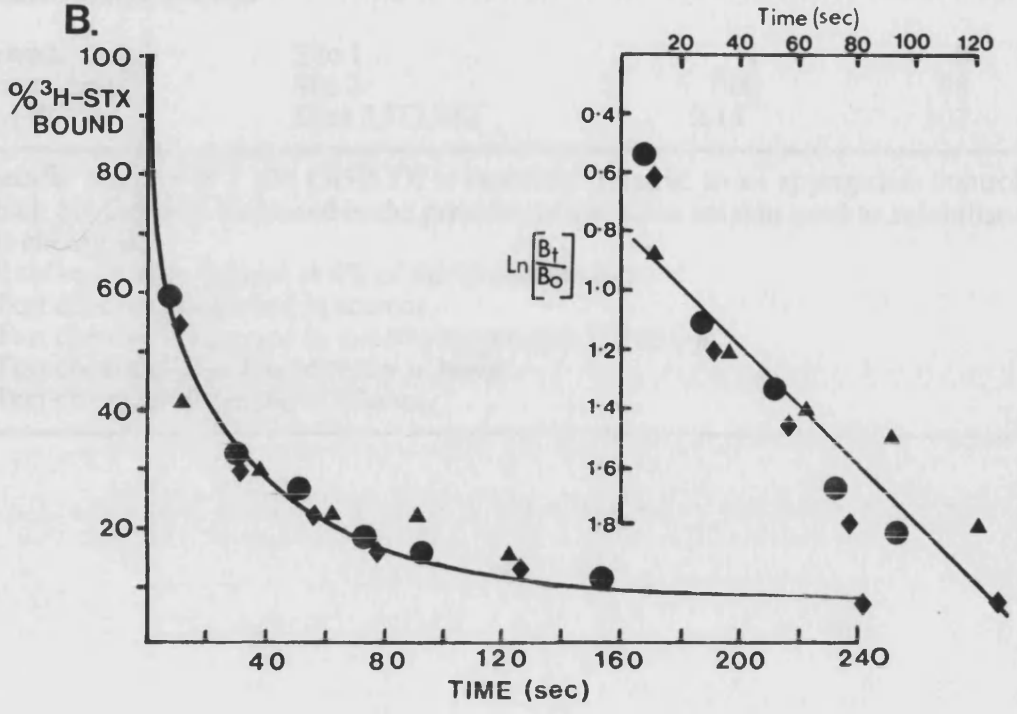

B.
% ^{3}H-STX BOUND
100
80
60
40
20
40 80 120 160 200 240
TIME (sec)
Time (sec)
20 40 60 80 100 120
$Ln\left[\frac{B_t}{B_o}\right]$
0·4
0·6
0·8
1·0
1·2
1·4
1·6
1·8

TABLE 3.1

The effect of insecticides and other chemicals on specific [3H]STX binding to *Manduca* CNS

Compound	*Class*	*Conc.(μM) (* mg/ml)*	*Binding (%control)*
Insecticide			
DDT[1]	organochloride	10	85
Lindane[1]	organochloride	10	98
Chloropyrifos[1]	organochloride	10	106
Dieldrin[1]	cyclodiene	10	97
Methomyl[1]	carbamate	10	97
Diflubenzuron[1]	acylurea	10	94
Cyromazine[2]	triazine	100	92
Permethrin[1]	pyrethroid (type I)	10	89
Tetramethrin[1]	pyrethroid (type I)	10	96
Cypermethrin[1]	pyrethroid (type II)	10	89
Deltamethrin[1]	pyrethroid (type II)	10	85
Fenpropathrin[1]	pyrethroid (type II)	10	88
Sodium channel toxins			
Tetrodotoxin[3]	Site 1	1	0
Veratridine[4]	Site 2	300	88
Lqq-ScVn[3]	Sites 3,IT1,IT2	0.15*	102

Specific binding of 1 nM [3H]STX is expressed relative to an appropriate control in which binding was measured in the presence of the same solvent used to solubilise the test chemical.
All solvents were present at 4% of the incubation mix.
[1] Test chemical dispersed in acetone.
[2] Test chemical dispersed in dimethylsulphoxide (DMSO).
[3] Test chemical dissolved directly in buffer.
[4] Test chemical dispersed in ethanol.

CHAPTER 4

THE BINDING OF [^{3}H]BTX-B TO *MANDUCA SEXTA* NERVE CORD HOMOGENATE

4.1 Introduction

Site 2 on the voltage-dependant sodium channel binds a class of compounds collectively known as the alkaloid toxins, which include batrachotoxin (BTX), veratridine (VTD) and aconitine (ACN) (see Catterall, 1980 and General introduction, Section 1.4). These toxins modify the channel in a number of ways, but their primary action is to completely inhibit the inactivation mechanism, causing a steady-state sodium conductance through the channel once it has been opened. The binding of these effectors at site 2 has received particular attention, because this site has been shown to interact with other toxin binding sites on the channel. The best defined of these is the apparent cooperativity between sites 2 and 3. Ion flux studies with neuroblastoma cells have shown that an α-scorpion toxin (α-ScTX) from *Leiurus quinquestriatus* enhances the $^{22}Na^+$ uptake caused by the alkaloid toxins (Catterall, 1975, 1977). The examination of this interaction was confined almost exclusively to ion flux studies until the development of [^{3}H]Batrachotoxinin A-20-α-benzoate ([^{3}H]BTX-B), a synthetic analogue of BTX with virtually identical pharmacological properties (Brown *et al.*, 1981). This ligand has proved to be an important tool in the study of sodium channel pharmacology. The cooperative effects between sites 2 and 3 observed in the ion flux studies are caused by an increase in the affinity of

[^{3}H]BTX-B for its receptor during α-ScTX occupancy of site 3. In binding studies this interaction manifests itself as an enhancement of specific [^{3}H]BTX-B binding at non-saturating concentrations (Catterall *et al.*, 1981).

This same effect is now used as part of the characterisation of new sites for recently discovered sodium channel toxins, for example, a polypeptide toxin from the marine snail *Conus striatus* (Gonoi *et al.*, 1987) and a polyether from the dinoflagellate *Ptychodiscus brevis* (Sharkey *et al.*, 1987). The enhancing effect of these toxins on [^{3}H]BTX-B binding affinity presumably reflects the fact that BTX interacts preferentially with the open configuration of the sodium channel, as demonstrated in electrophysiological studies, where repetitive depolarisation of nerve preparations causes the much more rapid appearance of BTX modified channels (Khodorov, 1985). As is the case for α-ScTX, novel toxins which increase [^{3}H]BTX-B binding affinity also considerably depress channel inactivation (Gonoi *et al.*, 1987; Huang *et al.*, 1984), allowing the channels to remain open for longer.

Interpretation of binding data involving radiolabelled pyrethroids has proved to be difficult due to the very large non-specific binding component observed with these highly lipophilic molecules (Soderlund *et al.*, 1983). However, their primary action is very similar to α-ScTX in that they depress sodium channel inactivation (see General Introduction, Section 1.2.2.1). It is therefore perhaps not surprising that pyrethroids have recently been shown to considerably enhance specific [^{3}H]BTX-B binding in mammalian microsomes (Lazdunski *et al.*, 1988; Lombet *et al.*, 1988), and the exploitation of this interaction is proving to be important in different areas of pyrethroid research. Autoradiographic studies have employed this effect to map for the first time pyrethroid binding sites in sections of rat brain (Lazdunski *et al.*, 1988; Lombet *et al.*, 1988). Furthermore the increase in [^{3}H]BTX-B binding affinity is only apparent with the toxic pyrethroid stereoisomers (Brown *et al.*, 1988). There is also

some suggestion that type I and type II pyrethroids can be distinguished on the basis of their effects on [^{3}H]BTX-B binding (Brown and Olsen, 1984).

The technique has now been extended to the study of insect neuronal tissue. Comparison between data from insect and mammalian preparations reveals that on the basis of the concentration of pyrethroid required to produce half-maximal enhancement of [^{3}H]BTX-B binding, the apparent affinity of pyrethroids for housefly head membranes (Pauron *et al.*, 1989) is two orders of magnitude greater than their affinity for rat brain synaptosomes. This information could have important implications regarding the relative pyrethroid toxicity in mammals and insects, which has been generally assumed to be mainly a function of differences in rates of metabolism (Abernathy and Casida, 1973; Casida *et al.*, 1983).

Using this technique, Pauron *et al.* (1989) have also made a significant contribution to the study of the mechanism of *kdr* resistance, since they have shown that compared to preparations from susceptible housefly strains, the synergistic effect of pyrethroids on [^{3}H]BTX-B binding is dramatically reduced in *kdr* resistant strains. This is probably the first direct evidence to indicate that *kdr* resistance is due to an alteration of the sodium channel pyrethroid binding site.

Studies using mammalian cell cultures and rat brain synaptosomes have shown that pyrethroids enhance the $^{22}Na^+$ uptake stimulated by the alkaloid toxins BTX and VTD (Jaques *et al.*, 1980; Bloomquist and Soderlund, 1988). Furthermore deltamethrin has been shown to potentiate VTD stimulated transmitter release in synaptosomes prepared from *Periplaneta americana* nervous tissue (Nicholson *et al.*, 1987). These results are entirely consistent with the pyrethroid induced increase in [^{3}H]BTX-B binding affinity described above. However, although there was good evidence that pyrethroids and VTD both acted at the level of the sodium channel at

the neuromuscular junction of *Manduca sexta*, there was no apparent synergism between these agents in that preparation (see Chapter 2).

In view of these unexpected results in *Manduca* neuromuscular junction, it seemed appropriate to attempt to characterise [^{3}H]BTX-B binding in that insect. For this, the *Manduca* nerve cord homogenate preparation, which has already been shown to possess a relatively high density of sodium channels (see chapter 3), was used.

4.2. Methods

4.2.1. Chemicals

Chemicals were obtained as follows: *Leiurus quinquestriatus* scorpion venom (ScVN), purified Toxin-II from the anemone *Anemonia sulcata* (ATX-II), tetrodotoxin (TTX) and veratridine (VTD) were purchased from Sigma Chemical Company. [^{3}H]BTX-B (specific activity 42.7 Ci/mmol) was from New England Nuclear. Cypermethrin and permethrin were gifts from Shell Research Limited.

4.2.2. [^{3}H]BTX-B binding to rat brain

Experiments were initially carried out to ensure that specific [^{3}H]BTX-B binding could be achieved using the filtration assay procedures which had already been developed and successfully employed in the study of [^{3}H]STX binding to *Manduca* nerve cord and heart homogenate (see Chapter 3).

Rat brain synaptosomes were prepared following the procedure of Creveling *et al.* (1983). Approximately 1 gram of freshly dissected rat cerebral cortex was homogenised in two volumes of incubation buffer (130 mM choline chloride, 5.5 mM

glucose, 0.8 mM $MgSO_4$, 5.4 mM KCl and 50 mM Hepes buffer, adjusted to pH 7.4 with Tris base) using ten strokes of a hand-held loose fitting glass-glass homogeniser. The homogenate was diluted with a further two volumes of incubation buffer and centrifuged at 1000*g* for 15 minutes at 4°C. The supernatant was discarded and the pellet resuspended in 5 ml of fresh incubation buffer by repetitive pipetting using a pasteur pipette.

The incubation conditions and assay media used were also those of Creveling *et al.* (1983). Binding was initiated by the addition of 10 nM [^{3}H]BTX-B to aliquots of the rat brain preparation to make up a total volume of 250 µl. The effect of including 0.15 mg/ml scorpion venom (ScVN) and both 0.15 mg/ml ScVN and 1 µM TTX together was also assessed. All treatments were prepared in triplicate and incubated at 37°C for 30 minutes. The binding reaction was terminated by rapid dilution of the incubation mixture with 3 ml of ice cold wash medium (163 mM choline chloride, 5 mM Hepes, 1.8 mM $CaCl_2$ and 0.8 mM $MgSO_4$, adjusted to pH 7.4 with Tris base). Bound ligand was separated from free by filtration under vacuum through Whatman GF/C glass fibre filters (pre-soaked in 0.3% polyethylenimine) which were washed immediately with a further 5 ml of wash medium. The filters were then suspended in 5 ml scintillation fluid (Optiphase Safe) and radioactivity counted using the tritium window of a Rackbeta liquid scintillation spectrometer.

Non-specific binding was determined in parallel experiments in which 300 µM VTD was added. Specific binding is defined as the difference between total and non-specific binding.

4.2.3. [^{3}H]BTX-B binding to Manduca *CNS homogenate*

Entire nerve cords from final instar larvae were prepared in a tight fitting hand-held homogeniser as described for [^{3}H]STX binding experiments (Chapter 3, Section

3.2.2). The protein concentration was adjusted to approximately 0.5-0.6 mg/ml in a final assay volume of 135 µl. A concentration of 10 nM [^{3}H]BTX-B was initially shown to be too low to obtain a clear signal, and so 50 nM was routinely used in these experiments.

Experiments to find conditions for optimal binding were carried out by systematically determining specific binding under a series of different conditions, including changes in incubation buffers, incubation temperature and manipulation of the nerve cord preparation. In each case assessments were also made of the effects of including presumed saturating concentrations of ScVN (0.15 mg/ml), TTX (1µM) and a mixture of both toxins together. All buffers were adjusted to pH 7.4. The assay procedures employed were the same as those already described for [^{3}H]BTX-B binding to rat brain vesicles.

Experiments to test the effects of pyrethroids employed the appropriate carrier solvent controls and were performed in 3 ml glass test tubes (rather than the polypropylene tubes normally used).

4.2.4. Measurement of protein quantity

The protein content of the homogenate was determined using a BCA Protein Assay Kit.

4.2.5. Electron microscopy of Manduca *nerve cord homogenate*

Two nerve cords were dissected from fifth instar *Manduca* larvae as described and prepared in the same glass-glass homogeniser as was used for the binding assay. The homogenate was suspended in 50 mM potassium phosphate buffer (pH 7.4) containing 2.5% gluteraldehyde at 4°C for 18 hours. The sample was washed twice

(by briefly spinning the homogenate in an eppendorf centrifuge and resuspending the pellet in fresh buffer), and then resuspended in buffer containing 1% osmium tetroxide for 1 hour at room temperature. The sample was washed as described and the pellet resuspended in 100 mM cacodylate (pH 7.4) containing 1% tannic acid for 30 minutes. After a further wash the pellet was dehydrated through a series of increasing acetone concentrations and finally embedded in LR white resin (London Resin Co, Woking, Surrey). Sections were made on a Reichert ultramicrotome using a diamond knife, floated onto copper grids (Balzers Union, Berkhamsted, Herts) and stained with 2% uranyl acetate for 7 minutes in the dark. After washing with water, sections were further stained with Reynolds' lead citrate (7 minutes). After a final wash, sections were allowed to dry at room temperature. They were examined and photographed using a JEOL 1200EX transmission electron microscope operated at 100 KV.

4.3. Results

4.3.1. [^{3}H]BTX-B binding to rat brain vesicles

The results illustrated in Fig. 4.1 show that under the conditions employed in these experiments, [^{3}H]BTX-B is specifically bound to rat brain membranes, and that considerable enhancement of this specific [^{3}H]BTX-B binding occurs in the presence of scorpion venom (0.15 mg/ml). Addition of both ScVN and TTX (1 μM) together caused a further potentiation of the response. The effect of TTX is presumably due to prevention of depolarisation by the exclusion of sodium ions still present in the vesicular preparation. These results are consistent with those of Creveling *et al.* (1983), and show that the filtration assay employed in this study is effective in detecting specific [^{3}H]BTX-B binding.

4.3.2. [^{3}H]BTX-B binding to Manduca *CNS homogenate*

4.3.2.1. Effect of incubation medium

Table 4.1 shows the effects of various incubation media on the proportion of specifically bound [^{3}H]BTX-B to *Manduca* CNS homogenate incubated at 37°C for 40 minutes. [^{3}H]BTX-B did not bind specifically in 50 mM potassium phosphate buffer. The use of sodium phosphate buffer allowed specific binding to be measured, but only in the presence of TTX (1 μM). The proportion of specifically bound [^{3}H]BTX-B was increased in a higher concentration of sodium phosphate buffer. The incubation and wash media of Creveling *et al.* (1983) proved to be most successful, with specific binding being about 37% of the total in the presence of TTX, and only marginally lower in its absence. The results imply that [^{3}H]BTX-B is only capable of specific binding in media which can support a membrane potential, which is consistent with the current theory of batrachotoxin mode of action. The Creveling incubation and wash media were used in all subsequent experiments with the addition of 1 μM TTX in the incubation medium.

4.3.2.2. Other treatments

[^{3}H]BTX-B did not bind specifically to CNS homogenate prepared from nerve cords which had been previously frozen, possibly indicating that either sodium channel function or the ion-pumping mechanisms necessary for maintaining membrane potential (or both) are disrupted on freezing and thawing.

No specific [^{3}H]BTX-B was evident when reaction mixtures were incubated at 22°C (room temperature) for up to 55 minutes, suggesting that either more time was required for equilibrium to occur, or that a critical temperature must be reached before

the specific binding interaction can take place. However this was not investigated further and a temperature of 37°C was used in all subsequent experiments.

There was no specific [^{3}H]BTX-B binding to CNS homogenates which had been further manipulated by centrifugation at 1500*g* for 15 minutes and then resuspended in fresh binding medium. This was probably due to the reduction in the quantity of CNS material (which was detected later by protein assay) as a result of this extra procedure. This was not investigated further.

4.3.2.3. Optimum tissue concentration and time to equilibrium

Having found an incubation medium which would allow measurement of specific [^{3}H]BTX-B binding it was necessary to establish the time needed to reach equilibrium for the interaction. Binding was found to be maximal after 30 minutes and so the incubation time of 45 minutes (which had been used in the previous experiments) was adequate for the reaction to equilibrate.

A protein concentration of 0.2-0.3 mg/ml gave an excellent specific binding signal for [^{3}H]STX binding to the *Manduca* CNS preparation used here (Verdon *et al.*, 1988; see also Chapter 3, Section 3.3.1.1). With [^{3}H]BTX-B experiments, however, this protein concentration proved to yield an inadequate ratio of specific to non-specific binding. The [^{3}H]BTX-B binding signal was improved when the tissue concentration was increased two-fold from that used for [^{3}H]STX binding to the *Manduca* nerve cord preparation. Specific [^{3}H]BTX-B binding is linear over this range (Fig. 4.2) and so a protein concentration of 0.5-0.6 mg/ml (corresponding to approximately a quarter of a nerve cord per assay tube) was used in all experiments.

4.3.2.4. Saturation experiments

The concentration dependence of specific [^{3}H]BTX-B binding to *Manduca* CNS homogenate was investigated in an attempt to establish the affinity of [^{3}H]BTX-B for its receptor site and the specific [^{3}H]BTX-B binding capacity of the tissue. The binding curves for two such experiments are illustrated in Fig. 4.3, and it is evident that saturation could not be achieved at [^{3}H]BTX-B concentrations of up to 150 nM (Fig. 4.3B). Caution is necessary in interpreting such data but some analysis can nevertheless be made. Both the initial shape of the incomplete binding isotherms and the Scatchard representations of this data (insets) are indicative of positive cooperativity. B_{max} values are impossible to predict, but at the highest concentrations tested (150 nM) the quantity of [^{3}H]BTX-B specifically bound was 0.77 pmol/mg protein. The standard concentration of 50 nM [^{3}H]BTX-B used in most experiments was presumably well below the value of K_D for the binding reaction. Unfortunately it was not possible to extend the range of [^{3}H]BTX-B concentrations examined to higher concentrations.

4.3.2.5. The interaction of [^{3}H]BTX-B with scorpion venom (ScVN) and anemone toxin II (ATX-II)

Specific [^{3}H]BTX-B binding was not observed under any conditions which included ScVN at presumed saturating concentrations (Table 4.1). Specific [^{3}H]BTX-B binding in media containing TTX was abolished by the further addition of ScVN, indicating that the inability of [^{3}H]BTX-B to bind to its receptor site is not a secondary effect caused by α-ScTX-induced vesicular depolarisation. The effect of ScVN was primarily to reduce the total [^{3}H]BTX-B binding, rather than to increase the non-specific binding, which shows that the active component in the venom exerts

its effects directly at the [^{3}H]BTX-B binding site. Due to the limited availability of [^{3}H]BTX-B, the concentration dependence of this effect could not be evaluated.

In contrast to these results, purified ATX-II at a concentration of 1.5 μM had no significant effect on [^{3}H]BTX-B binding (Table 4.2).

4.3.2.6. Effects of pyrethroid insecticides

Table 4.2 shows that permethrin (type I pyrethroid) and cypermethrin (type II) at a concentration of 10 μM had no significant effect on the specific binding of [^{3}H]BTX-B to *Manduca* nerve cord homogenate. Mixtures of pyrethroids (10 μM) and ATX-II (1.5 μM) appeared to be similarly ineffective. For each treatment the value represents the mean of a single experiment performed in triplicate. It would have been preferable to confirm these results by repeating this set of important experiments. Again, a shortage of [^{3}H]BTX-B prevented further investigation.

4.3.3. Electron microscopy of Manduca *nerve cord homogenate.*

Electron microscopic examination of the *Manduca* CNS preparation identical to those used in the binding studies revealed the presence of numerous membrane-bound vesicles (Fig. 4.4). These were mostly spheroidal in shape, and in the size range 200-500 nm in diameter, although smaller vesicles were also present.

4.4. Discussion

The results indicate that [^{3}H]BTX-B has a relatively low affinity for its receptor site in *Manduca* CNS, since saturation was not approached at concentrations of up to 150 nM. Preparations from other species have also been shown to have high K_D values

for the [^{3}H]BTX-B-receptor interaction in the absence of α-ScTX or other effectors known to enhance [^{3}H]BTX-B binding affinity. For example, K_D values of 140 nM in electric eel electroplax (McNeal and Daly, 1986), 460 nM in rat brain vesicles (Lazdunski *et al.*, 1988), 700 nM in mouse brain homogenate (Brown *et al.*, 1981) and 140 nM for a housefly head membrane preparation (Soderlund *et al.*, 1989) have been reported.

Analysis of the incomplete binding isotherms in this study suggests positive cooperativity of [^{3}H]BTX-B binding to *Manduca* CNS tissue. Data from all previous work on [3H]BTX-B binding has yielded linear Scatchard plots, indicating a single class of non-interacting binding sites in both mammalian (Creveling *et al.*, 1983; Catterall *et al.*, 1981; Brown, 1986; Sheldon *et al.*, 1986) and insect tissues (Soderlund *et al.*, 1989). These plots normally give B_{max} values which are in agreement with the [^{3}H]STX binding capacity of the same tissue (although in some cases they yield a rather lower B_{max}: see Catterall *et al.*, 1981). The estimated B_{max} for [^{3}H]STX sites in *Manduca* CNS is 3.41 pmol/mg protein (see Chapter 3, Section 3.3.1.2) compared with a value of 0.77 pmol/mg of specifically bound [^{3}H]BTX-B at a concentration of 150 nM. This may indicate that only a small proportion of the specific binding sites are occupied at this concentration, especially if [^{3}H]BTX-B binding is indeed positively cooperative, in which case multiple binding sites would be expected on each channel.

The proportion of [^{3}H]BTX-B specifically bound in the different media tested, and the effect of TTX, suggests that a membrane potential is required across the sodium channel for specific [^{3}H]BTX-B binding to occur. The presence of membrane-bound vesicles in the *Manduca* CNS homogenate does not prove that such a membrane potential exists, but the existence of intact vesicles is a prerequisite for a membrane potential to be present. Dependence of binding on membrane potential implies that [^{3}H]BTX-B does not bind to the inactivated configuration of the sodium channel in

Manduca nerve tissue, as has been shown to be the case in other tissues by electrophysiological studies (Khodorov, 1985). Low temperature prevents specific binding in caterpillar CNS as is the case with mammalian tissue (Brown, 1986). In both rat brain and caterpillar CNS preparations, the binding of [^{3}H]BTX-B alone is of low affinity (see above) and includes a large non-specific component. However the similarities between the binding properties of [^{3}H]BTX-B to *Manduca* CNS and other nerve tissue preparations appear to end here.

The inclusion of *Lqq* scorpion venom in the incubation buffer completely inhibited specific [^{3}H]BTX-B binding to *Manduca* CNS under all conditions tested. This is in marked contrast to the situation in mammalian preparations, where a saturating concentration of α-ScTX (which is a component of *Lqq*-ScVN) increases the specific binding of [^{3}H]BTX-B dramatically (10 to 20-fold) by reducing the K_D of the interaction (Catterall *et al.*, 1981). In the present study, crude venom, rather than pure α-ScTX, was used. However, in rat brain, the same effect is achieved without fractionation of the crude venom (Creveling *et al.*, 1983).

Since the specific to non-specific binding ratio is otherwise very low (Brown *et al.*, 1981) the enhancement of site 2 binding by a site 3 toxin has been exploited in the study of the pharmacology of the [^{3}H]BTX-B receptor site (e.g. Postma and Catterall, 1984; Willow and Catterall, 1982). Models had been proposed before the development of [^{3}H]BTX-B which predicted this interaction on the basis that in $^{22}Na^+$ flux studies the effect of BTX was synergized by the presence of α-ScTX (Catterall, 1977). The primary action of α-ScTX is to inhibit sodium channel inactivation (Catterall, 1980), thus prolonging the open time of the channel. That BTX interacts much more rapidly with the conducting state of the sodium channel (due to a much greater affinity) would appear to be fundamental to its mode of action (Khodorov, 1985). By developing this argument it is possible to speculate on the reason why *Lqq* scorpion venom inhibited [^{3}H]BTX-B binding to the *Manduca* CNS

homogenate. As well as an α-ScTX, *Lqq* venom contains at least two distinct insect specific components (Zlotkin *et al.*, 1985; see also General Introduction, Section 1.5). An "excitatory" toxin (*Lqq*-IT1) acts in much the same way as α-ScTX, but there is also a "depressant" insect toxin (*Lqq*-IT2), which, in cockroach axonal preparations, apparently acts by suppressing the Na^+ current (Zlotkin *et al.*, 1985; see also Zlotkin, 1986). If this action of *Lqq*-IT2 is caused by the inhibition of sodium channel activation, then [^{3}H]BTX-B would not be expected to bind with high affinity. However, this possibility still does not explain the complete absence of specific [^{3}H]BTX-B binding in the presence of ScVN.

Other feasible explanations for this phenomenon include the competitive displacement of [^{3}H]BTX-B at site 2, although this seems improbable due to the structural dissimilarity between [^{3}H]BTX-B and the polypeptide components of scorpion venom. However, the insecticidal N-alkylamides, which bear no chemical resemblance to [^{3}H]BTX-B, do appear to displace [^{3}H]BTX-B competitively (Ottea *et al.*, 1989, 1990). Inhibition of specific binding may otherwise occur via an allosteric mechanism. Such a direct allosteric displacement of [^{3}H]BTX-B by TTX and STX has been demonstrated under certain conditions (Brown, 1986), and this is probably the mechanism of [^{3}H]BTX-B displacement by local anaesthetics (Creveling *et al.*, 1983). However, although any of these explanations may be possible, it is important to note that even if one of the components of *Lqq* scorpion venom is the cause of the abolition of [^{3}H]BTX-B binding in *Manduca* CNS, this cannot be a general insect phenomenon, since specific [^{3}H]BTX-B binding to housefly head membranes is enhanced in the presence of *Lqq*-ScVN (Soderlund *et al.*, 1989).

Although there remains some controversy regarding the precise binding site of ATX-II (Vincent *et al.*, 1980; see also Strichartz *et al.*, 1987), it is generally accepted that this toxin binds to the same receptor (site 3) as α-ScTX (Catterall, 1980). Whatever

the case, ATX-II has a similar effect to α-ScTX in enhancing specific [^{3}H]BTX-B binding in mammalian preparations (Catterall *et al.*, 1981; Sheldon *et al.*, 1986; Lazdunski *et al.*, 1988). It has been shown in this study that ATX-II has no significant effect on [^{3}H]BTX-B binding to *Manduca* CNS homogenate. The concentration of ATX-II used in this experiment (1.5 μM) is somewhat lower than that normally added to ensure saturation (for example, Lazdunski *et al.*, 1988) but is nevertheless within the range required for enhancement of [^{3}H]BTX-B to rat brain synaptosomes (Catterall *et al.*, 1981) and cardiac tissue (Sheldon *et al.*, 1986). The results may therefore indicate that *Manduca* sodium channels lack binding site 3. This is consistent with the observation that neither sea anemone toxins nor α-scorpion toxins have any potentiating effect on [^{3}H]BTX-B binding to housefly head membranes (Pauron *et al.*, 1989). Furthermore, α-scorpion toxins are inactive in blowfly larvae injection experiments (Zlotkin, 1986). If these species do not possess a conventional site 3, then the scorpion venom induced enhancement of [^{3}H]BTX-B binding to housefly head preparations (Soderlund *et al.*, 1989) could be explained by the presence of the insect "excitatory" toxin *Lqq*-IT1, which acts in a similar manner to the α-ScTX, but interacts with a different binding site (Gordon *et al.*, 1984). However the fact that ATX-II slows sodium channel inactivation in cockroach giant interneurones (Pelhate and Sattelle, 1982) shows that the absence of site 3 cannot be a general phenomenon in insects.

The pyrethroid insecticides permethrin and cypermethrin are the only toxins known to inhibit sodium channel inactivation which have been shown to act directly at the level of the *Manduca* sodium channel (see Chapter 2). Therefore, the finding that neither of these insecticides is capable of enhancing [^{3}H]BTX-B binding to *Manduca* CNS would suggest that allosteric enhancement of site 2 binding is not possible in this preparation. If this is the case, the evidence that *Manduca* sodium channels do not possess site 3 is weakened, since the lack of interaction with pyrethroids indicates that [^{3}H]BTX-B has no greater affinity for the open configuration of the *Manduca* sodium

channel. In contrast, concentration dependent enhancement of [^{3}H]BTX-B binding has been demonstrated with both type I and II pyrethroids in rat brain microsomes (Lazdunski *et al.*, 1988; Lombet *et al.*, 1988). Such effects have also been reported for DDT in similar preparations (Payne and Soderlund, 1989). Furthermore, the effects are amplified by the addition of site 3 toxins (Lazdunski *et al.*, 1988; Lombet *et al.*, 1988). The results reported for similar experiments performed on housefly head membranes are rather confusing. Pauron *et al.* (1989) showed that pyrethroids enhance [^{3}H]BTX-B binding but that this effect was not potentiated by the presence of site 3 toxins. However, using essentially the same preparation, Soderlund *et al.* (1989) demonstrated a ScVN induced increase in [^{3}H]BTX-B binding which is unaffected by pyrethroids. Further work is therefore required to investigate these discrepancies.

It is feasible that specific [^{3}H]BTX-B binding to this preparation is to a site not associated with sodium channels, since it has been shown that both BTX and VTD interact with calcium channels in mammalian neuroblastoma cells (Romey and Lazdunski, 1982). This possibility cannot at present be dismissed, although no specific [^{3}H]BTX-B binding to *Manduca* muscle homogenate was detected (results not shown). This tissue contains at least one class of calcium channel.

The electron microscopy studies described here show that the *Manduca* CNS homogenate used in this study is vesicular in nature. However, despite the fact that [^{3}H]BTX-B binding is optimal in conditions capable of maintaining a membrane potential, the presence of such a potential is still assumed and is not under experimental control. This is particularly relevant to the study of batrachotoxin binding, since a membrane potential has always been thought to be a prerequisite for the open state of the sodium channel. It remains possible therefore that the results reported here are unusual because the specific [^{3}H]BTX-B binding is to an inactivated state of the sodium channel, with an unfavourable conformation at site 2.

Although the results indicate that this is unlikely (specific [^{3}H]BTX-B binding was entirely TTX-dependent in sodium phosphate buffer) the existence of a membrane potential was not determined experimentally. This could be done using techniques with radiolabelled synthetic cations (see Breer and Knipper, 1985). Recent evidence, however, casts doubt on the necessity of a membrane potential for [^{3}H]BTX-B to bind to its receptor. In housefly head membrane preparations, binding occurs equally well in both high potassium and sodium based buffers. [^{3}H]BTX-B binding was also unaffected by TTX in high sodium based buffers (Soderlund *et al.*, 1989). These results suggest that this preparation consisted of membrane fragments or depolarised vesicles. Furthermore, both scorpion venom (Soderlund *et al.*, 1989) and pyrethroid insecticides (Pauron *et al.*, 1989) potentiate this binding considerably.

This evidence, coupled with the fact that the assay conditions employed in the present study were capable of facilitating conventional [^{3}H]BTX-B binding in rat brain vesicles, and that the *Manduca* nerve cord homogenate is a rich source of sodium channels as determined by [^{3}H]STX binding (see Chapter 3), implies that the unusual [^{3}H]BTX-B binding found in this study is not artefact. If this is the case, site 2 of the *Manduca* CNS sodium channel is functionally distinct from that of the other species so far studied. This would presumably imply a structural difference.

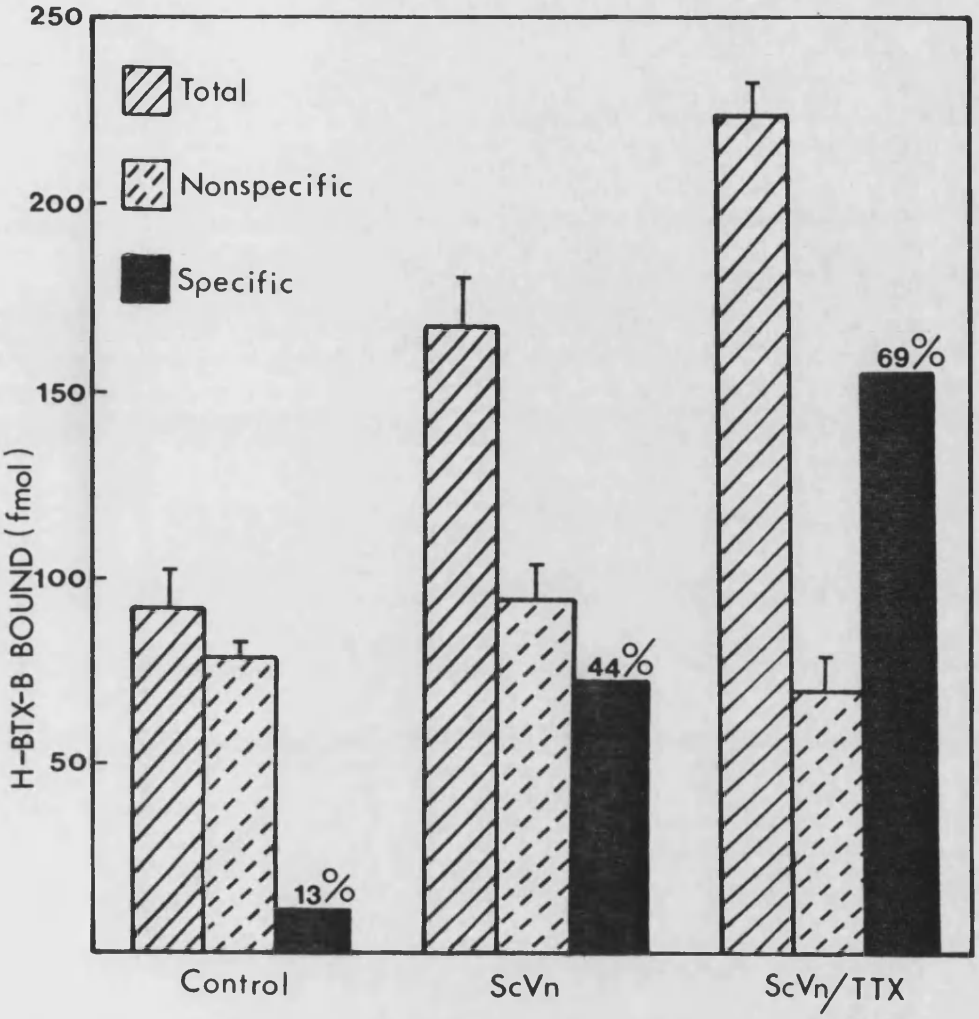


FIGURE 4.1. *The effects of ScVN (0.15 mg/ml) and ScVN plus TTX (1 μM) on the binding of 10 nM [^{3}H]BTX-B to rat brain synaptosomes.* Percentage values indicate the proportion of specifically bound [^{3}H]BTX-B.

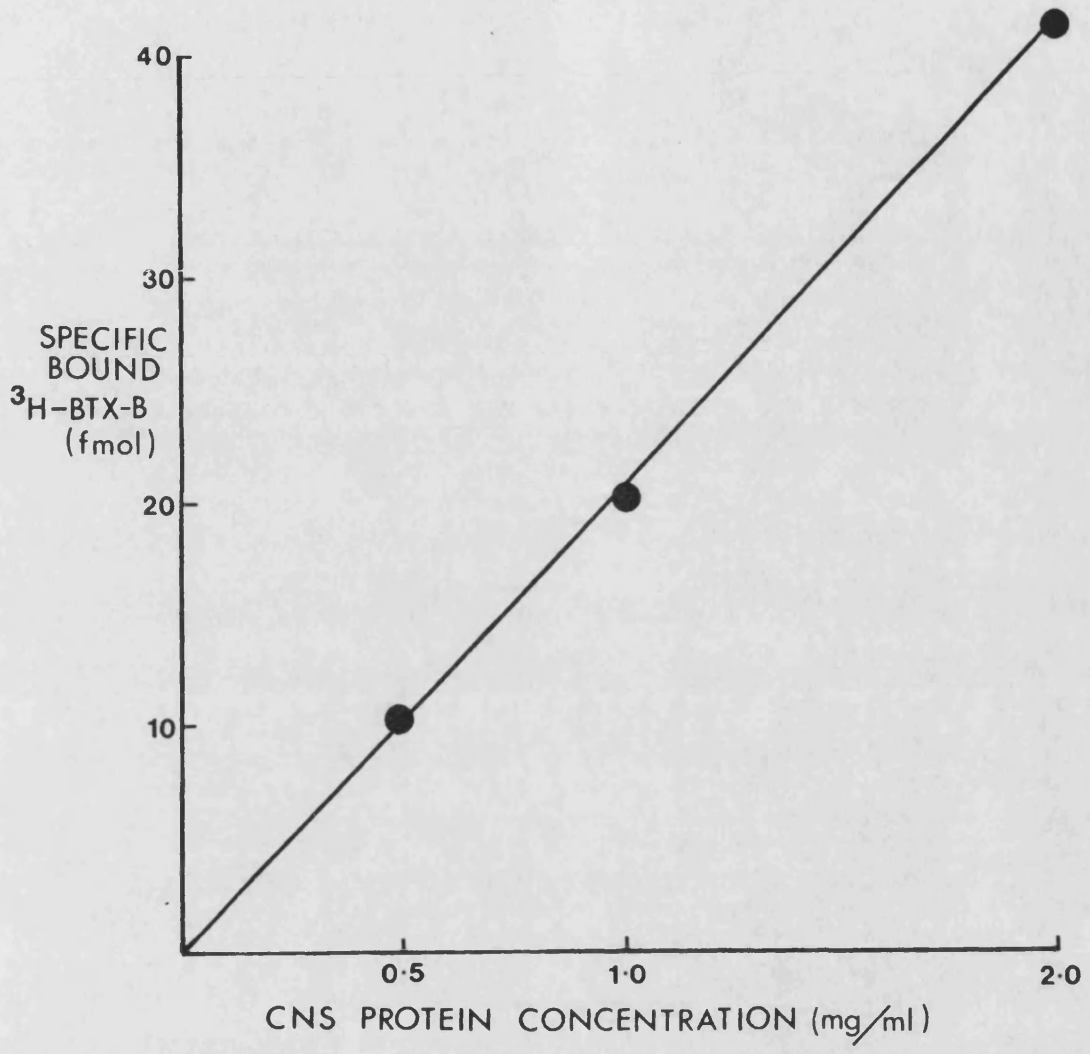
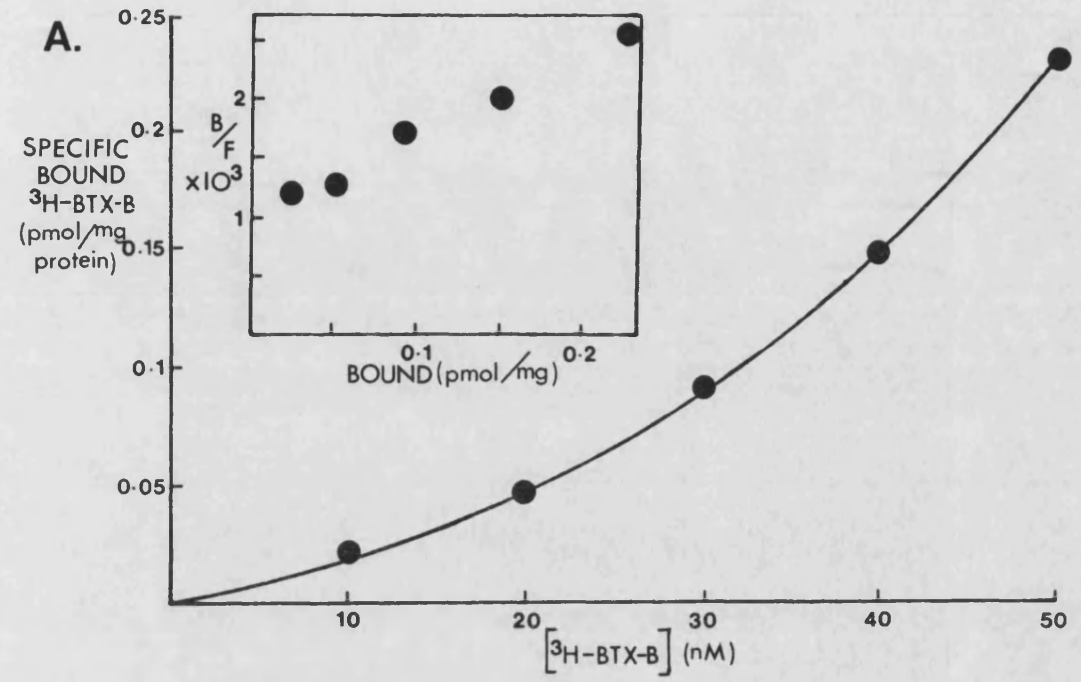
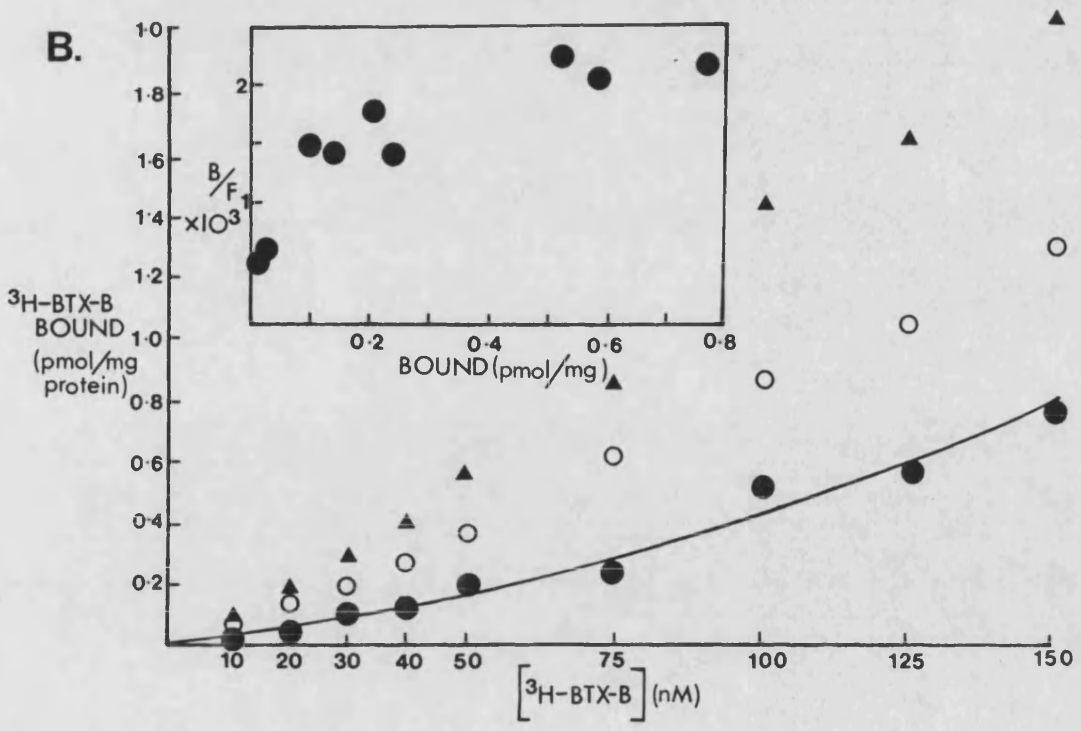


FIGURE 4.2. *Dependence of the specific binding of [^{3}H]BTX-B (50 nM) to* Manduca *CNS on total protein concentration.* The assay is independent of protein concentrations up to at least 2.0 mg/ml. A concentration of 0.5-0.6 mg/ml was routinely used.

FIGURE 4.3. *The dependence of specific [^{3}H]BTX-B binding on [^{3}H]BTX-B concentration.* (A) and (B) represent two different experiments. Binding was not saturable at concentrations of up to 150 nM. Scatchard transformations (insets) appear to indicate positive cooperativity. Specific binding (●) is the difference between the total (▲) and non-specific (○) binding.


A.
SPECIFIC BOUND ^{3}H-BTX-B (pmol/mg protein)
0·25
0·2
0·15
0·1
0·05
B/F ×10^3
2
1
0·1
0·2
BOUND (pmol/mg)
10
20
30
40
50
[^{3}H-BTX-B] (nM)


B.
^{3}H-BTX-B BOUND (pmol/mg protein)
1·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
B/F ×10^3
2
1
0·2
0·4
0·6
0·8
BOUND (pmol/mg)
10
20
30
40
50
75
100
125
150
[^{3}H-BTX-B] (nM)

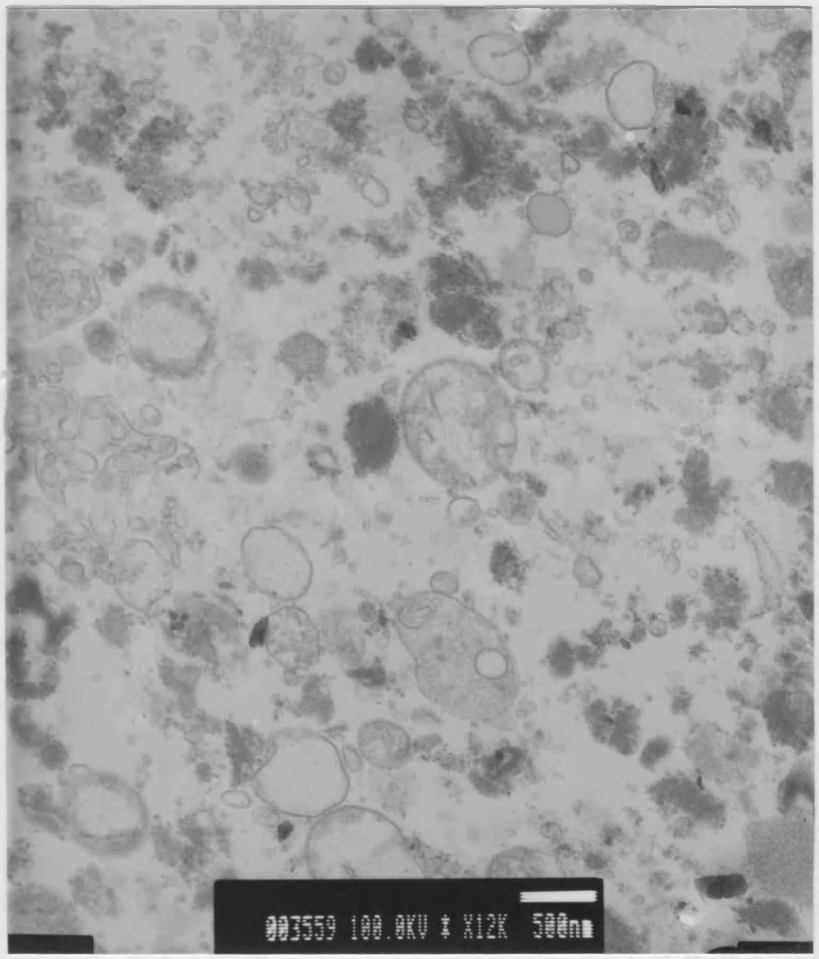


FIGURE 4.4. *Electron micrograph of the crude* Manduca *CNS homogenate.* The preparation apparently contains numerous membrane bound vesicles.

TABLE 4.1

Effect of incubation medium and sodium channel toxins on specific [^{3}H]BTX-B binding to *Manduca* CNS.

Incubation Medium	*Control*	*TTX (1μM)*	*ScVN (1.5mg/ml)*	*TTX+ScVN*
Potassium (100mM)	0.66(±2.2)	3.0(±5.1)	0.4(±0.58)	1.1(±4.6)
Sodium (50mM)	4.3(±5.8)	**16.0(±3.7)**	0.6(±3.6)	3.6(±2.1)
Sodium (100mM)	N.D	**21.4(±3.5)**	N.D	N.D
Creveling	**35.2(±2.3)**	**36.6(±4.5)**	1.0(±3.5)	2.4(±2.7)

Values indicate the proportion of specifically bound [3H]BTX-B (50 nM) expressed as a percentage of the total binding. Standard deviations result from three independent experiments done under identical conditions, except for the Creveling medium, where experiments were repeated four times.

TABLE 4.2

Effect of pyrethroids and ATX-II on specific [^{3}H]BTX-B binding to Manduca CNS.

Treatment	*Specific [^{3}H]BTX-B binding (% control)*
Cypermthrin (10μM)	93.8 (±19.1)
Permethrin (10μM)	107.2 (±22.0)
ATX-II (1.5μM)	89.7 (±6.3)
ATX-II + cypermethrin	83.7(±13.0)
ATX-II + permethrin	74.6 (±5.6)

Experiments with ATX-II employed [^{3}H]BTX-B at a concentration of 10 nM. The effects of pyrethroids alone were measurd with 50 nM [^{3}H]BTX-B.
Standard deviations result from a single experiment performed in triplicate.

CHAPTER 5

STUDIES ON THE GENE ENCODING THE VOLTAGE-SENSITIVE SODIUM CHANNEL OF *MANDUCA SEXTA*

5.1. Introduction

Hodgson and Huxley's (1952) original analysis of the squid giant-axon action potential, from which several important features of the functional nature of the sodium channel were inferred, depended upon the newly developed voltage-clamp technique. A significant advance since that time has been the introduction of single channel recording (Sakmann and Neher, 1983). This powerful technique can be used to study purified sodium channels which have been reconstituted into artificial lipid bilayers. In combination with the use of specific sodium channel neurotoxins, it has allowed for the detailed and unambiguous analysis of channel gating and ion permeation events (Hartshorne *et al.*, 1986). However, although these new techniques represent a quantum leap when compared to conventional voltage-clamp technology, the additional information concerning the molecular mechanisms of channel function which can be inferred from such measurements is limited. To make further progress it is necessary to know something about the protein structure of the channel.

A considerable breakthrough therefore occurred when Noda *et al.* (1984) isolated and sequenced a cDNA encoding the eel electroplax sodium channel. Fragments of this cDNA have been used in homology hybridization studies to isolate three similar but

distinct cDNAs which are thought to be sodium channel α-subunits from rat brain (Noda *et al.*, 1986a; Kayano *et al.*, 1988) and partial sequences of a putative sodium channel from *Drosophila* (Salkoff *et al.*, 1987a,b; Okamoto *et al.*, 1987). A number of homologous sodium channel cDNAs have recently been isolated from mammalian excitable tissues using similar techniques (Auld *et al.*, 1988; Trimmer *et al.*, 1989; Rogart *et al.*, 1989; Sills *et al.*, 1989; Kallen *et al.*, 1990). Those which have been expressed in *Xenopus* oocytes and analysed under voltage-clamp conditions display the unique biophysical characteristics expected of sodium channels, thus proving that the isolated gene actually encodes a sodium channel, and demonstrating that the presence of this single subunit (the α-subunit) is sufficient to produce a functional channel (*e.g.* Noda *et al.*, 1986b; Goldin *et al.*, 1986; Suzuki *et al.*, 1988).

The deduced primary sequences of these sodium channel proteins, each about 2000 amino acids in length, gave the first insights into the possible structural conformation of the channel (see Fig 5.1). Within each sequence, computer aided analysis revealed the presence of four internally homologous domains (referred to here as I, II, III and IV). Each is composed of about 300 amino acids, and they are connected together by "linker" sequences of varying length. Hydropathy profiles reveal that each homology domain contains an arrangement of six relatively hydrophobic segments (referred to as S1-S6) each of which could be predicted to span the membrane as an α-helix. Direct comparisons made between sodium channel primary sequences of different species have shown that the most highly conserved regions are situated within these homology domains and the III-IV linker sequence (see Salkoff *et al.*, 1987b), and so it is within these regions that the structures responsible for the unique functioning of sodium channels are thought to lie.

Several models have been proposed concerning the secondary and tertiary structure of the sodium channel peptide (Noda *et al.*, 1986a; Greenblatt *et al.*, 1985; Guy and Seetharamulu, 1986; Guy, 1988), which contain certain common features. Most

models propose six or eight membrane spanning segments in each homology domain. Potential sites for glycosylation and phosphorylation assign the segments containing them as being extracellular and intracellular, respectively, and the four homology domains are assumed to adopt a pseudo-symmetrical arrangement around a central transmembrane pore. However, the models are based on empirical predictions of secondary structure adjusted to accommodate for functional expectations of the channel, and are therefore inherently uncertain. An advantage of theoretical analysis is that the resulting models can be tested experimentally and subsequently modified as more information becomes available, thus giving an increasingly accurate representation of the molecular functioning of the sodium channel.

One such experimental approach to this is channel-protein engineering. Greenblatt *et al.* (1985) have proposed that the S3 segments of each homology domain form the lining of the channel pore. To test this hypothesis, a 22 amino acid sequence from the region IS3 was synthesized and subsequently shown to spontaneously form cation selective channels in artificial lipid bilayers, with a single channel conductance similar to that of the authentic rat brain sodium channel (Oiki *et al.*, 1988).

In another approach, site-directed mutagenesis (in conjunction with voltage- and patch-clamp analysis in the oocyte expression system) has been employed in the study of several aspects of sodium channel function. For example, the S4 segments of the homology domains contain highly conserved positively charged residues at every third position, and a common feature of all models proposed so far is that these regions represent the voltage sensing mechanism associated with channel activation. Mutating the channels by reducing positive charge in this region (neutralising arginine or lysine by replacing with glutamine, or reversing the charge by substituting with glutamic acid) reduces the steepness of the voltage dependence of activation (Stühmer *et al.*, 1989). This would be expected if positive charges in this region represent gating charges, as predicted by molecular models. However, such results

are not necessarily restricted to mutations involving charge. A neutral amino acid change (leucine to phenylalanine) in S4 of domain III causes a shift in the current-voltage relationship towards more positive potentials. This too probably arises from a change in the voltage dependence of channel activation (Auld *et al.*, 1990). This discovery was not predicted, but resulted from a fortuitous cloning error, and illustrates the complexity of the mechanisms involved in channel activation.

Research into the sodium channel inactivation mechanism has also been furthered by the use of primary sequence data. It had been proposed that the structure required for inactivation is a cytoplasmic domain, based on the knowledge that in squid giant-axons, sodium channel inactivation is rapidly abolished by the internal perfusion of pronase, and so it must therefore be readily accessible to proteolytic degradation (Armstrong *et al.*, 1973). Of all the regions designated as being cytoplasmic by the molecular models, only the III-IV linker sequence is highly conserved. In fact, most other putative internally exposed regions are very poorly conserved. The involvement of the III-IV linker in inactivation has been confirmed using several approaches. Voltage-clamp studies on rat muscle showed that an internally applied antibody directed against an 18 amino acid sequence from this region slows sodium channel inactivation (Vassilev *et al.*, 1988). No such effects are observed with antibodies directed against other cytoplasmic regions. This strategy has been extended to patch-clamp analysis of cultured rat brain neurons, where the same antibody almost completely inhibits the inactivation of single sodium currents (Vassilev *et al.*, 1989). In both cases, the effects were greatly reduced when the membranes were depolarised before exposure to this antibody, indicating that the sequence recognised by the antibody is inaccessible when the channel adopts the inactivated conformation. The evidence that this part of the channel peptide is required for inactivation is also supported by site-directed mutagenesis experiments. Cleavage of the III-IV linker causes a drastic reduction in the inactivation of sodium currents when the mutated channels are expressed in *Xenopus* oocytes (Stühmer *et al.*, 1989). The effects on

macroscopic currents are very similar to those reported for channels treated with internally applied peptidases (Armstrong *et al.*, 1973).

Although the evidence for the involvement of the III-IV linker with inactivation is compelling, the molecular mechanism involved remains speculative. The generally held view was first proposed by Armstrong and Bezanilla (1977). On the basis of their results they suggested that inactivation is caused by a positively charged cytoplasmic domain that is electrostatically attracted to a negatively charged site which becomes exposed after activation, thus blocking the channel from the cytoplasmic side. In accord with this, the III-IV linker includes a highly positively charged region with one triplet and three pairs of lysines which are entirely conserved in most sodium channel primary sequences so far isolated. However, when this idea was tested by Moorman *et al.* (1990), reducing positive charge by replacing groups of two or three lysines with asparagines actually resulted in faster rather than slower inactivation of the sodium current. Furthermore, conversion of the only arginine in the region to glutamic acid (thereby reversing the charge) delayed activation. Most of the lysine residues which were substituted in this study were included in the peptide used to generate the antibody which prevents inactivation (Vassilev *et al.*, 1988, 1989). These results were therefore contrary to predictions and so the III-IV linker presumably has a more complicated effect on channel gating than had previously been expected.

Inactivation of the sodium channel is also modified by some polypeptide toxins, including α-scorpion toxin (α-ScTX) which bind to receptor site 3 (Catterall, 1980; see also General introduction, Section 1.4). Immunoprecipitation of cleaved sodium channel fragments with antibodies raised against various specific segments of the channel primary sequence have been used to identify the general location of the site of covalent attachment of photo-affinity labelled α-ScTX (Tejedor and Catterall, 1988). This site has now been characterised more precisely, since antibodies which

recognise the sequences between the transmembrane segments S5 and S6 of domains I and IV inhibit radiolabelled α-ScTX binding in an apparently competitive manner (Thomsen and Catterall, 1989). This finding suggests that these sequences comprise at least part of receptor site 3 and supports models of membrane topology in which homology domains I and IV are adjacent, and the short sequences between S5 and S6 are located externally. Extracellularly applied antibodies also modify inactivation, although the results are at present difficult to interpret since the antibodies were specific for the S4 region, and channel activation was virtually unaffected (Meiri *et al.*, 1987).

Taken together, the evidence to date indicates that the molecular mechanism of sodium channel gating is much more complicated than previously anticipated. However, the cyclical process of experimentation and theoretical analysis will undoubtedly lead to a more rapid understanding of sodium channel molecular structure and function than could ever have been achieved before the development of techniques to isolate specific genes, and thereby deduce primary sequence data.

Pyrethroid insecticides act in a similar manner to α-ScTX, in that they retard sodium channel inactivation (see General Introduction, Section 1.2.2.1). Due to their hydrophobic properties, it is likely that pyrethroids bind to a site in the lipid phase adjacent to the sodium channel, but little else is known about the pyrethroid receptor site. However there is now good evidence that *kdr* resistant insects are insensitive to pyrethroids due to a modification of the binding site (Pauron *et al.*, 1989). Site-directed mutagenesis experiments have shown that minor alterations in sodium channel structure can lead to distinct changes in functional properties. If the modification at the pyrethroid binding site in *kdr* insects is relatively minor, then the location of the pyrethroid receptor might easily be determined by comparison of sodium channel sequences from susceptible and *kdr* resistant insects. This would yield information concerning the molecular mechanism of channel inactivation, but

moreover, the detection of this site and the amino acid alterations which render *kdr* sodium channels inaccessible to pyrethroids has enormous implications in the field of insecticide research. In conjunction with the development of precise molecular models for the structure and function of the sodium channel, such information could be used in the rational design of novel insecticides. This may prove to be the only viable approach in overcoming the increasing problem of pyrethroid resistance.

Attempts to isolate the *kdr* resistant sodium channel gene from the housefly *Musca domestica* have so far been unsuccessful, and in fact the cloning strategies employed have proved to be unworkable at very early stages of these research programmes (Soderlund and Knipple, 1988; Soderlund *et al.*, 1989). To date, the only putative sodium channel genes isolated from insects are from *Drosophila* (Salkoff *et al.*, 1987b; Okamoto *et al.*, 1988; Ramaswani and Tanouye, 1989; Loughney *et al.*, 1989). It would be useful to obtain such sequence data from a Lepidopteran species, because there is growing evidence that economically important representatives from this order are developing *kdr*-type resistance to pyrethroids (Gammon, 1980; Nicholson and Miller, 1985; Hama *et al.*, 1987; Ahmad *et al.*, 1989).

An attempt was therefore made here to isolate the sodium channel gene from a *Manduca* cDNA library, since such a clone would prove useful in screening libraries prepared from other Lepidoptera due to the high degree of conservation which would be expected. Furthermore, the primary structure of the *Manduca* sodium channel is of interest in its own right, because there is electrophysiological (see Chapter 2) and pharmacological evidence (see Chapter 4) that it may be functionally and therefore structurally distinct from other sodium channels.

5.2. Methods

5.2.1. Chemicals

All enzymes were purchased from Northumbria Biologicals Ltd. All other chemicals were of the highest grade available. Recipies for media and solutions not shown in the text are given in Appendix III.

5.2.2. Preparation of DNA

5.2.2.1. Preparation of rat genomic DNA

Rat genomic DNA was isolated using a modification of the method described by Herrmann and Frischauf (1987) for the preparation of DNA from whole organs. The spleen (approximately 1 gram in weight) from a freshly killed rat was cut into small pieces and immediately frozen over dry ice, then crushed to powder under liquid nitrogen using a pre-cooled (-70°C) pestle and mortar. This was transferred to a 400 ml beaker containing 20 ml of buffer solution (100 mM EDTA, 200 mM NaCl, 100 µg/ml DNase-free RNase, and 50 mM Tris-Cl; pH 7.9). The mixture was swirled repeatedly to ensure uniform distribution, transferred to a 50 ml polypropylene tube (Falkon) and shaken vigorously for 5 minutes, and then mixed further on a rocking platform for 10 minutes at room temperature. 2 ml of 10% SDS was added and mixed for a further 10 minutes. 1 ml of freshly prepared proteinase K (10 mg/ml in water) was added, mixed first by inversion and then on a rocking platform at 37°C overnight. The solution was then added to a flat-sided vessel containing an equal volume of pre-equilibrated phenol and mixed gently on a rocking platform for 2 hours at room temperature, and then spun in a bench-top centrifuge at 3000 rpm for 10 minutes. The upper aqueous layer was removed and re-extracted with phenol. The upper layer was then centrifuged in a Sorvall SS34 rotor at 9000 rpm for 20 minutes at 25°C, and the

supernatant was dialysed against 2 litres of TE (pH 8.0) at room temperature for two hours and then against fresh TE at 4°C overnight. The solution was then transferred to 50 ml polypropylene tubes containing 0.1 volumes 3 M sodium acetate (pH 6.5) and 0.8 volumes of isopropanol, and mixed by inversion. The rat spleen DNA (which precipitated immediately) was spooled out using a flame sealed pasteur-pipette and dissolved in 3 ml TE by mixing gently on a rotating wheel overnight. A dilute sample was analysed spectrophometrically which indicated a total yield of 6 mg DNA based on the assumption that 1 OD/ml is equivalent to 50 μg of double stranded DNA at 260 nm (Maniatis *et al.*, 1982).

5.2.2.2. Preparation of Manduca *genomic DNA*

DNA extraction from the flight muscles of 25 newly emerged adult moths was performed in exactly the same manner as for rat spleen, except that the precipitate formed by the addition of isopropanol was uniformly dispersed throughout the solution and could not be spooled. The precipitate was therefore gently spun down in an SS34 rotor (5000 rpm for 1 hour) in order to minimise shearing. The pellet was washed in 70% ethanol, allowed to dry and resuspended in 1 ml of TE. The estimated yield was 410 μg.

The extraction procedure described above provided the best quality DNA which could be obtained from *Manduca*, as alternative methods yielded inferior DNA in comparison. However, some doubts were raised as to its suitability for use in Southern blot analysis (see Results). Two further attempts to extract *Manduca* genomic DNA from eviscerated larvae using exactly the same procedure were unsuccessful, yielding no DNA at all in one case and DNA which could not be cut with restriction enzymes in the other.

Alternative methods that were attempted included high speed centrifugation on a caesium chloride cushion following guanidinium thiocyanate lysis of eviscerated larvae (Chirgwin *et al.*, 1979), and urea extraction of DNA from larval body wall muscle using a modified version of a technique used for DNA isolation from plant material (Shure *et al.*, 1983) which has been used successfully for honeybee DNA (J.R. Beeching, University of Bath, personal communication). A method for the extraction of DNA from whole *Drosophila* (Jowett, 1986) also failed to yield DNA from *Manduca* larval nerve cord and pharate adult brain.

5.2.3. Preparation of probes

5.2.3.1. Design and synthesis of oligonucleotide probe (Oligo SC2)

Preliminary studies involved the use of a degenerate oligonucleotide probe (synthesized by V.B. Cockroft, University of Bath) based on a seven amino acid sequence from region IS3 which was completely conserved between rat, eel and the putative fly sodium channel (Salkoff *et al.*, 1987b). This probe however failed to detect any positive clones when used to screen the *Manduca* cDNA library. Oligo(dT), which is used in the construction of cDNA libraries, primes the synthesis of DNA that is heavily biased toward sequences at the 3' end of the template. It is therefore possible that this library contained only partial cDNA sodium channel transcripts, in which case IS3 (near the 5' end of the sodium channel sequence) would not be represented. This factor was taken into consideration in the design of an alternative probe.

Thus, *Oligo SC2* was based on an amino acid sequence from part of IVS6, since this represents the conserved region closest to the 3' end of the sodium channel primary sequence (see Fig. 5.2). The chosen segment was highly conserved between rat and eel, but less so between these species and *Drosophila*. However, due to the

phylogenetic distance between vertebrates and insects, the inferred amino acid sequence from the putative *Drosophila* sodium channel (DSC1) was chosen as the basis for oligonucleotide design (Salkoff *et al.*, 1987b). At the time this effort was initiated, this was the only putative insect sodium channel sequence data available.

A table of *Manduca* codon usage was compiled from the published information on nucleotide and deduced amino acid sequences of *Manduca* genes (Table 5.1). Although only seven such sequences were available at that time it is interesting to note that the codon most frequently utilised for any given amino acid was exactly the same as for *Drosophila*, according to a table derived from over 150 published sequences (*Drosophila* Codon Table Version 5. Supplied by M. Ashburner, University of Cambridge). Using Table 5.1 to predict the most likely *Manduca* codons for the *Drosophila* amino acid sequence in segment IVS6, an antisense oligonucleotide probe of 50 nucleotides in length was designed (*Oligo SC2*, see Fig. 5.2). This was synthesized on an Applied Biosystems 380A DNA Synthesizer, and a yield of approximately 60 nmol was obtained (as measured by absorbence at 260 nm). *Oligo SC2* was sized by electrophoresis of a ^{32}P 5' end-labelled sample (see Section 5.2.4) on a 20% acrylamide gel. After autoradiography, comparison with standards showed it to be approximately 50 nucleotides in length. No further characterisation was attempted.

5.2.3.2. cDNA probes

The cDNA clone *NA8.4* was a gift from Dr. J. Marshall (University of Cambridge). This is a 2,500 base pair (2.5 Kb) fragment of the rat brain cDNA (Rat IIA) isolated by Auld *et al.* (1988), which spans homology domains III and IV (see Fig. 5.1). The sample of *NA8.4* which was received had been subcloned into the plasmid pEMBL.

5.2.3.2.1. Amplification of plasmid DNA and isolation of cDNA NA8.4

Competent TG1 cells were transformed with pEMBL/*NA8.4* plasmid and selected on LB agar plates containing 50 µg/ml ampicillin. Single isolated colonies were grown overnight at 37°C with constant agitation in LB media containing 100 µg/ml ampicillin. The cells were harvested and a plasmid "miniprep" prepared using an alkaline lysis protocol (Miller, 1987). After purification through a Sepharose CL6B column, *NA8.4* was isolated by digestion with *Eco*R1. Insert and vector were separated by electrophoresis on a 1% Low Melting Point agarose gel. The 2.5 Kb fragment was sliced from the gel, and the DNA extracted by the "freeze-squeeze" technique (Tautz and Renz, 1983). Recovery was estimated by electrophoresis of a small sample on an ethidium bromide stained gel and visual comparison of ultraviolet fluorescence intensity with bacteriophage λ standards.

5.2.3.2.2. Isolation of purified NA8.4 *(*NA8.4p*)*

On the basis of results obtained when screening the *Manduca* cDNA library with *NA8.4* as isolated above (see Results, Section 5.3.4.2) it was appropriate to obtain a sample of higher purity *NA8.4* (*NA8.4p*).

A pEMBL/*NA8.4* "maxiprep" yielded large quantities (730 µg) of plasmid DNA after CsCl-centrifugation and DNA isolation according to the procedures of Miller (1987). 30 µg was digested with *Eco*R1 and electrophoresed on a 1% agarose gel run at 1 V/cm for 18 hours to achieve a good separation. The 2.5 Kb fragment was excised, packed into the wells of a 1% Low Melting Point agarose gel and and run once more. The 2.5 Kb bands were removed as before and diluted in water in small aliquots to a final agarose concentration of 0.25%. These were then boiled for 7 minutes and immediately frozen at -20°C. The final concentration of *NA8.4p* was estimated at 4 ng/µl.

5.2.3.3.3. Isolation of the III-IV linker sequence (Li3-4)

A 316 base pair (bp) sequence which comprises both the III-IV homology domain linker sequence and the highly conserved IIIS6 segment is contained within restriction sites for *Pst*I and *Bst*EII (see Fig. 5.1). 40 µg of the pEMBL/*NA8.4* "maxiprep" was therefore treated with these endonucleases and the 316 bp fragment isolated and prepared as described for *NA8.4p* (see above). Recovery was estimated at 2 ng/µl.

5.2.4. Radiolabelling of probes

Oligo SC2 was 5' end-labelled by the transfer of the [^{32}P]phosphate from [γ-^{32}P]ATP using T4 polynucleotide kinase (T4 PNK). 20 pmol of *Oligo SC2*, 5 µl of 10x kinase buffer and 2 units of T4 PNK were added to 100 µCi dried down [γ-^{32}P]ATP (>5000 Ci/mmol) in a final volume of 50 µl. After incubation at 37°C for 30 minutes, 1 µl of 1 mM cold ATP was added and the reaction incubated for a further 5 minutes.

cDNA probes were multi-primer labelled (Fienberg and Vogelstein 1983, 1984). In principle, the denatured cDNA is hybridized to a mixture of random hexanucleotides, and incubated with a mixture of the four deoxynucleotide triphosphates, one of which is radiolabelled ([α-^{32}P]dATP). A complementary strand of DNA is then enzymically synthesized from the 3' end of the hexanucleotide primer by the addition of DNA polymerase I (Klenow), thus incorporating the ^{32}P label. cDNAs which were labelled following the exact methods described by Fienberg and Vogelstein produced probes with very low levels of ^{32}P incorporation, and so in most cases, cDNA labelling was done with a Multi-Prime Labelling Kit (Amersham) using 100 pmol cDNA and 100 µCi [α-^{32}P]dATP (>3000Ci/mmol).

^{32}P incorporation into both cDNA and oligonucleotide probes was determined by allowing 1 µl aliquots of the completed labelling reaction to dry on Whatman DE-81 discs and comparing radioactivity between discs washed in 0.3 M Na_2HPO_4 and unwashed discs. ^{32}P incorporation into probes used for screening was normally greater than 50%. Separation of the probe from unincorporated radiolabel was achieved using a Sephadex G-25 spun column (see Maniatis *et al.*,1982).

5.2.5. Southern blot analysis of genomic DNA

Preliminary small scale studies were carried out using a number of different restriction enzymes to determine their relative ability to digest genomic DNA isolated from rat spleen. Representative examples requiring different salt concentrations for optimum activity were screened to accommodate for the probability of salt contamination in the DNA sample. The reaction volume was then scaled up for the three chosen restriction enzymes such that 10 µg of DNA was digested with 100 units of enzyme in a total volume of 100 µl. The same reaction mixture in double the volume was similarly incubated at 37°C (the optimum temperature for all enzymes chosen) for approximately 6 hours. The reaction was stopped by the addition of 10 mM EDTA and the DNA size-fractionated electrophoretically on an 8 mm thick 1% agarose gel at 1 V/cm for up to 24 hours. Excess agarose was trimmed from the gel such that only DNA fragments larger than the 2 Kb *Hind*III digested λ marker were retained. The gel was then soaked in 0.25 M HCl for 15 minutes to allow for partial DNA hydrolysis. DNA was then denatured in 1.5 M NaCl, 0.5 M NaOH for 30 minutes with constant agitation, and then neutralised in 1.5 M NaCl, 0.5 M Tris-Cl (pH 7.5) for 15 minutes followed by a final treatment in 5x SSC. DNA was transferred overnight by capillary action to nylon membrane (Genescreen-Plus, Du Pont) as described by Maniatis *et al.* (1982) using 5x SSC as the transfer buffer. The gel was then restained with 1 mg/ml ethidium bromide and viewed under an ultraviolet transilluminator to ensure complete transfer of the DNA. The membrane

was allowed to dry at room temperature for 30 minutes, then at 37°C for 3 hours, before being heat sealed in a polythene bag containing 1% SDS, 1 M NaCl, 10% Dextran sulphate and 50 mM Tris-Cl (pH 7.5). Hybridization and wash conditions were the same as those described for library screening (see Section 5.2.8.1).

Southern transfers of *Manduca* genomic DNA (isolated from adult flight muscles using the same methods as for rat spleen DNA) were done in exactly the same manner. Rat and *Manduca* Southern blots were screened in parallel using the same preparation of radiolabelled probe.

5.2.6. Ontogenic appearance of sodium channels as an indicator of mRNA activity

The *Manduca* cDNA library (a gift from Dr. F. Horodyski, University of Washington, Seattle, USA) was constructed using mRNA extracted from day 9-12 developing adult *Manduca* brains (Horodyski *et al.*, 1989). Before screening this library for sodium channel cDNAs it was necessary to test whether sodium channel mRNA is being synthesized at this stage of brain development. This was done by monitoring the expression of sodium channels by measuring [^{3}H]STX binding site density in *Manduca* brains over the corresponding developmental period.

5.2.6.1. Preparation

Pupae were decapitated and the cuticle removed from the region between the eyes to expose the developing adult brain. The preparation was flushed with 50 mM sodium phosphate buffer (pH 7.4) before removing the brain which was transferred to a petri-dish containing fresh buffer on ice. The optic lobes and any fat body were separated under a binocular microscope and discarded. Brains were blot dried, rapidly frozen over dry ice and stored at -40°C. Staging of developing adults was by days after

pupal ecdysis (following Horodyski *et al.*, 1989), and ten dissections were performed for each stage. Dissections took place over a period of about a week until sufficient material was available to determine the development of sodium channel density in a single experiment, in order to eliminate experimental variation.

5.2.6.2. [^{3}H]STX binding assay

The assay was performed in exactly the same manner as described for the determination of [^{3}H]STX capacity in morphologically distinct regions of the nerve cord (see Chapter 3, Section 3.3.1.5).

Protein quantities were determined by the modified Lowry method as described (see Chapter 3, Section 3.2.3).

5.2.7. Preliminary Experiments With Manduca *cDNA library*

The *Manduca* brain cDNA library had been packaged into λZAP II (Stratagene) as described by Horodyski *et al.* (1989). It was supplied with a stab culture of strain XL1-Blue *E. Coli*. The cDNA library and the XL1-Blue cells were transported by air from Seattle on ice. Both were stored at 4°C.

5.2.7.1. Preparation of XL1-Blue cells

A stab of XL1-Blue cells was streaked for single colonies on LB agar plates containing 12.5 μg/ml tetracycline and incubated overnight at 37°C. Plates were sealed and stored at 4°C for no more than two weeks before being discarded. Liquid cultures were prepared by picking a single colony from the LB-tetracycline plate and shaking vigorously overnight at 37°C in 10 ml LB medium supplemented with 0.2% maltose and 10 mM $MgSO_4$. The cells were harvested by spinning in a bench-top

centrifuge at 1000*g* for 10 minutes and resuspended in 5 ml of 10 mM $MgSO_4$. Cells prepared in this manner were always used immediately.

5.2.7.2. Determination of library titre and proportion of recombinants

Assuming a titre of 1.5 x 10^9 plaque forming units per ml (pfu/ml) for the cDNA library stock (data supplied by F. Horodyski) a sample was diluted to 10,000 pfu/ml in SM and then serially diluted through 1000 and then 100 pfu/ml. 100 µl aliquots from each dilution were incubated in 10 ml glass tubes (Oxoid) with 200 µl freshly prepared plating cells at 37°C. After 15 minutes, 50 µl of 0.5 M IPTG (isopropyl-ß-D-thiogalactoside) plus 50 µl of 250 mg/ml X-GAL (5-bromo-4-chloro-3-indoyl-ß-D-galactoside) were added, followed by 2.5 ml of top-agarose. The mixture was then plated immediately onto 90 mm LB-agar plates, allowed to set, then incubated overnight at 37°C. The procedure was carried out in triplicate.

5.2.8. Screening the cDNA library

The library was plated out as above, except that X-GAL and IPTG were not included in the protocol. 240x240 mm plates were used, so the volume of top-agarose and plating cells was scaled up 10-fold to accommodate for the increase in surface area. The cDNA library was plated out at a density of 100,000 pfu per plate, and after an overnight incubation at 37°C the plaques had reached confluence. Master plates were sealed and stored at 4°C. Three master plates were prepared for each round of screening.

5.2.8.1. Primary screen

Nylon filters (Hybond N, Amersham) were cut to size and laid carefully onto the agarose surface of the master plates for 1 minute to allow DNA transfer to occur. The filter was keyed to the plate by means of holes pushed through the filter and agarose with a needle. The DNA which had adsorbed to the filter was denatured (by placing the filter DNA side up on a pad of Whatman 3MM soaked in 1.5 M NaCl, 0.5 M NaOH), neutralised (by transferring filters to 1.5 M NaCl, 0.5 M Tris-Cl; pH 8.0) and then washed in 2x SSC to remove any residual agarose. DNA was fixed to the filter by baking at 80°C for 2 hours. Lifts were taken in duplicate for each master plate. Filters were incubated in glass hybridization tubes with 10 ml prehybridization solution (6x SSC, 5x Denharts solution, 0.5% SDS, 200 μg/ml denatured salmon sperm DNA) in a hybridization oven (Hybaid) at 55°C overnight. ^{32}P labelled probe was mixed with fresh prehybridization solution and added to the hybridization tube containing the filter. This was incubated overnight at 55°C, after which time the temperature was lowered slowly over a period of 4-6 hours to 42°C (i.e. drop hybridization). Filters were removed and washed for 30 minutes in 2x SSC at room temperature, then in 2x SSC, 0.1% SDS for 30 minutes at 45°C. Filters were then wrapped in "clingfilm" to prevent them from drying completely. Autoradiography was done using pre-flashed X-ray film (Fugi) and intensifying screens. If the background signal was high the filters were washed under more stringent conditions and autoradiography repeated.

5.2.8.2. Secondary screen

The hybridization signal was designated positive if it was of much greater intensity than background and appeared on autoradiographs from duplicate filters. Positive plaques were picked with the wide-end of a pasteur-pipette after careful alignment of the autoradiograph with the master plate. Each agar/agarose plug was added to 1 ml

SM containing 50 µl chloroform and allowed to stand at 4°C overnight to allow the phage to diffuse out of the agarose. Phage were then titred onto 90 mm petri-dishes (assuming 5 x 10^8 pfu/ml) and a plate with clearly discrete plaques was chosen for secondary screening (approximately 500 plaques/plate). Nylon filters lifted from these plates were screened with ^{32}P labelled probe and analysed by autoradiography as described.

5.3. Results

5.3.1. Southern blot analysis of genomic DNA

Oligo SC2 did not hybridize to rat Southern blots under any circumstances. However, all of the cDNA probes hybridized to specific fragments of rat genomic DNA with high stringency. Fig. 5.3 shows a Southern blot from rat DNA after hybridization with *Li3-4*, but identical banding patterns were obtained with *NA8.4* and *NA8.4p*. These experiments indicate the presence of several different sodium channel genes in rat genomic DNA, which implies that the different sodium channel subtypes are the products of distinct genes rather than the result of alternative mRNA splicing. The results also show that the protocol used for DNA transfer produced successful Southern blots; that the procedures used to isolate and radiolabel cDNAs produced functional probes; and that the hybridization techniques employed were capable of detecting specific DNA sequences.

None of the probes employed in this study hybridized specifically to Southern blots constructed from *Manduca* genomic DNA, even under conditions of very low stringency. This result could indicate that *Manduca* genomic DNA does not contain sequences which are homologous to the rat IIA sodium channel cDNA, but this possibility seems unlikely.

Analysis of the DNA electrophoresis gels prior to Southern transfer shows that compared to rat DNA, there is a considerable quantity of endonuclease treated *Manduca* DNA which is of comparable molecular weight to the uncut reference DNA (Fig. 5.4). This indicates that even the best quality *Manduca* DNA which could be obtained could not be digested to completion. Under such circumstances, specific DNA sequences would not be expected to aggregate in discrete bands. However, there is visible banding of repeat sequences in digested DNA (Fig 5.4), which indicates that at least some *Manduca* genomic DNA does cut to completion. Perhaps therefore, this quantity of DNA is too little to allow for a distinct hybridization signal.

5.3.2. Ontogenic appearance of sodium channels

Changes in density of [^{3}H]STX binding sites in the developing *Manduca* pharate adult brain are shown in Fig. 5.5. Assuming [^{3}H]STX binding sites represent sodium channels, it can be seen that sodium channel density increases progressively from day 9 to day 16. Although the data suggests that the rate of sodium channel biosynthesis is more rapid after day 12, it nevertheless indicates that sodium channel mRNA is present in pharate adult brains in the developmental stages utilised to make the *Manduca* cDNA library which was used in this study, and so it is likely that this library contains sodium channel transcripts.

5.3.3. Determination of library titre and proportion of recombinants

The titre of the cDNA library stock averaged 3×10^9 pfu/ml, of which 46% were recombinant (as measured by the ratio of blue/white plaques). These values compare favourably with those obtained by Horodyski (personal communication) indicating that the library had been unaffected during transportation and confirming that the

techniques employed in manipulating the library and plating cells could duplicate results obtained independently from another laboratory.

5.3.4. Screening the cDNA library with ^{32}P labelled probes

5.3.4.1. Oligonucleotide probe SC2

Oligo SC2 identified 13 positive plaques from the *Manduca* cDNA library. Autoradiographs of secondary screen filters washed at the same stringency as those in the primary screen (2x SSC, 0.1% SDS, 50°C) showed a uniform background signal, and did not display any distinct plaques with outstanding hybridization signal intensity. Increasing the stringency of washing by raising the temperature to 58°C completely stripped the filters of probe. Since no cross-hybridization occurred at all at this relatively low stringency all "positive" plaques originally isolated from the primary screen were considered to have been false.

5.3.4.2. cDNA probes

Primary screening using sodium channel cDNAs as probes was initially carried out using *NA8.4*. Because the level of ^{32}P incorporation which had been achieved by radiolabelling this probe was relatively low (<30%), hybridization was allowed to continue for 60 hours. Autoradiography of filters washed at reasonably high stringency (0.2x SSC 0.1% SDS, 65°C) identified over 30 strong positive hybridization signals (none of these corresponded to any of those identified by screening the same filters with *Oligo SC2*). 10 positive plaques of varying signal intensity and from different plates were re-screened as described. In the case of each secondary screen filter, strong hybridization had occurred at positions corresponding to every plaque. The possibility that each of these contained a real positive clone could be ruled out, since the plaque density of the primary screen plates would have

ensured that with each positive plaque, 30-50 others in close proximity would have been picked also.

It is feasible that the secondary screen results were caused by pEMBL vector contamination of the labelled probe, as strong hybridization signals of uniform intensity remained after washing the filters at high stringency, suggesting base sequence identity between hybrids. Both pEMBL and the cDNA library vector λZAP II contain the *LacZ* and ampicillin resistance genes, so that pEMBL vector contamination of the probe would cause false positives. Such vector contamination may not have been detected in the primary screen since the much higher plaque density would have diluted the contamination signal to very low levels. In the absence of pure pEMBL, which could have been added to the hybridization mixture in excess to compete with labelled pEMBL, a probe of much greater purity was required. It was for this reason that the purified version of *NA8.4* (*NA8.4p*) was prepared. *NA8.4p* however, failed to identify any positive plaques from the original primary screen filters or from the *NA8.4* secondary screen filters. The reasons for this result remain unclear. Furthermore, this probe did not identify positive signals from filters lifted from a freshly plated library. Probe *Li3-4* proved to be similarly unsuccessful.

5.4. Discussion

The work reported here was unsuccessful in cloning a *Manduca* sodium channel, and unfortunately there was insufficient time to identify the reasons for this. However, the following discussion attempts to draw at least some conclusions from this work.

The hybridization of sodium channel cDNAs to rat genomic DNA Southern blots has not been previously reported. Incubation of ^{32}P labelled cDNA *Li3-4* with such blots

reveals the presence of eight to ten specific hybridization fragments. Do all of these sequences correspond to sodium channels? Sequence analysis between different sodium channel subtypes from rat as well as the two putative *Drosophila* sodium channels (DSC1 and Para) shows that the sequence which links homology domains III and IV is highly conserved among sodium channels, but this is not a feature of the putative mammalian calcium channel α-subunit, which otherwise maintains overall structural homology with sodium channels (Tanabe *et al.*, 1987; Mikami *et al.*, 1989). This indicates that cDNA probe *Li3-4* is indeed specific for sodium channel sequences and the hybridization bands must therefore represent either multiple copies of specific sodium channel genes or genes for different sodium channel subtypes.

To date, several sodium channel mRNAs have been isolated from various excitable tissues of the rat, namely Rat Brain I and II (Noda *et al.*, 1986a) Rat Brain IIA (Auld *et al.*, 1988) Rat Brain III (Kayano *et al.*, 1988) Skeletal Muscle I (Trimmer *et al.*, 1989) Skeletal Muscle II (Kallen *et al.*, 1990) Rat Heart I (Rogart *et al.*, 1989) and a 326 amino acid fragment of a different but highly conserved rat heart sodium channel (Sills *et al.*, 1989). Rat Brain IIA is so similar to Rat Brain II that it probably results from alternative splicing events or sequence polymorphisms between the different strains of rat used (Auld *et al.*, 1988), and Skeletal Muscle II has an identical amino acid sequence to Rat Heart I. Therefore six distinct rat sodium channel isoforms have been isolated to date. The results of the Southern blot analysis suggest that provided none of these isomers is represented as multiple copies in the rat genome, further sodium channel subtype sequences have yet to be identified.

It was not possible to demonstrate hybridization of any of the available sodium channel probes to Southern blots prepared from *Manduca* genomic DNA. However, comparison of agarose gels prior to Southern transfer with those prepared from rat DNA suggested that *Manduca* DNA produced Southern blots of poor quality, due to an inferior efficiency of restriction endonuclease digestion. Despite attempts at

extracting *Manduca* genomic DNA using a variety of methods, yields were always low and the inadequate cutting efficiency of the broad range of restriction enzymes which were tested could not be improved upon. This difficulty in preparing good quality DNA does not appear to be an inherent property of *Manduca*, since the production of functional genomic Southern blots from this insect has been reported elsewhere (Rebers *et al.*, 1987). Furthermore, the method of Maniatis *et al.* (1982) which was used to extract the DNA utilised in that report, is almost identical to the protocols involving proteinase K digestion and phenol extraction which were used in this study.

In order to confirm that the *Manduca* Southern blots are incapable of hybridizing to homologous probes, the possibility of synthesizing an oligonucleotide probe corresponding to part of one of the known *Manduca* gene sequences was considered, but it was decided that this was impractical due to the time and cost involved.

Despite the uncertainty surrounding the *Manduca* genomic Southern blot analysis, it was nevertheless considered worthwhile to undertake a comprehensive programme to screen the *Manduca* cDNA library for the following reasons. First, cDNA *NA8.4* spans homology domains III and IV. Analysis of the amino acid sequences of the corresponding regions in both DSC1 (Salkoff *et al.*, 1987b) and para (Loughney *et al.*, 1989) reveals a considerable degree of conservation within both the putative membrane spanning domains and the cytoplasmic sequence linking them. *NA8.4* was used as the probe to isolate large fragments of both the para and DSC1 genes from a *Drosophila* genomic library (Ramaswani and Tanouye, 1989). Secondly, according to models proposed by Guy and Seetharamulu (1986) and Guy (1988), the TTX/STX receptor site is located in the extracellular loop between S5 and S6 in each homology domain. A single point mutation replacing a conserved glutamic acid with glutamine in this region (amino acid 387) prevents the blockade of the Rat II sodium channel with TTX (Noda *et al.*, 1989) thus providing convincing evidence that this aspect of

the model is correct. Since the *Manduca* CNS possesses [^{3}H]STX receptors which have binding properties very similar to rat brain sodium channels (Verdon *et al.*, 1988; see also Chapter 3) it is reasonable to assume that the amino acid sequences which constitute the [^{3}H]STX receptor are highly conserved. Third, site-directed antibodies raised to a conserved region of the III-IV linker sequence have been used to immunoprecipitate sodium channels from a number of vertebrate excitable tissues (Gordon *et al.*, 1988) and have also been shown to immunoprecipitate sodium channels from solubilized membranes prepared from the CNS of species representing four orders of insects, including the lepidopteran *Spodoptera littoralis* (Gordon *et al.*, 1990). There is therefore good evidence of significant conservation between rat and Lepidopteran sodium channel primary sequence, at least in certain regions of *NA8.4*.

The fact therefore that the cDNA probes, particularly *Li3-4*, failed to isolate homologous sequences from the *Manduca* cDNA library is puzzling. However, Soderlund *et al.* (1989) reported screening a *Drosophila* genomic library with oligonucleotide probes derived from several conserved sequences, including the III-IV linker. Although isolation of part of a gene homologous to DSC1 using another oligonucleotide is discussed, no further reference is made to the oligonucleotide derived from the III-IV linker, suggesting that the ineffectiveness of probes derived from this region is not limited to the present study.

Although the DSC1 sequence used in the design of *Oligo SC2* is homologous to the corresponding amino acid sequence of the Rat II sodium channel, it nevertheless deviates from this sequence (see Fig. 5.2) which is otherwise almost entirely conserved between all other sodium channels so far cloned from rat excitable tissues. It was therefore perhaps not surprising that *Oligo SC2* did not hybridize to rat genomic Southern blots. *Oligo SC2* was based on one of the most hydrophobic segments of DSC1, which is presumed to be a membrane spanning region in all models proposed to date. Before embarking on the screening programme it was

thought that the most likely problem with this probe would be its lack of specificity, since other quite distinct transmembrane proteins may coincidentally possess similar arrangements of hydrophobic residues suited to this function. This may have been the reason why a shorter oligonucleotide probe to the same region used by Soderlund and Knipple (1988) isolated a gene from a *Drosophila* genomic library which bore no resemblance to the putative sodium channel gene. The reason why *Oligo SC2* failed to isolate any true positive plaques therefore remains unclear.

There is however no information regarding the functional significance of the DSC1 gene isolated by Salkoff *et al.* (1987b) on which *Oligo SC2* was based. This putative *Drosophila* sodium channel gene maps cytogenetically to region 60D-E, which is not associated with any of the loci thought to be involved in sodium channel function (Hall, 1986). Furthermore, embryonic neuronal cell cultures which are deficient in the 60D-E region show normal sensitivity to veratridine which is reversed by tetrodotoxin (Sakai *et al.*, 1989). This suggests either that DSC1 does not code for a sodium channel, that DSC1 codes for a less prominent sodium channel subtype, or that DSC1 is expressed at a later stage. In addition the para locus has recently been cloned by "P Element Transposon Tagging" techniques, and the deduced amino acid sequence is very similar to the rat brain sodium channel (Loughney *et al.*, 1989). The argument that this gene codes for a sodium channel is supported by the observation that changes in para dosage lead to corresponding changes in neuronal excitability (Stern *et al.*, 1990). The para sequence was unfortunately not published in time to use it in the cloning strategy reported here.

However, the deduced structural features of DSC1 are also very similar to those of vertebrate sodium channels. Furthermore, fragments of an identical sequence have also been isolated independently from *Drosophila* genomic libraries using rat brain sodium channel cDNAs (Ramaswami and Tanouye, 1989), as well as synthetic oligonucleotides corresponding to both IS4 of DSC1 (Soderlund *et al.*, 1989) and eel

electroplax region IVS6 (Okamoto *et al.*, 1986). These independent isolations using probes derived from different sources confirm that significant homology exists between DSC1 and vertebrate sodium channels.

It would therefore appear that there may be more than one class of sodium channel in *Drosophila*, and that these have undergone greater divergence than the rat brain sodium channel isoforms, which are all very similar. Furthermore, the para locus of *Drosophila* appears to undergo alternative splicing to produce several distinct sodium channel subtypes (Loughney *et al.*, 1989). If multiple and diverse sodium channel genes are a common phenomenon in insects, then this represents a considerable complication in identifying the molecular mechanism of pyrethroid insecticide resistance.

Comparison of the base composition of *Oligo SC2* with the gene sequence for DSC1 (Salkoff *et al.*, 1987a) shows that the same codons are shared with 10 of the 17 amino acids from which *Oligo SC2* was derived. The same comparison between *Oligo SC2* and para (Loughney *et al.*, 1989) reveals that of the 13 amino acids which are conserved between DSC1 and para over this region, only 4 have identical codons to those used in the synthesis of *Oligo SC2*. This observation is of relevance, since on the basis of the most commonly utilised codon for a given amino acid, codon usage is identical in *Drosophila* and *Manduca* (see Methods, Section 5.2.3.1). This therefore raises a general point of caution in designing oligonucleotide probes to target libraries constructed from a particular species. Choosing bases to accommodate ambiguous codon positions from a codon usage table is never more than a "best guess" strategy, and could lead to a probe of very poor hybridization potential. This may account for the negative results encountered using *Oligo SC2* in this study.

The library screened in this study has proven to be a functional cDNA library, since eclosion hormone genes have already been isolated and sequenced from it (F.

Horodyski *et al.*, 1989). It was also apparently in good condition, since the determination of library titre and the proportion of recombinant plaques yielded similar values to those obtained soon after its construction (Horodyski, personal communication). Determination of the number of [^{3}H]STX binding sites at different times during development indicated that the Horodyski cDNA library should have contained sodium channel cDNAs, as there is a progressive increase in [^{3}H]STX binding sites in *Manduca* pharate adult brains during the period day 9 to day 12, which was the developmental stage used to provide the tissue from which the library was constructed. Furthermore, comparison of studies on embryonic rat brains shows that Rat I and II mRNA levels (Grubman *et al.*, 1988) begin to increase significantly two days before the corresponding increase in [^{3}H]TTX binding sites (Lombet *et al.*, 1983). Such a time lag between the appearance of mRNA and [^{3}H]STX binding sites in *Manduca* pharate adult brains would mean that the cDNA library was fortuitously constructed at a time when the levels of sodium channel mRNA would be expected to be at their highest. However, the evidence suggests that with rat brain sodium channels, this delay may be due to post-translational assembly of the sodium channel subunits (Schmidt *et al.*, 1985), and the insect sodium channel appears to be composed only of a single subunit (Gordon *et al.*, 1990). However, even if translation and appearance of sodium channels occurred over a much shorter time scale, the increase in [^{3}H]STX binding sites during days 9 to 12 would still demonstrate the presence of sodium channel mRNA at this stage, and so the presence of sodium channel cDNA in the Horodyski library can be predicted with confidence.

The cloning strategy employed in this study relied heavily on the use of probes derived from the clone *NA8.4*. The inferred amino acid sequence of this cDNA is highly conserved between all rat sodium channel isoforms as well as the para and DSC1 sodium channels. The initial optimism that this would be sufficient to isolate a sodium channel cDNA from a library which was likely to contain sodium channel transcripts proved to be unfounded.

Both electrophysiological (see Chapter 2) and pharmacological evidence (see Chapter 4) suggest that *Manduca* sodium channels have novel properties. It is therefore possible that the sequences which predict the structural and functional properties of the channel have diverged from rat sodium channel to such an extent that they are not sufficiently complementary to hybridize to rat brain sodium channel probes. In this context, it is worth noting that Noda *et al.* (1986a) using cDNA probes from Rat Brain I and II failed to detect hybridizable RNA species in Northern blots derived from adult skeletal muscle, although it was later shown that this tissue contains abundant quantities of RNA from the highly conserved Skeletal Muscle 1 (Kallen *et al.*, 1990).

The work described in this chapter was performed during the period 1988-90. The advent of the Polymerase Chain Reaction (Mullis and Faloona, 1987) has recently provided an alternative class of cloning strategies which were not available at the time. Although the para locus contains at least 26 exons distributed over more than 60 Kb of genomic DNA (Loughney *et al.*, 1989), the position of the introns is indicated in the published sequence data. Most of the exons include several highly conserved sequences. It should therefore be possible to synthesize two short degenerate oligonucleotides which do not flank known introns and use these for PCR amplification. If *Manduca* genomic DNA was used as a template, this should result in the generation of one or more sequences specific for the *Manduca* sodium channel. As well as providing information concerning the structure of the *Manduca* sodium channel over this region, these sequences could be used to probe the *Manduca* cDNA library with high stringency. This alternative cloning strategy would hopefully prove to be more effective.

FIGURE 5.2. *Design of Oligo SC2.* The amino acid sequence of segment S6 in Domain IV is highly conserved between the Rat II sodium channel, the *Drosophila* para sodium channel and the putative *Drosophila* DSCI sodium channel. The presumed *Manduca* cDNA corresponding to this region of DSCI was inferred from the *Manduca* codon usage data (Table 5.1). *Oligo SC2*, the antisence of this presumed cDNA, was synthesized on an automatic DNA synthesizer.

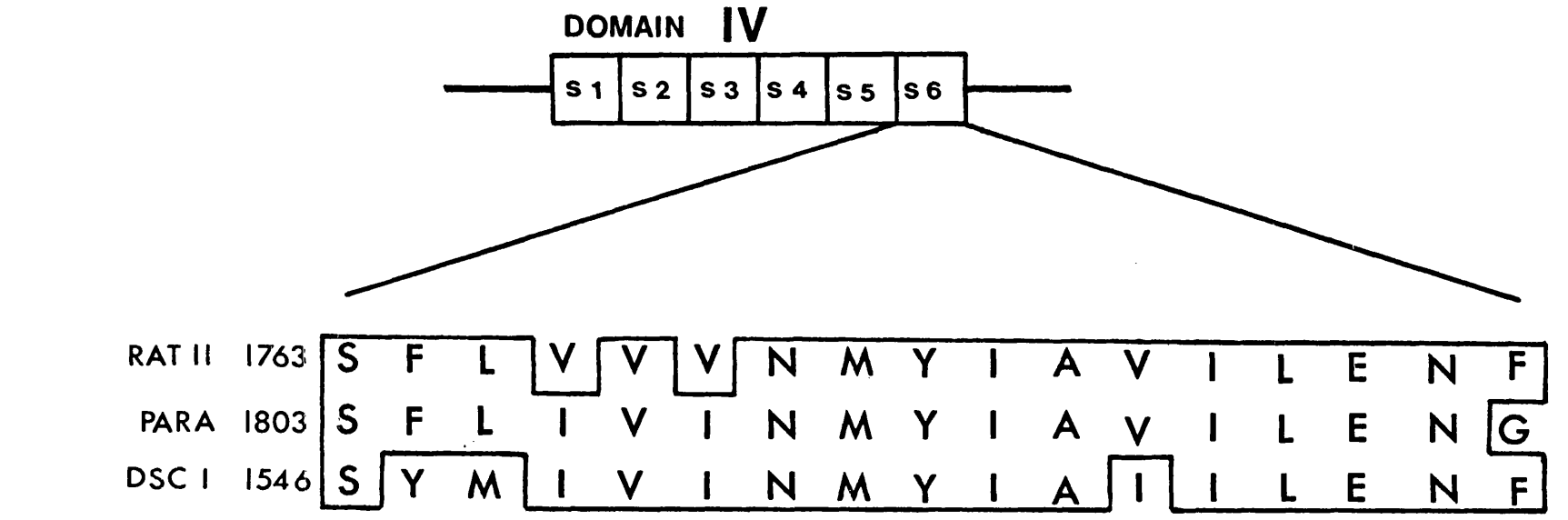
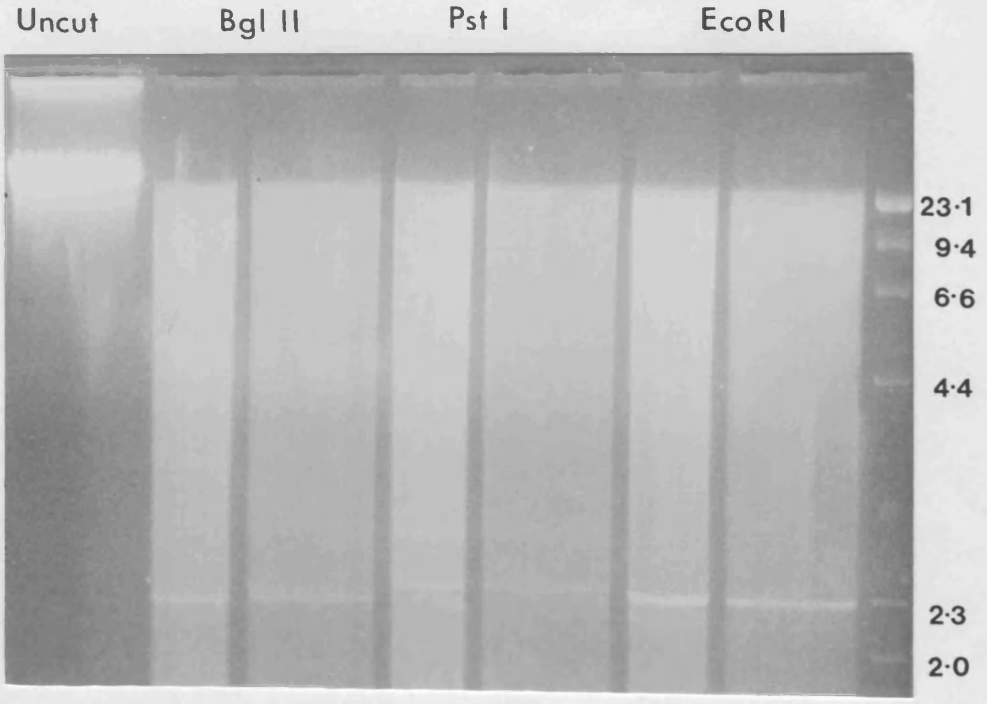

DOMAIN IV
S1
S2
S3
S4
S5
S6
RAT II 1763 S F L V V V N M Y I A V I L E N F
PARA 1803 S F L I V I N M Y I A V I L E N G
DSC I 1546 S Y M I V I N M Y I A I I L E N F
MANDUCA cDNA 5'-TCC TAC ATG ATC GTC ATC AAC ATG TAC ATC GCC ATC ATC CTG GAG AAC TT-3'
OLIGO SC2 3'-AGG ATG TAC TAG CAG TAG TTG TAC ATG TAG CGG TAG TAG GAC CTC TTG AA-5'

FIGURE 5.3. *Southern blot analysis of rat genomic DNA.* (A) Photograph of agarose gel containing rat DNA after digestion with the indicated restriction endonucleases followed by electrophoresis. The sizes of the *Hind*III digested λ markers are indicated to the right (in kilobases). (B) Autoradiograph of the Southern transfer from the same gel after incubation with radiolabelled probe *Li3-4*. This specific sodium channel probe hybridized to several fragments of the digested rat DNA, probably indicating the presence of several sodium channel subtypes.
Wash stringency: 0.2x SSC, 1% SDS, 60°C. Autoradiograph exposed overnight.

A.

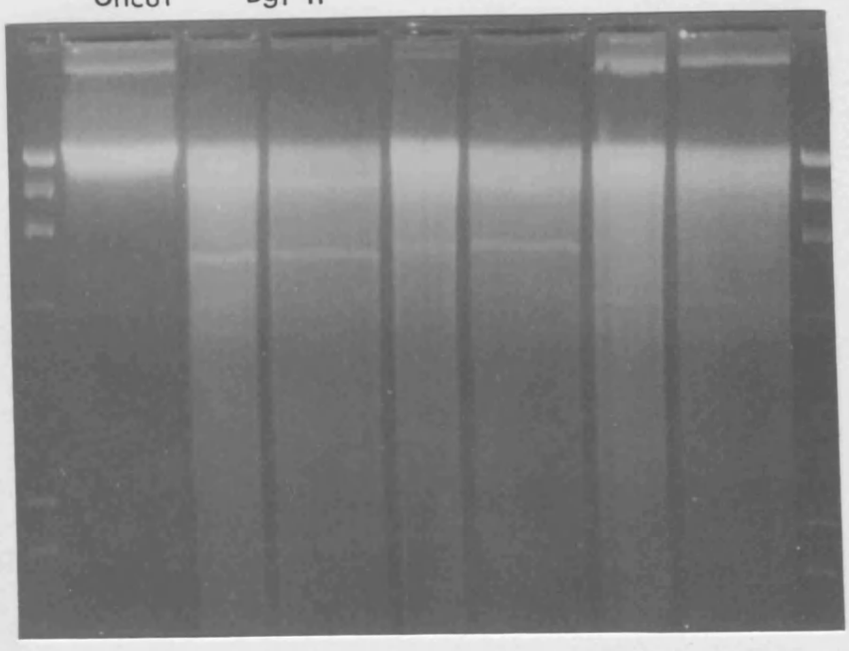


B.

FIGURE 5.4. *Comparison of rat and* Manduca *genomic DNA after digestion with restriction endonucleases followed by agarose gel electrophoresis.* The photographs were taken under ultraviolet illumination after ethidium bromide staining. Much of the endonuclease treated *Manduca* DNA is of high molecular weight (being comparable with the uncut reference DNA), indicating incomplete digestion. In comparison, the endonuclease treated rat DNA appears to have digested to completion. (see Fig. 5.3 for λ marker sizes).

MANDUCA

Uncut Bgl II Pst I Sal I



RAT

Uncut Bgl II Pst I Eco RI

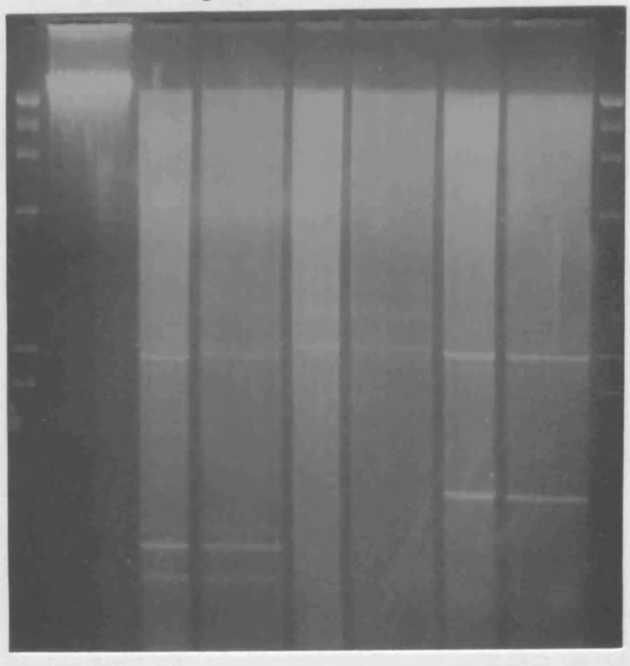


FIGURE 5.5. *Ontogeny of [^{3}H]STX binding sites in developing adult* Manduca *brain.* The data shows that the density of [^{3}H]STX binding sites increases rapidly after day 9 of pupal development, indicating the presence of high levels of sodium channel mRNA after this time. The shaded area indicates the developmental stages utilised in the construction of the *Manduca* brain cDNA library.

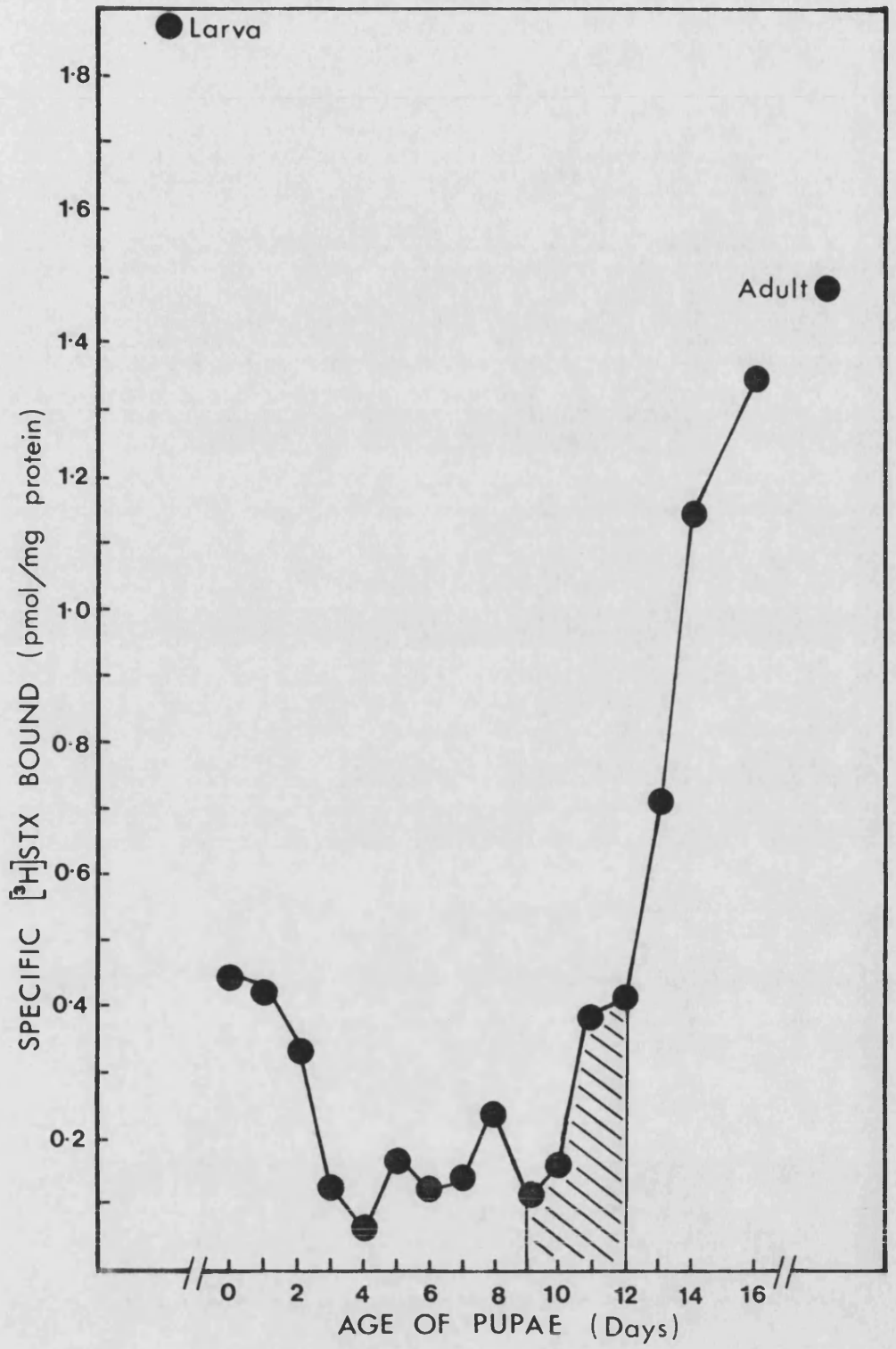

Larva
Adult
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
SPECIFIC [3H]STX BOUND (pmol/mg protein)
0
2
4
6
8
10
12
14
16
AGE OF PUPAE (Days)

TABLE 5.1

Codon usage data for *Manduca sexta*

Phe	TTT	5	Ser	TCT	11	Tyr	TAT	5	Cys	TGT	5
	TTC	**44**		**TCC**	**22**		**TAC**	**20**		TGC	15
Leu	TTA	2		TCA	3		(TAA)	4		(TGA)	1
	TTG	10		TCG	4		(TAG)	1	Trp	TGG	10
Leu	CTT	11	Pro	CCT	5	His	CAT	2	Arg	CGT	2
	CTC	16		CCC	17		CAC	14		CGC	9
	CTA	5		CCA	6	Gln	CAA	9		CGA	2
	CTG	**23**		CCG	0		CAG	28		CGG	2
Ile	ATT	10	Thr	ACC	7	Asn	AAT	11	Ser	AGT	3
	ATC	**31**		ACC	16		**AAC**	**44**		AGC	12
	ATA	5		ACA	7	Lys	AAA	25	Arg	AGA	8
Met	**ATG**	**31**		ACG	3		AAG	51		AGG	8
Val	GTT	15	Ala	GCT	34	Asp	GAT	14	Gly	GGT	8
	GTC	**30**		**GCC**	**48**		GAC	42		GGC	21
	GTA	9		GCA	13	Glu	GAA	19		GGA	18
	GTG	22		GCG	27		**GAG**	**40**		GGG	5

Table constructed using *Manduca* gene sequences published by Bradford and Keeley (1989), Cole *et al.* (1987), Dickinson *et al.* (1988), Gyorgyi *et al.* (1988), Horodyski *et al.* (1989), Rebers and Riddiford (1988) and Wang *et al.* (1988).
Codons indicated in bold type were used for the synthesis of the oligonucleotide probe *Olig-SC2*.

CHAPTER 6

GENERAL OVERVIEW

The possibility that insect pests will develop widespread *kdr*-like resistance to pyrethroid insecticides is a cause for serious concern. Traditionally, resistance in agricultural pests has been overcome by an increase in the application dose of the insecticide, and eventually by superceding it with a novel class of compound. These strategies are of limited value today, since pyrethroid insecticides are expensive, and alternatives are simply not available.

Until now the route to pesticide discovery has involved the random screening of synthetic chemicals directed towards target species. It now seems obvious that a more informed and rational approach is appropriate, in which the design of novel compounds is based on the study of the molecular interactions between the pesticide and its target site. A complete understanding of such an interaction will be required in order to elucidate the mechanism of *kdr* resistance.

Considering the problem, there is a surprisingly poor understanding of fundamental aspects of insect neuroscience in general, and of insect sodium channels in particular. Therefore, as a first step in this direction, a multidisciplinary study was initiated in an attempt to characterise an insect voltage-sensitive sodium channel. The larva of *Manduca sexta* provided an ideal model system for these investigations: the final instar is large and so compared to other insects is capable of yielding a relatively large

quantity of neuronal tissue for pharmacological investigation. The early third instar larva proved to be ideal for the electrophysiological examination of the neuromuscular junction, and a developing *Manduca* brain cDNA library was available for screening. A Lepidopteran larva is also the most suitable insect to study in this context, since pyrethroid insecticides are targeted most heavily against this order (Ruight, 1985).

Electrophysiological experiments on the neuromuscular junction of the body wall muscle demonstrated that pyrethroid insecticides cause an increase in the frequency of miniature synaptic potentials. Under certain circumstances this effect could be abolished by TTX, implicating the sodium channel as the probable target site. The site 2 sodium channel toxin veratridine was shown to induce similar effects, although the response was distinguishable, in that the time course of the effects was typically more rapid. The overall similarity in the response suggests that VTD and pyrethroids have essentially the same mode of action, in that they both stabilise the open configuration of the sodium channel. The induced increase in transmitter release therefore probably resulted from a depolarisation of the nerve terminal. These effects are well documented for the *Musca* neuromuscular junction (see Salgado *et al.*, 1983a,b). However, it is difficult to interpret the effect of simultaneous application of toxin and insecticide in terms of this scheme. When VTD was added to a cypermethrin poisoned muscle fibre, no change was observed in mepsp frequency, and transmitter release continued for much longer than would have been the case for VTD alone. Thus, cypermethrin seemed either to pre-empt or suppress the effect of VTD. This is not in accord with previous observations: pyrethroids produce a potentiation of the VTD stimulated neurotransmitter release from synaptosomes prepared from both whole houseflies and cockroach ganglia (Clark and Matsumura, 1991; Nicholson *et al.*, 1987), and enhance VTD induced $^{22}Na^{+}$ uptake into mammalian neuroblastoma cells (Jaques *et al.*, 1980).

There are two possible explanations for the response at the *Manduca* neuromuscular junction, although neither are particularly convincing. First, there is some evidence that the facilitation of transmitter release caused by pyrethroids is in part due to a direct release of Ca^{2+} from intracellular presynaptic storage sites (Enan and Matsumura, 1991). It is therefore plausible that pyrethroids may act by regulating transmitter release independently of presynaptic depolarisation, and so the addition of sodium channel activators would not be expected to have any potentiating effect. However, in the present work, TTX was found to be capable of completely reversing the pyrethroid induced effects at the *Manduca* neuromuscular junction, confirming that the sodium channel is the primary lesion in this system. Secondly, it is possible that occupancy of the pyrethroid receptor site on the sodium channel prevents VTD from binding. This seems unlikely however, since in these experiments the VTD concentration was 1000-fold higher than the pyrethroid concentration. Moreover, interaction between [3H]BTX-B binding and pyrethroid was not seen in the Manduca nerve cord preparation.

[^{3}H]BTX-B binding is enhanced by pyrethroids in *Musca* membrane preparations (Pauron *et al.*, 1989), demonstrating that the binding sites for pyrethroids and alkaloid toxins must be distinct, although a functional link exists between them. In *kdr* resistant *Musca* larvae, cross resistance to aconitine (another site 2 toxin) is observed in both electrophysiological experiments (Salgado *et al.*, 1983a) and by injection assay (Bloomquist and Miller, 1986). This may indicate that the pyrethroid and alkaloid binding sites are very close, such that an alteration in the pyrethroid binding site in *kdr* flies affects site 2 binding also. Binding studies with [^{3}H]BTX-B fail to show any alteration of this site in *kdr* flies (Pauron *et al.*, 1989), but this may reflect a difference between the [^{3}H]BTX-B and aconitine sites.

Further characterisation of neuroreceptor site 2 on the *Manduca* voltage-sensitive sodium channel using [^{3}H]BTX-B did not prove to be very successful. In most of the

preparations in which [^{3}H]BTX-B pharmacology has been characterised, binding of higher affinity occurs in the presence of ligands which stabilise the sodium channel in the open configuration. Of particular interest was the allosteric modulation of [^{3}H]BTX-B binding to mammalian synaptosomes by pyrethroids (Lombet *et al.*, 1988). It was hoped that the characterisation of this interaction in *Manduca* would provide an interpretation of the unusual results gained from the electrophysiology studies, and ultimately provide insight into the mechanism of *kdr*. However, [^{3}H]BTX-B binding to *Manduca* CNS homogenate could not be enhanced in the presence of *Lqq* scorpion venom, anemone toxin II or pyrethroids, in contrast to the case in vertebrate preparations. This meant that the affinity of the BTX receptor for the labelled toxin was unacceptably low, making a full characterisation of binding kinetics not only difficult, but unrealistically costly ([^{3}H]BTX-B is very expensive and available in only limited amounts).

At the time this work was carried out, [^{3}H]BTX-B binding had only been demonstrated in mammalian brain preparations, and so it was considered possible that the differences in binding properties were due to the fact that the toxin binding sites on insect sodium channels were inherently different. Another possible explanation was that although electron microscopy studies of the *Manduca* nerve cord preparation showed that it contained vesicles, the presence of a membrane potential (a prerequisite for high affinity [^{3}H]BTX-B binding) was not demonstrated. Since that work was completed however, [^{3}H]BTX-B has been shown to bind conventionally to housefly head membranes, in that the binding affinity is increased in the presence of *Lqq* scorpion venom and is comparable to that measured in rat brain synaptosomes. Furthermore, binding is unaffected under depolarising conditions (Soderlund *et al.*, 1989). The difference in the results obtained in housefly and in *Manduca* remain unresolved. It is quite possible that there are real differences between the pharmacological properties of the [^{3}H]BTX-B binding site on *Manduca* sodium channels and the other species so far investigated. In particular, the apparent

inhibition of [^{3}H]BTX-B binding by *Lqq* scorpion venom is unparalleled. Isolation and characterisation of the scorpion venom component which prevents [^{3}H]BTX-B binding to *Manduca* sodium channels may provide the first specific probe for a Lepidopteran sodium channel, which will be of relevance to the insecticide industry.

Characterisation of the [^{3}H]STX receptor site in this preparation demonstrated that the pharmacology of [^{3}H]STX binding was very similar to that of the mammalian sodium channel, thus neuroreceptor site 1 is highly conserved. The binding capacity of nerve cord homogenate for [^{3}H]STX is high considering it is a crude preparation, being comparable to rat brain synaptosomes. It would be interesting to use this assay to determine B_{max} values in insect populations which display *kdr*-like resistance to investigate the proposal that this type of resistance can be conferred by a reduction in the number of sodium channels.

It was unfortunate that such little progress was made in the attempt to clone the *Manduca* sodium channel. If *kdr* is the result of an alteration in sodium channel structure, then this approach offers the best hope for the elucidation of the precise molecular mechanism involved, and may offer insight into ways of counteracting it. However, even this approach may be by no means straightforward, since the limited information available from insect sodium channel cloning to date suggests that insects, in common with mammals, may possess multiple sodium channel subtypes. Indeed, the [^{3}H]STX binding studies carried out in this project appear to have isolated a sodium channel subtype in *Manduca* heart tissue. The existence of multiple sodium channel subtypes would complicate the search for the gene product conferring *kdr* resistance. Furthermore, the existence of such subtypes raises the possibility that the *kdr* mechanism simply involves a change in their proportions such that intrinsically pyrethroid insensitive subtypes predominate.

On a more optimistic note, proteolytic maps have recently shown that purified sodium channels are structurally distinct in different insect species (Gordon *et al.*, 1990). This fact may explain the differences between the electrophysiological and [^{3}H]BTX-B binding results presented here, and those obtained in other insect species. A complete understanding of the insecticide receptor sites may thus pave the way for the design of novel compounds which not only select specifically for insects, but which can also be effectively targeted towards particular insect species.

APPENDIX I

Preparation of physiological saline for *Manduca sexta.*

Stock solutions.

10 x Salts			100 x Buffer		
	mM	gl^{-1}		*mM*	gl^{-1}
NaCl	40	2.34	Na_2HPO_4	150	21.3
KCl	400	29.84	NaH_2PO_4	150	18.0
$MgCl_2$	180	17.15			
CaCl	30	3.33			

Method

51.35 g sucrose was added to 500 ml H_2O (for a final sucrose concentration of 150 mM).

100 ml stock salts and 10 ml stock buffer solutions was then added.

H_2O was added to a final volume of 1 litre.

The saline was adjusted to pH 6.5 (with 0.1 M HCl or KOH).

Stock solutions were stored in the refrigerator. Physiological saline was prepared fresh daily. Stock salts solution was discarded every fortnight. Stock buffer solution remained viable for longer periods.

(Saline composition modified from Weevers, 1966)

APPENDIX II

Analysis of binding data

Assuming a homogeneous species of ligand and a single non-interacting population of receptors, the binding of radioligand, L, to its receptor, R, to form the radioligand-receptor complex, LR, can be represented as:

$$L + R \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} LR \qquad (1)$$

where K_1 and K_{-1} are the kinetic association and dissociation constants respectively.

At equilibrium:

$$K_D = [L][R]/[LR] \qquad (2)$$

where K_D is the equilibrium dissociation rate constant, [L] is the concentration of unbound ligand, [R] is the concentration of free receptor sites and [LR] the concentration of the ligand-receptor complex.

(A) *Equilibrium equations*

The equilibrium binding equation (2) can be rearranged to give the Scatchard equation:

$$[LR]/[L] = -[LR]/K_D + [R]_T/K_D \qquad (3)$$

where $[R]_T$ is the total concentrating of receptor sites.

For analysis of binding data it is common to substitute Bound, or [B], for [LR]; Free or [F] for [L]; and B_{max} for $[R]_T$

Thus the Scatchard equation (3) is normally represented as:

$$[B]/[F] = -[B]/K_D + B_{max}/K_D \qquad (4)$$

Thus from a saturation curve, a plot of the concentration of bound ligand divided by the concentration of free ligand [B]/[F] versus the concentration of bound ligand [B], yields a slope equal to the negative reciprocal of the equilibrium dissociation constant ($-1/K_D$), and the intercept on the abscissa provides a measure of the total concentration of binding sites (B_{max}).

Once the B_{max} has been calculated by Scatchard analysis the saturation data can be represented as a Hill plot:

$$\log[B]/ (B_{max}-[B]) = n\log[F] - n\log K_D \qquad (5)$$

A plot of $\log[B]/B_{max}-B$ versus log[F] yields a slope equal to the Hill coefficient (n) and the intercept on the abscissa provides a measure of K_D. Values of n close to 1.0 imply a simple bimolecular reaction between ligand and receptor.

(B) ***Kinetic equations***

Assuming pseudo first-order conditions ($[L]_T >> [B]_{eq}$) then association time course data can be expressed as:

$$\ln [B]_{eq}/([B]_{eq}-[B])_t = K_1 t.[L]_T[R]_T/[B]_{eq} \qquad (6)$$

where $[L]_T$ is the total ligand concentration, $[B]_{eq}$ is binding at equilibrium (the total ligand-receptor concentration at equilibrium), $[B]_t$ binding at time *t*, and $[R]_T$ the total concentration of receptor sites.

A plot of $\ln[B]_{eq}/([B]_{eq}-[B]_t)$ versus *t* yields a slope equal to $K_1[L]_T[R]_T/[B]_{eq}$.

The kinetic association constant, K_1, can be calculated by determining $[R]_T$ (using Scatchard values of B_{max}) and estimating $[B]_{eq}$ from the association time course data.

Dissociation can be described by the following equation:

$$\ln[B]_t/[B]_o = -K_{-1}t \qquad (7)$$

where $[B]_o$ is the concentration of the ligand-receptor complex immediately prior to dilution (thus eliminating the forward reaction) and $[B]_t$ the concentration of the complex at time *t* after the initiation of dissociation.

A plot of $\ln[B]_t/[B]_o$ versus *t* yields a slope equal to the kinetic dissociation constant, K_{-1}.

For a simple bimolecular interaction, the equilibrium and kinetic rate constants are related such that:

$$K_D = K_{-1}/K_1 \qquad (8)$$

Therefore, K_D can be calculated from both equilibrium and kinetic equations (although Scatchard analysis is required to determine K_1).

(For derivation of integrated rate equations, see Weiland and Molinoff, 1981).

APPENDIX III

Media and solutions used in molecular biology experiments.

LB medium (per litre)

Bacto tryptone	10 g
Bacto yeast extract	5 g
NaCl	10 g

Adjust to pH 7.4 with NaOH. Sterilise by autoclaving.

Agar Plates: LB medium plus 15 g/litre Bacto-agar.

Top-agarose: LB medium plus 7 g/litre agarose.

TE solution

10 mM Tris.Cl

1 mM EDTA (pH 8.0)

Adjust to appropriate pH with HCl. Autoclave.

SM solution (per litre)

NaCl	5.8 g
$MgSO_4.7H_2O$	2.0 g
1M Tris.Cl (pH 7.5)	50 ml
2% gelatin	5 ml

Sterilise by autoclaving.

Denhart's solution (50x)

Ficoll	5 g
polyvinylpyrrolidone	5 g
BSA (Pentax Fraction V)	5 g

Make up to 500 ml with water.

Filter through a disposable Nalgene filter.

20x SSC (per litre)

NaCl	175.3 g
Sodium citrate	88.2 g

Adjust to pH 7.0 with NaOH.

All glassware was baked at 180°C before use.

Glass and plasticware used in the manipulation of phage was made detergent free by rinsing in 2% acetic acid.

All water was double distilled and filtered through a Milli-Q (Millipore) water purification system.

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